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### INSTRUCTIONS FOR AUTHORS

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## Detection of *Acinetobacter* spp. in Blood Cultures by an Improved Fluorescent *in Situ* Hybridization Assay

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### Abstract

Fluorescent *in situ* hybridization (FISH) allows rapid detection of microorganisms. We aimed (i) to evaluate the sensitivity and specificity of FISH for the detection of *Acinetobacter* spp. in blood culture specimens and (ii) to test the simultaneous application of two genus-specific probes labeled with the same fluorochrome to increase the fluorescent signal intensity and improve the detection of *Acinetobacter* spp. Three hundred and twenty blood culture specimens were tested *via* both the conventional laboratory methods and FISH to detect *Acinetobacter* spp. The specimens were examined separately with each genus-specific probe Aci and ACA, and also using a mixture of the both probes Aci and ACA. In all examinations, probe EUB338 was used accompanied by Aci and ACA. The specificity of FISH was 100% (97.5% confidence interval [CI] = 98.7% – 100%). The sensitivity of FISH by the use of probe Aci was 96.4% (95% CI = 81.7% – 99.9%), whereas, the sensitivity of this technique by the use of probe ACA as well as by the combination of both probes Aci and ACA was 100% (97.5% CI = 87.7% – 100%). Moreover, simultaneous hybridization by probes Aci and ACA increased the fluorescent signal of *Acinetobacter* spp. cells to 3+ in 13 specimens. In conclusion, FISH, particularly using a combination of Aci and ACA, is a highly accurate method for the detection of *Acinetobacter* spp. in blood cultures. Furthermore, simultaneous hybridization by the both probes Aci and ACA can increase the fluorescent signal intensity of *Acinetobacter* spp. cells in some blood culture specimens and facilitate the detection of these microorganisms.

**Key words:** *Acinetobacter*, bacteremia, blood culture, FISH, simultaneous hybridization

### Introduction

*Acinetobacter* spp. are aerobic, oxidase negative, and nonfermentative Gram-negative bacteria that have been reported to cause various nosocomial infections such as bacteremia (Phillips, 2015; Endo *et al.*, 2014). *Acinetobacter* bloodstream infection is typically associated with intravascular devices (Phillips, 2015). The mortality rate of *Acinetobacter baumannii* bacteremia can be 40.2% (Gu *et al.*, 2016). Development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *A. baumannii* is an increasing concern in the healthcare sector. In an investigation on *A. baumannii* isolates from a referral hospital in Southern Iran, 53% and 44% of

isolates were identified as having MDR and XDR phenotypes, respectively (Alaei *et al.*, 2016). Use of appropriate antimicrobial drugs is thus crucial in the management of *Acinetobacter* spp. infections, particularly bacteremia. Detection of *Acinetobacter* spp. in blood culture specimens using conventional cultural and biochemical methods is time-consuming and requires at least two days, while, rapid detection of causative organism is essential for immediate selection of appropriate antibiotics and quick start of proper therapy of the patients. A rapid detection can improve prognosis and decrease the length of hospitalization (Peters *et al.*, 2006). Therefore, rapid detection of *Acinetobacter* spp. in blood cultures is required.

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Fluorescent *in situ* hybridization (FISH) using rRNA-targeted fluorescently labeled probes is a helpful rapid method that has been used for the identification of various microbes (Peters *et al.*, 2006; Tajbakhsh *et al.*, 2011; Poppert *et al.*, 2010; Tajbakhsh *et al.*, 2013b). Also, application of FISH using DNA probe for the rapid identification of *Acinetobacter* spp. from colony and blood culture specimens has been reported by Frickmann *et al.* (2011); in their study both the sensitivity and specificity of FISH were 100%. Although numerous reference strains and clinical isolates of *Acinetobacter* spp. and non-target organisms were tested *via* FISH by these authors, only seven *Acinetobacter*-positive blood culture specimens were found and investigated in their work (Frickmann *et al.*, 2011). However, for a more precise evaluation on the sensitivity of FISH for the detection of *Acinetobacter* spp. in blood cultures, further investigation using a higher number of *Acinetobacter*-positive blood culture specimens is required.

A probable limitation of FISH technique is the low signal intensity of some microbial cells that may make difficulties for the detection of microorganisms. A reason for the weak fluorescent signal is the low ribosome content found in some bacterial cells (Moter and Göbel, 2000; Zwirgmaier, 2005). Moreover, materials surrounding the bacteria in samples as well as blood cells such as erythrocytes and eosinophile granulocytes can exhibit a background fluorescence which may mask the specific fluorescent signal of microorganisms (Peters *et al.*, 2006; Moter and Göbel, 2000). One solution to enhance the specific fluorescent signal can be to use two or more specific probes labeled with the same fluorochrome and targeting different regions of the rRNA to increase the number of fluorescent molecules per microbial cell (Moter and Göbel, 2000; Zwirgmaier, 2005).

Our objectives in this study were (i) to evaluate the FISH for the detection of *Acinetobacter* spp. in blood culture specimens and (ii) to investigate the simultaneous application of two genus-specific probes labeled with the same fluorochrome in order to increase the fluorescent signal intensity and improve the detection of *Acinetobacter* spp. in these specimens.

## Experimental

### Materials and Methods

**Bacterial strains and cell fixation.** The American Type Culture Collection (ATCC) and the Persian Type Culture Collection (PTCC) reference strains, as well as other bacterial strains used in our investigation were *A. baumannii* (ATCC 19606 and three clinical isolates), *Acinetobacter calcoaceticus* (PTCC 1318), *Acinetobacter*

*haemolyticus* (two clinical isolates), *Acinetobacter* spp. (five clinical isolates), *Stenotrophomonas maltophilia* (ATCC 13637 and six clinical isolates), *Pseudomonas aeruginosa* (PTCC 1707), *Pseudomonas* sp. (environmental isolate), *Microbacterium (Flavobacterium) aborescens* (ATCC 4358), *Flavobacterium* spp. (three clinical isolates), *Neisseria meningitidis* (ATCC 13090), *N. meningitidis* (PTCC 1507), *Brucella abortus* (S19 and one clinical isolate), *Brucella melitensis* (ATCC 23456), *Shewanella* sp. (environmental isolate), *Aeromonas* sp. (clinical isolate), *Plesiomonas shigelloides* (clinical isolate), *Vibrio parahaemolyticus* (ATCC 17802), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) (ATCC 14028), *Salmonella enterica* subsp. *enterica* serovar Typhi (*Salmonella* Typhi) (PTCC 1609), *Escherichia coli* (ATCC 8739), *Yersinia enterocolitica* (PTCC 1477), *Serratia marcescens* (clinical isolate), *Enterobacter aerogenes* (clinical isolate), *Citrobacter diversus* (clinical isolate), *Providencia rettgeri* (clinical isolate), *Proteus penneri* (environmental isolate), and *Streptococcus pneumoniae* (ATCC 49619). These strains were used to check the specificity of probes.

The bacterial strains outlined above were grown, harvested while in the exponential growth phase, and fixed with 4% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) at 4°C for 1 h. The fixation protocol has been described elsewhere (Tajbakhsh *et al.*, 2008). All fixed bacterial strains were then examined *via* FISH, as explained below.

**Blood culture specimens.** This project was approved by the Ethical Committee of Bushehr University of Medical Sciences with reference number B-93-16-13. Between December 2014 and October 2015, a total of 320 positive blood culture specimens determined to contain Gram-negative bacteria or Gram-positive cocci by Gram staining, were collected from a major university hospital in the city of Bushehr, south west of Iran. The specimens were examined *via* conventional laboratory methods and FISH to detect genus *Acinetobacter*. Due to a tendency to retain crystal violet, *Acinetobacter* organisms may initially appear as Gram-positive cocci in direct smears made from blood culture specimens (Doughari *et al.*, 2011), and that is why the specimens containing Gram-positive cocci were also included in this study.

**Conventional laboratory methods.** An aliquot of each positive blood culture specimen was subcultured on blood agar (Merck, Darmstadt, Germany) and MacConkey agar (Merck, Darmstadt, Germany) plates and incubated for 24 h. Identification of the grown colonies was carried out by conventional laboratory methods such as Gram staining, culturing in triple sugar iron (TSI) agar (Merck, Darmstadt, Germany), oxidase, catalase, lysine decarboxylase, nitrate reduction, esculin

Table I  
Probes used for FISH.

Probe	Sequence (5'-3')	Fluorochrome	Target	Reference
Aci	TTA GGC CAG ATG GCT GCC	Cy3	<i>Acinetobacter</i> spp.	(Frickmann <i>et al.</i> , 2011)
ACA	ATC CTC TCC CAT ACT CTA	Cy3	<i>Acinetobacter</i> spp.	(Wagner <i>et al.</i> , 1994)
EUB338	GCT GCC TCC CGT AGG AGT	Fluo	Bacteria	(Amann <i>et al.</i> , 1990)

hydrolysis, indole, and motility tests (Doughari *et al.*, 2011), as well as DS-DIF-NONFERM (Yablonevaya, Nizhny Novgorod, Russia) or API 20 E (bioMérieux SA, Marcy-l'Etoile, France) kits. The DS-DIF-NONFERM was used for the identification of nonfermenters including *Acinetobacter* spp. The API 20 E was used for the identification of fermentative bacteria.

**FISH.** To fix the blood culture specimens, 200 µl of each specimen was mixed with 3 volumes of 4% paraformaldehyde and the next steps of the fixation procedure were performed as described previously (Tajbakhsh *et al.*, 2008).

The oligonucleotide probes used in the present study (Table I) were synthesized and 5'-labeled with fluorochromes Cy3 or Fluo (Metabion, Planegg/Steinkirchen, Germany). The probes Aci (Aci-16S 729) (Frickmann *et al.*, 2011) and ACA (Wagner *et al.*, 1994) that each targets a different position of the 16S rRNA of *Acinetobacter* spp., were used for the detection of the genus *Acinetobacter*. The 5' ends of the probes Aci and ACA were labeled with fluorochrome Cy3, which exhibits a red fluorescent signal. The probe EUB338, that targets and hybridizes a region of the 16S rRNA of almost all bacteria (Amann *et al.*, 1990), was 5'-labeled with fluorochrome Fluo, which emits a green signal. All of the control bacterial strains and blood culture samples were examined by FISH using three different mixtures of the probes on separate glass slides: (i) Aci-Cy3 and EUB338-Fluo, (ii) ACA-Cy3 and EUB338-Fluo, and (iii) Aci-Cy3, ACA-Cy3, and EUB338-Fluo.

The FISH procedure was performed as follows: 10 µl of each fixed control bacterial strain or each fixed blood culture sample were put on glass slides and air dried. For the dehydration, the slides were submerged for 3 min in each 50%, 80%, and absolute ethanol (Tajbakhsh *et al.*, 2008). In the hybridization step, specimens or bacterial strains were covered with 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% SDS, 30% formamide) containing a mixture of the probes. As mentioned above, each strain or blood culture specimen was tested separately with the three different mixtures of the probes: Aci and EUB338, ACA and EUB338, as well as Aci, ACA, and EUB338. The slides were then incubated at 46°C for 90 min in the moisture chambers for the hybridization. Subsequently, the slides were immersed into a washing buffer (20 mM Tris-HCl [pH 8], 0.01% SDS, 112 mM NaCl) and incu-

bated at 48°C for 15 min. DNA was then stained with 1 µg/ml 4', 6-diamidino-2'-phenylindole dihydrochloride (DAPI; Roche, Mannheim, Germany) for 5 min. Afterwards, the slides were rinsed with phosphate buffered saline, left to air-dry, and mounted with a fluorescent mounting medium (DAKO, Glostrup, Denmark) (Tajbakhsh *et al.*, 2011; Moosavian *et al.*, 2007). The slides were observed and analyzed with an epifluorescence microscope (Nikon 80i, Tokyo, Japan) equipped with a DS-5Mc-L1 digital camera system. Microscopy was performed in a blinded manner by two investigators. The tests were carried out twice. In this study, the positive results of FISH were categorized based on the fluorescent signal intensity as follows: 1+ (weak fluorescent signal), 2+ (moderate fluorescent signal), and 3+ (strong fluorescent signal).

**Analysis of assay.** The results of FISH were compared with the results of the conventional laboratory methods of identification. The sensitivity and specificity of FISH were calculated with the formulas  $(a/(a+c)) \times 100$  and  $(d/(b+d)) \times 100$ , respectively, where  $a$  = true positive,  $b$  = false positive,  $c$  = false negative, and  $d$  = true negative. Ninety five percent confidence interval (95% CI) was calculated using Exact Binomial method. If calculated sensitivity or specificity was 100%, one-sided 97.5% confidence interval was calculated using the same method. Statistical analyses were performed using StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.

## Results

A set of bacterial strains was used to check the specificity of the probes (Table II). The probe EUB338 hybridized all bacterial strains. However, both the probe Aci and ACA hybridized exclusively to *Acinetobacter* species but not to any of the negative controls, which indicates the high specificity of these probes (specificity 100% [97.5% CI = 89.1% – 100%]).

In this project, 320 positive blood culture specimens were tested by both conventional laboratory identification and FISH. By conventional culturing, *Acinetobacter* spp. were detected in 28 of 320 specimens. FISH using probe Aci detected *Acinetobacter* spp. in 27 of these 28 *Acinetobacter*-positive blood culture specimens, whereas FISH by the use of probe ACA, and also by

Table II  
Examination of bacterial strains *via* FISH.

Bacteria	Source	Results of hybridization with probe		
		EUB338	Aci	ACA
<i>A. baumannii</i>	ATCC 19606	+	+	+
<i>A. baumannii</i>	3 clinical isolates	+	+	+
<i>A. calcoaceticus</i>	PTCC 1318	+	+	+
<i>A. haemolyticus</i>	2 clinical isolates	+	+	+
<i>Acinetobacter</i> spp.	5 clinical isolates	+	+	+
<i>S. maltophilia</i>	ATCC 13637	+	-	-
<i>S. maltophilia</i>	6 clinical isolates	+	-	-
<i>P. aeruginosa</i>	PTCC 1707	+	-	-
<i>Pseudomonas</i> sp.	Seawater	+	-	-
<i>M. aborescens</i>	ATCC 4358	+	-	-
<i>Flavobacterium</i> spp.	3 clinical isolates	+	-	-
<i>N. meningitidis</i>	ATCC 13090	+	-	-
<i>N. meningitidis</i>	PTCC 1507	+	-	-
<i>B. abortus</i>	S19	+	-	-
<i>B. abortus</i>	clinical isolate	+	-	-
<i>B. melitensis</i>	ATCC 23456	+	-	-
<i>Shewanella</i> sp.	Seawater	+	-	-
<i>Aeromonas</i> sp.	clinical isolate	+	-	-
<i>P. shigelloides</i>	clinical isolate	+	-	-
<i>V. parahaemolyticus</i>	ATCC 17802	+	-	-
<i>S. Typhimurium</i>	ATCC 14028	+	-	-
<i>S. Typhi</i>	PTCC 1609	+	-	-
<i>E. coli</i>	ATCC 8739	+	-	-
<i>Y. enterocolitica</i>	PTCC 1477	+	-	-
<i>S. marcescens</i>	clinical isolate	+	-	-
<i>E. aerogenes</i>	clinical isolate	+	-	-
<i>C. diversus</i>	clinical isolate	+	-	-
<i>P. rettgeri</i>	clinical isolate	+	-	-
<i>P. penneri</i>	Seawater	+	-	-
<i>S. pneumoniae</i>	ATCC 49619	+	-	-

the mixture of both probes ACA and Aci, could detect *Acinetobacter* spp. in all of the mentioned 28 specimens. In other words, probe Aci, but not ACA, failed to detect *Acinetobacter* sp. in one specimen. The remaining 292 samples were negative for *Acinetobacter* spp. according to both the conventional identification and FISH. Therefore, based on the results of our study, the sensitivity of FISH for the detection of *Acinetobacter* spp. in blood culture specimens using the probe Aci was 96.4% (95% CI = 81.7% – 99.9%), whereas, the sensitivity of this technique by the use of probe ACA and by the mixture of both probes ACA and Aci was 100% (97.5% CI = 87.7% – 100%). The specificity of FISH for the detection of *Acinetobacter* spp. in blood cultures was 100% (97.5% CI = 98.7% – 100%).

Furthermore, in this study, we attempted to improve the specific fluorescent signal of *Acinetobacter* organ-

isms. The simultaneous hybridization by two genus-specific probes, Aci and ACA, labeled with the same fluorochrome (Cy3), increased the fluorescent signal intensity of *Acinetobacter* spp. cells from 1+ or 2+ to 3+ in 13 of 28 *Acinetobacter*-containing blood culture specimens and facilitated the observation and detection of these microorganisms (Fig. 1). No change in fluorescent signal intensity was observed in the remaining 15 *Acinetobacter*-containing blood culture specimens by application of the mixture of the probes Aci and ACA.

It should be noted that in three of the 28 *Acinetobacter*-containing blood cultures, *Acinetobacter* spp. initially appeared as Gram-positive cocci in direct smears prepared from the specimens. However, the organisms in these three specimens were successfully identified as *Acinetobacter* spp. *via* both FISH and further conventional biochemical identification.

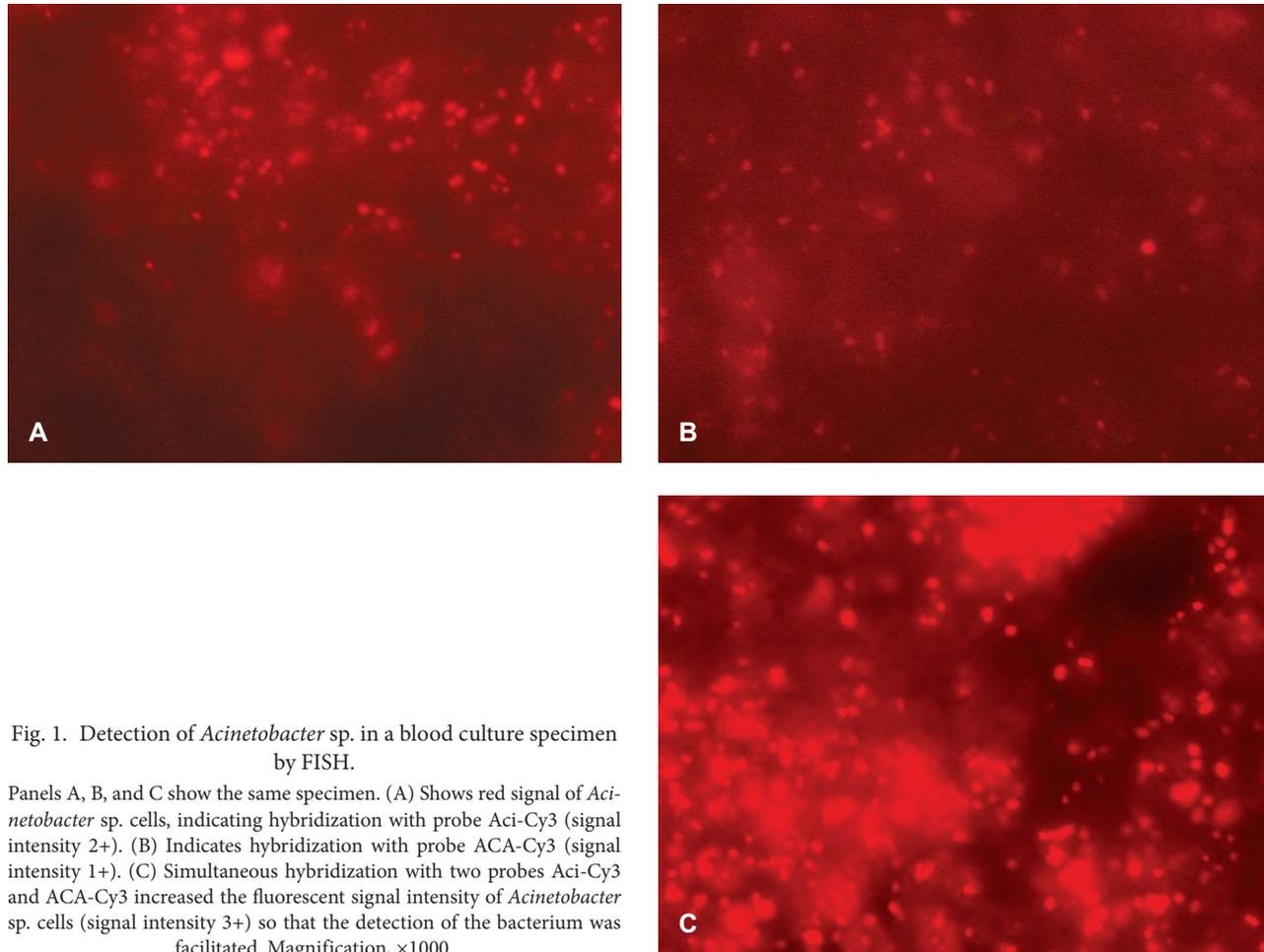


Fig. 1. Detection of *Acinetobacter* sp. in a blood culture specimen by FISH.

Panels A, B, and C show the same specimen. (A) Shows red signal of *Acinetobacter* sp. cells, indicating hybridization with probe Aci-Cy3 (signal intensity 2+). (B) Indicates hybridization with probe ACA-Cy3 (signal intensity 1+). (C) Simultaneous hybridization with two probes Aci-Cy3 and ACA-Cy3 increased the fluorescent signal intensity of *Acinetobacter* sp. cells (signal intensity 3+) so that the detection of the bacterium was facilitated. Magnification,  $\times 1000$ .

## Discussion

Reducing the time required for identification of microorganisms in positive blood cultures is important to enable satisfactory pathogen-based antibiotic therapy at an early phase and to improve outcome (Peters *et al.*, 2006; Frickmann *et al.*, 2011). In this paper, we have designed a study to evaluate the FISH technique and to use of a combination of two genus-specific probes to improve the FISH procedure for the detection of *Acinetobacter* spp. in blood culture samples.

In this study, the oligonucleotide probes Aci and ACA were used to identify genus *Acinetobacter*. In the study conducted by Frickmann *et al.* (2011), the probe Aci which was tested with many bacterial species, correctly identified all *Acinetobacter* spp. and excluded all non-target bacterial species, and therefore found to be highly specific. In the present study, we added more bacterial strains including *Flavobacterium* spp., *B. abortus*, *B. melitensis*, *N. meningitidis*, *Shewanella* sp., *Aeromonas* sp., *P. shigelloides*, *V. parahaemolyticus*, *P. rettgeri*, and *S. pneumoniae*, that may cause bacteremia and be present in blood cultures (Hall, 2015; Wellinghausen *et al.*, 2006; Carroll and Hobden, 2016; Hochedez *et al.*, 2010; Chen *et al.*, 2013; Cheng *et al.*, 2015; Choi *et al.*, 2015;

Tajbakhsh *et al.*, 2013a) and were not examined by Frickmann *et al.* (2011). These strains were not hybridized with probe Aci and thus we confirmed the high specificity of this probe for the detection of *Acinetobacter* spp. The following reasons help to explain why these strains were added in our study to check probe specificity. *Flavobacterium* spp. are isolated from a few blood culture samples in the city of Bushehr. Also, brucellosis is prevalent in our geographic area and blood is one of the specimens in which *Brucella* spp. are often found. Consequently, the correct negative results with these bacteria were important for us. The reason for examination on *N. meningitidis* was that *Acinetobacter* spp. resemble *Neisseria* spp. on conventional smears, so that *Acinetobacter* spp. recovered from bacteremic patients have been mistaken for *N. meningitidis* (Carroll and Hobden, 2016); however, Aci could successfully differentiate *Acinetobacter* spp. from this bacterium. Regarding examination on *V. parahaemolyticus*, *Aeromonas* sp., and *Shewanella* sp., it should be mentioned that Bushehr is a seaport with a vast coastal region and its people have much contact with microorganisms in marine water. Since *Vibrio* spp., *Aeromonas* spp., and *Shewanella* spp. are commonly found in aquatic environment such as marine water (Hochedez *et al.*, 2010; Janda and Abbott,

2014), it was needed to use of these bacteria as negative control for the probe. Also, because seafood is a natural reservoir of *P. shigelloides* (Chen *et al.*, 2013), and occupational exposure can be a source of bacteremia for fish handlers, we also decided to test the probe on this organism. Furthermore, although *S. pneumoniae* is a Gram-positive organism, it was used for the evaluation of probe specificity. We previously showed that the oligonucleotide probes can penetrate into the *S. pneumoniae* cells without enzymatic treatment, *i.e.*, the FISH procedure for this bacterium is similar to the procedure for Gram-negative organisms (Tajbakhsh *et al.*, 2013a). The probe Aci also produced a correct negative result with *S. pneumoniae* as mentioned above. It should be emphasized that the results of the examination of bacterial strains with probe ACA were same to the results of the probe Aci, and both probes were highly specific for the detection of *Acinetobacter* spp. ACA was developed by Wagner *et al.* (1994) and applied for *in situ* monitoring of *Acinetobacter* spp. in activated sludge. We used the probe ACA in the field of clinical microbiology.

Three hundred and twenty blood cultures were examined to evaluate the sensitivity and specificity of FISH for the detection of *Acinetobacter* spp. No false-positive results were observed and the specificity of FISH was 100%. By conventional identification, 28 specimens were positive for *Acinetobacter* spp., of which 27 specimens were FISH positive using probe Aci. Thus, the sensitivity of FISH in blood culture specimens by the use of probe Aci was 96.4%. Our results are close to the results of the investigation performed by Frickmann *et al.* (2011); in their work the sensitivity and specificity of FISH using probe Aci were 100%. In the present study, no false-negative results were observed by the use of probe ACA, as well as by the combination of probes ACA and Aci, and thus a 100% sensitivity was achieved. FISH is therefore a highly accurate method for the detection of *Acinetobacter* spp. in positive blood cultures. A benefit of the simultaneous application of probes Aci and ACA is that if one probe failed to identify *Acinetobacter*, the organism may be identified by the other one.

Wong *et al.* (2007) used DNA probe for the detection of *Acinetobacter* spp. from positive blood cultures. Although they did not state the number of blood culture specimens used for the evaluation of FISH, both sensitivity and specificity have been reported to be 100%. Our results are in accordance with the results of the investigation performed by Wong *et al.* (2007). Also, the potential of peptide nucleic acid (PNA) probe for the detection of *Acinetobacter* spp. from blood cultures has been shown (Peleg *et al.*, 2009). However, PNA probes are expensive.

There are reports concerning the other rapid methods for the detection of *Acinetobacter* spp. in positive

blood cultures. Rapid identification of *A. baumannii*, *A. nosocomialis*, and *A. pittii* with a multiplex PCR assay showed a sensitivity of 92.4% and specificity of 98.2%. False-positive results were observed in this method so that blood culture samples containing bacteria such as *Aeromonas hydrophila*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, or *Pseudomonas putida* were detected as *Acinetobacter*-positive by multiplex PCR. Moreover, false-negative results of multiplex PCR were reported, however, altogether it has been reported as a convenient assay (Chen *et al.*, 2014). Also, rapid and accurate identification of *A. baumannii* in positive blood cultures using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been shown (Bazzi *et al.*, 2017), but it is expensive and requires specific equipment.

In this investigation, the FISH assay was improved by the combined use of two probes Aci and ACA, so that the intensity of fluorescent signal of *Acinetobacter* spp. in 13 blood cultures was increased and detection of the organism was facilitated. For this procedure, careful selection of probe sequences should be considered in order that the probes target to independent sites in the rRNA molecule and also probe-probe interaction should not occur because cross-hybridization of probes to each other results in reduced signals (Lee *et al.*, 1993). The probes Aci and ACA have the mentioned characteristics, *i.e.*, they (i) target independent sites and (ii) do not bind to each other. Meanwhile, it should also be said that combination of the probes Aci and ACA did not show any adverse effect on the FISH results. All of these conditions support the idea of using the combination of Aci and ACA for the improving the fluorescent signal of *Acinetobacter* spp. cells. The organism in the remaining 15 *Acinetobacter* – containing specimens emitted a strong fluorescent signal by hybridization with each probe, and no change in signal intensity was observed in these 15 specimens by combination of Aci and ACA. The lack of a background fluorescence in these 15 specimens might be the reason for the exhibition of a strong specific fluorescent signal of *Acinetobacter* spp. cells, even with each probe alone. In other studies, application of probe combinations to increase the signal intensity of natural planktonic bacteria (Lee *et al.*, 1993), *Desulfobacter hydrogenophilus* (Amann *et al.*, 1990), and *P. aeruginosa* (Hogardt *et al.*, 2000) has been reported.

In our study, *Acinetobacter* organisms appeared as Gram-positive cocci in direct smear prepared from three blood cultures. This is an important point and may influence on antimicrobial management and lead to administration of inappropriate antibiotics, because blood culture Gram stain results are used to guide initiation of antimicrobial regimens (Munson *et al.*, 2003).

However, FISH correctly detected *Acinetobacter* spp. in these three specimens on the same day. Therefore, we strongly recommend the application of *Acinetobacter* probes for Gram-positive cocci observed in blood cultures besides for Gram-negative bacteria. Such blood cultures containing Gram-positive cocci were not examined in the previous studies (Frickmann *et al.*, 2011; Wong *et al.*, 2007).

In conclusion, FISH, particularly by the use of a combination of probes Aci and ACA, is a highly accurate technique for the detection of *Acinetobacter* spp. in positive blood cultures. A benefit of the simultaneous application of the probes Aci and ACA is that if one probe failed to identify *Acinetobacter*, there is still a possibility for the other one to identify the organism. Furthermore, simultaneous hybridization by the both probes Aci and ACA can increase the fluorescent signal intensity of *Acinetobacter* spp. cells at least in some blood culture specimens and facilitate the observation and detection of these microorganisms. FISH can also be a method of rapid identification when *Acinetobacter* organisms appear as Gram-positive cocci in direct smears from blood cultures.

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## Non-invasive Diagnostic of *Helicobacter pylori* in Stools by Nested-qPCR

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### Abstract

The aim of this study was to develop a non-invasive diagnostic test for the detection of *Helicobacter pylori* in stool samples from digestive symptomatic patients, using a new protocol of nested-qPCR. A total of 143 patients were invited to participate in the study. A gastric biopsy of each patient was collected for Rapid Urease Testing (RUT) and histology by Giemsa stain. A fecal sample for nested-qPCR analysis was also obtained. DNA was extracted from the fecal samples, and conventional PCR followed by qPCR of the ureC gene of *H. pylori* was carried out. We evaluated the presence of *H. pylori*, in 103 females and 40 males, mean ( $\pm$ SD) age of  $56.5 \pm 14.18$ . The sensitivity of RUT to detect the infection was 67.0% (95% C.I.: 57.2–75.8) and specificity was 92.3% (95% C.I.: 76.5–99.1). Histology by Giemsa stain, commonly used as a reference for *H. pylori* detection, showed a sensitivity of 98.6% (95% C.I.: 92.5–100.0) and a specificity of 89.7% (95% C.I.: 72.7–97.8). In contrast, detection of *H. pylori* infection in stools by nested-qPCR showed a sensitivity of 100% (95% C.I.: 94.9–100.0) and a specificity of 83.9% (95% C.I.: 66.3–94.6). Our test, based in nested-qPCR is a better diagnostic alternative than conventional RUT, and is similar to histology by Giemsa stain in the detection of *H. pylori*, by which the test could be used for non-invasive diagnosis in clinical practice.

**Key words:** *Helicobacter pylori*, molecular diagnostics, nested-qPCR, stools

### Introduction

*Helicobacter pylori* is responsible for gastritis and peptic ulcers; moreover, it is one of the most studied causal agents of gastric cancer (GC) in the last years (Misra *et al.*, 2014), for which in 1994 it was considered as group I carcinogen by the International Agency for Research on Cancer (IARC, 2012). Infection is frequent during childhood and sometimes induces superficial gastritis, which can progress to atrophic gastritis, intestinal metaplasia, dysplasia, and finally GC (Philippe *et al.*, 2016). The gram-negative bacterium adheres and colonizes the gastric mucosa, with the participation of several virulence factors, including cytotoxin-associated gene A antigen (CagA) and vacuolating cytotoxin (VacA), as well as: induced by contact with epithelium (IceA), blood group antigen-binding adhesion (BabA), sialic acid-binding adhesion (SabA), duodenal ulcer-promoting gene (DupA), and outer inflammatory protein (OipA) (Cadamuro, 2014).

In South America, and particularly in Chile, more than 70% of population is positive for *H. pylori* (Coelho and Coelho, 2014), a rate that has been significantly stable during the last 10 years. Different studies have shown a prevalence of infection ranging from 60% to 79%, according to socio-economic, educational and health conditions of the population studied (Ministerio de Salud, 2013). Chile has one of the highest rates of *H. pylori* infection in the world (Ferrecchio *et al.*, 2007; Porras *et al.*, 2014), making it necessary to develop a fast, reliable and non-invasive method to detect the pathogen, before the infected patient develops any gastric pathology, including cancer.

Currently there are two basic genres of tests to detect infection by *H. pylori*: invasive and non-invasive. Invasive tests including culture, histology and the rapid urease test (RUT) (Hunt *et al.*, 2011; Lee *et al.*, 2013; Ministerio de Salud, 2013) are inconvenient, costly, and uncomfortable because a patient is required to go to a hospital or clinic for an endoscopic gastric biopsy. Of

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the non-invasive tests, the Urea Breath Test ( $C^{13}$ -UBT) and the fecal antigen analysis stand out as the most valuable (Tamadon *et al.*, 2013).  $C^{13}$ -UBT is a fast and simple method that detects the presence of *H. pylori* in the gastric mucosa through urease activity of the pathogen (Di Rienzo *et al.*, 2013). Moreover, the test shows high sensitivity and specificity, with sensitivity between 81–100%, and specificity between 80–98% (Honar *et al.*, 2016), but the high initial investment of a isotope ratio mass spectrometer for obtaining results from  $C^{13}$ -UBT is not feasible in most public health centers in developing countries. Furthermore, the use of antisecretory drugs or antibiotics can influence the results of the test (Di Rienzo *et al.*, 2013).

Tests that detect *H. pylori* antigens in stool samples show high levels of specificity and sensitivity, similar to those for UBT (Dore *et al.*, 2016), and lately the costs have become more practical for the population. However, the accuracy of these tests decreases when the stools are aqueous because *H. pylori* antigens become diluted. These methods are also not recommended for patients with gastric ulcers (Shimoyama, 2013).

In this context, the aim of the present study was to show and evaluate the efficacy of a new non-invasive diagnostic method based on nested-qPCR, using *ureC* as a gene marker to detect *H. pylori* in stools samples, even in patients with gastric ulcers or watery stools.

## Experimental

### Materials and Methods

**Patients.** For this study 143 patients with digestive symptoms were considered: 103 females and 40 males, who were attended to by the Hospital San Pablo, Coquimbo, Chile for a routine gastrointestinal endoscopy. The mean ( $\pm$ SD) age of our patients was 56.5 years ( $\pm$  14.18). The inclusion criteria of the patients were as follows: adults over 18 years old, with digestive symptomatology, who had been tested for RUT and histology with Giemsa stain. The Bioethical Committee of the Health Service of Coquimbo, Chile, approved the protocol and patients voluntarily signed their consent.

A patent was requested for this protocol, with the N° 2016–01214 in INAPI (National Institute of Industrial Property, Chile).

**Endoscopy and biopsy samples.** The endoscopic procedure was performed in Hospital San Pablo, Coquimbo, Chile. Gastric biopsy samples were obtained from each patient for RUT and histology by Giemsa stain analyses, which were processed in the Service of Pathological Anatomy of the Hospital San Pablo according to standard protocols. The same pathologist performed the analysis of all biopsy samples.

**Stool samples.** Stools by normal evacuation were obtained from each patient before the endoscopic procedure. Each patient provided ~ 5 g of stools placed in a flask containing 3 ml of RNA Later® (Ambion), which were stored in a deep freezer ( $-80^{\circ}\text{C}$ ) until analysis.

**DNA purification and PCR amplification.** Approximately 200 mg of each stool sample was used to extract DNA, using QIAamp® Fast DNA Stool Mini Kit (QIAGEN) according to the manufacturer's protocol. Later, 120 ng of the extracted DNA was used to amplify the *H. pylori ureC* gene by nested-qPCR. DNA concentration was quantified by NanoDrop™ One (Thermo Scientific™). In brief, a first amplification with conventional PCR was performed in a Axygen® MaxyGene Thermal Cycler II, incubating 120 ng of DNA with 5  $\mu\text{l}$  Buffer 5x; 1.5  $\mu\text{l}$   $\text{MgCl}_2$  25 mM; 0.5  $\mu\text{l}$  dNTPs 10 mM; 1  $\mu\text{l}$  of each external primer (10  $\mu\text{M}$  each), and 0.2  $\mu\text{l}$  of Platinum Taq® DNA polymerase (5U/ $\mu\text{l}$ ) (Invitrogen), in a final volume of 25  $\mu\text{l}$ . Amplification conditions were as follows: a pre-denaturation of  $95^{\circ}\text{C}$  for 5 min, 25 cycles of  $95^{\circ}\text{C}$  for 45 s,  $57^{\circ}\text{C}$  for 45 s and  $72^{\circ}\text{C}$  for 45 s, followed by a final extension at  $72^{\circ}\text{C}$  for 10 min. Posteriorly, 2  $\mu\text{l}$  of a  $10\times$  dilution of this first PCR were used for subsequent amplification by qPCR in an Eco Real Time PCR (Illumina®). The qPCR mix contained 5  $\mu\text{l}$  of SYBR Green kit 2x (KAPA SYBR® FAST qPCR) and 0.1  $\mu\text{l}$  of each internal primer (10  $\mu\text{M}$  each), in a final volume of 10  $\mu\text{l}$ . Amplification conditions were: pre-denaturation at  $95^{\circ}\text{C}$  for 5 min, and 30 cycles of  $95^{\circ}\text{C}$  for 10 s and  $60^{\circ}\text{C}$  for 30 s. Sequences of primers are shown in Table I.

**Data analysis.** Data was analyzed using the Software Eco v4.1 PCR System and the program XLSTAT Version 2.06 to calculate sensitivity, specificity, and positive and negative ratio probability for each of the three techniques: nested-qPCR, RUT and histology by Giemsa stain for detection of *H. pylori* infection in symptomatic digestive patients. Cases for each technique were considered to be *H. pylori* infected according to a com-

Table I  
Primers used for qPCR assay.

	Sequence (5'-3') of primers	Amplicon size
External primers <i>ureC</i>	FExt: 5'-AGCTATAAAGTGGGCGAGAG-3' RExt: 5'-ATTGCACCCGTTAGGCTCAT-3'	224 bp
Internal primers <i>ureC</i>	FInt: 5'-GCGTTGGCAGTGCTAAAAGG-3' RInt: 5'-AGCCGTATCTAACACGATCC-3'	127 bp

bined gold standard of RUT/histology, RUT/qPCR or histology/qPCR, as applicable. *P* values < 0.05 were considered significant.

### Results

A total of 143 patients with digestive symptoms were evaluated by endoscopy, and the presence of *H. pylori* was evaluated by a novel method of nested-qPCR, and corroborated with RUT and histology by Giemsa stain. We use a combined gold standard for each test evalu-

ated. A patient was considered positive or negative for *H. pylori* when both of tests used as gold standard gave the same result for the infection. Results of the three tests are shown in Table II.

RUT, the standard method used in medical practice to detect the presence of this bacterium, only detected infection in 71/134 patients (53.0%), with a sensitivity of 67.0% (95% C.I.: 57.2 to 75.8) and a specificity of 92.3% (95% C.I.: 76.5% to 99.1); in turn, PPV was 97.3% (95% C.I.: 90.3 to 99.3) and NPV was 42.6% (95% C.I.: 35.7 to 49.8).

Table II  
Results of RUT, Histology and Real Time-PCR, for detect *H. pylori* in patients.

Gender	Diagnostic	RUT	Histology	qPCR UreC
Male	High antral lesion benign appearance	+	+	+
Female	Congestive and petechial gastropathy. Duodenal diverticulum	-	+	+
Female	Congestive gastropathy	-	-	+
Male	Petechial gastropathy, hiatal hernia	-	+	+
Male	Gastric ulcer, esophagitis b group, hiatal hernia	+	+	+
Female	Erosive gastropathy, erosive duodenitis, grade I esophagitis	+	+	+
Male	Barrett esophagus esophageal ulcer antral raised lesions	+	+	+
Male	Normal, hiatal hernia	-	+	+
Female	Normal	-	+	+
Female	Erosive gastropathy	+	+	+
Female	Congestive gastropathy	-	+	+
Female	Erosive gastropathy - Esophagitis group b of los angeles	+	+	+
Male	Normal	-	+	+
Female	Congestive gastropathy - Eosinophilic Esophagitis	+	+	+
Male	Normal	-	-	+
Male	Normal	-	+	+
Female	Erosive gastropathy antral multiple polyps fundus	-	+	+
Female	Obs. Celiac Disease	-	+	+
Male	Normal	-	-	-
Male	Antral erosive gastropathy	-	+	-
Female	Erosive Gastropathy	-	+	+
Female	Normal	-	-	-
Female	Normal	+	+	+
Female	Normal	+	+	+
Female	Normal	-	+	+
Female	Hiatal hernia, duodenopathy and congestive gastropathy	+	+	+
Female	Hiatal hernia, duodenopathy and congestive gastropathy	+	+	+
Female	Normal	+	+	+
Male	Hiatal hernia, duodenopathy and congestive gastropathy	+	+	+
Female	Without anormal findings	+	+	+
Female	Normal	+	+	+
Male	Normal	-	+	+
Female	Duodenal ulcer scar (Ulcerous Bulb)	-	+	+
Female	High Antral Lesion	+	+	+
Male	Petechial gastropathy	+	+	+
Female	Normal	+	+	+
Female	Hiatal hernia, erosive esophagitis servera, grade d of los angeles	-	-	-

Table II. Continued

Gender	Diagnostic	RUT	Histo- logy	qPCR UreC
Female	Normal	-	+	+
Female	Congestive gastropathy	+	+	+
Male	Congestive Duodenitis, Antral Erosive Gastritis	+	+	+
Female	Normal	-	-	-
Female	Ulcerous Bulb	-	+	+
Female	Antral Congestive Gastropathy, Nodular	+	+	+
Female	Antral Erosive Gastropathy, High Antral Lesion, Benign aspect	+	+	+
Male	Esophagitis Grade 1	+	+	+
Female	Chronic active gastritis, no evidence of malignant neoplasm is recognized	+	+	+
Male	Esophageal Candidiasis	+	+	+
Female	Congestive gastropathy	+	+	+
Female	Normal	+	+	+
Female	Atrophic Chronic Gastropathy	-	+	+
Female	Esophagitis Grade 1, Chronic Atrophic Gastritis	-	+	+
Female	Petechial gastropathy, Erosive Gastropathy.	+	+	+
Female	Gastritis Congestiva	-	+	+
Male	Normal	+	+	+
Male	Barrett's esophagus, antral ulcer on the anterior aspect of the minor curve	-	+	+
Female	Esophageal submucous lesion	-	-	-
Female	Gatritis congestive	-	-	-
Female	Normal	+	-	-
Female	Normal	+	+	+
Female	Normal	+	+	+
Female	Congestive and deformed bulb, micronodular gastropathy	+	+	+
Female	High Endoscopy Normal	+	-	+
Male	Endoscopy High Normal; Chronic non-specific gastritis	-	+	+
Female	Endoscopy High Normal; Chronic non-specific gastritis	-	+	+
Female	Gastropathy; Moderate non-specific esophagitis, Chronic atrophic gastritis	-	+	+
Female	Chronic gastritis with intestinal metaplasia, suggestive but not conclusive finding of Carcinoma	+	+	+
Female	Possible Congestive and Atrophic Arthropathy, Erosive Duodenopathy; Chronic atrophic gastritis with intestinal metaplasia and indeterminate atypia	-	+	+
Male	Congestive Gastropathy; Chronic Non-Specific Gastritis	-	+	+
Male	Polyps; Chronic Non-specific Gastritis, Tubular adenoma with moderate epithelial dysplasia	+	+	+
Male	Chronic gastropathy, hiatal hernia Esophagitis Moderate to severe erosion; Chronic atrophic gastritis with intestinal metaplasia.	-	-	-
Male	Antral Intestinal Metaplasia; Chronic atrophic gastritis with intestinal metaplasia, Chronic non-specific gastritis	-	+	+
Female	Control Antral Intestinal Metaplasia with important Regression; Chronic gastritis with intestinal metaplasia	+	+	+
Male	Chronic non-specific gastritis	-	-	-
Female	Congestive Duodenopathy; Chronic non-specific gastritis	-	+	+
Male	Moderate to severe erosive esophagitis, gastric ulcers; Chronic atrophic active gastritis, Intense esophagitis, Esophageal candidiasis	-	-	-
Female	Mild erosive esophagitis, erosive gastropathy and congestive duodenitis, increased duodenal papilla volume; Chronic active gastritis	+	+	+
Male	Erosive gastropathy, Antral intestinal metaplasia, Chronic gastritis with focal intestinal metaplasia	-	-	-
Male	Congestive and erosive gastropathy. Possible proliferative lesion (lymphoma or adenocarcinoma). Chronic active gastritis with intestinal metaplasia	+	+	+
Male	Intense gastropathy; Chronic gastritis with intestinal metaplasia and moderate dysplasia	+	+	+

Table II. Continued

Gender	Diagnostic	RUT	Histo- logy	qPCR UreC
Male	Antral intestinal metaplasia, gastric xanthoma, chronic gastritis with intestinal metaplasia	-	+	+
Female	Congestive and petechial gastropathy, sessile polyps; Chronic non-specific gastritis, hyperplastic type polyps	-	-	-
Male	Polyps; Chronic gastritis with intestinal metaplasia	-	+	+
Female	Chronic gastritis	-	-	+
Female	Mild erosive esophagitis; Fibrous polyps; Chronic gastritis with mild atrophy, mild intestinal metaplasia	-	-	-
Female	Active chronic gastritis with focal intestinal metaplasia	-	+	+
Female	Chronic atrophic gastropathy with intestinal metaplasia; Active chronic and atrophic gastritis	+	+	+
Female	Petechial gastropathy, Gastric scars; Active chronic gastritis	+	+	+
Male	Chronic non-specific gastritis	-	-	-
Female	Atrophic gastropathy, Gastric polyps Yamada I; Chronic gastritis	-	-	+
Female	Petechial gastropathy, antral erosions; Chronic gastritis	+	+	+
Male	Esophagitis Grade I; Chronic gastritis	-	-	-
Male	Hiatal hernia, antral congestive gastropathy, esophageal candidiasis; Chronic non-specific gastritis	-	+	-
Female	Gastric ulcer, Active chronic gastropathy with intestinal metaplasia	+	+	+
Female	Mild esophageal stricture; Chronic Gastritis with Intestinal Metaplasia	+	+	+
Female	Normal; Chronic gastritis	-	-	-
Male	Normal; Chronic gastritis Active mild with moderate atrophy	-	+	-
Female	Chronic gastropathy	-	+	+
Female	Chronic gastritis with moderate atrophy and extensive metaplasia, Chronic Active Gastritis with mild Intestinal metaplasia	+	+	+
Female	Gastric Polyps; Mild Chronic Gastritis with Moderate Atrophy and Focal Intestinal Metaplasia, Chronic Active Gastritis with Foveolar Hyperplasia	+	+	+
Female	Chronic gastropathy; Chronic mild gastritis	-	-	-
Male	Grade I esophagitis, chronic gastropathy, erosive duodenitis; Chronic non-specific active gastritis	+	-	-
Male	Chronic atrophic gastritis with mild intestinal metaplasia, moderately differentiated tubular adenocarcinoma	-	-	-
Female	Esophagitis type A of los angeles; Active Chronic Gastritis with mild atrophy, Active Chronic Gastritis with mild atrophy and focal intestinal metaplasia	+	+	+
Female	Atrophic gastropathy; Mild chronic gastritis with mild atrophy	-	-	+
Female	Antral Chronic Gastritis; Active Chronic Gastritis with mild atrophy and mild intestinal metaplasia	+	+	+
Female	Antral atrophic gastropathy, Hiatal hernia, Chronic gastritis with mild atrophy, on-specific chronic gastritis	-	-	-
Female	Ulcerous bulb; Mild chronic gastritis with mild atrophy	-	-	-
Female	Antral superficial gastropathy; Moderate atrophy and intestinal metaplasia, Chronic gastritis with mild inflammatory activity and mild atrophy	+	+	+
Female	Chronic gastropathy; Moderate chronic gastritis, Non-specific chronic gastritis, mild chronic gastritis	+	+	+
Female	Chronic gastropathy; Chronic gastritis with mild inflammatory activity, moderate intestinal metaplasia	-	-	-
Female	Nodular Gastropathy; Chronic active gastritis with moderate inflammatory activity and moderate atrophy.	+	+	+
Male	Antral Erosive Gastropathy; Moderate chronic gastritis with mild atrophy and focal metaplasia; Moderate atrophy and focal intestinal metaplasia	+	+	+
Female	Antral atrophic gastropathy; Moderate chronic gastritis with mild atrophy and moderate intestinal metaplasia	+	+	+
Female	Hiatal Hernia; Mild chronic gastritis, Chronic gastritis with mild inflammatory activity and mild atrophy	+	+	+

Table II. Continued

Gender	Diagnostic	RUT	Histo-logy	qPCR UreC
Female	Antral erosive gastropathy; Chronic gastritis with moderate inflammatory activity, mild intestinal metaplasia and mild atrophy	+	+	+
Female	Chronic atrophic lithiasis gastropathy; Mild chronic gastritis with mild atrophy, Chronic gastritis with moderate atrophy and extensive intestinal metaplasia	-	-	-
Female	Atrophic corpora-antral gastropathy; Active chronic gastritis with moderate atrophy	+	+	+
Female	Esophagitis type C Los angeles, Hiatal Hernia, Non-specific chronic gastritis	-	-	-
Female	Chronic gastritis with mild atrophy and focal intestinal metaplasia	-	+	+
Female	Chronic non-specific gastritis	-	+	+
Female	Nodular gastropathy; Chronic non-specific gastritis, Chronic gastritis with mild atrophy	-	-	-
Male	Chronic gastropathy; Chronic gastritis with mild activity, mild atrophy and focal intestinal metaplasia	+	+	+
Female	Hiatal Hernia; Chronic non-specific gastritis	-	-	-
Female	Petechial gastropathy; Active chronic gastritis with moderate atrophy	+	+	+
Female	Erosive Duodenitis, Duodenal ulcer scar; Chronic non-specific gastritis, Hyperplastic type polyps	+	+	+
Female	Duodenal polyps; Chronic non-specific gastritis	-	+	+
Female	Hiatal hernia, antral gastropathy, chronic gastritis with focal intestinal metaplasia	-	-	-
Female	Antral atrophic gastropathy, antral erosive gastropathy; Chronic non-specific gastritis, mild chronic gastritis	-	+	+
Female	Chronic gastritis with mild atrophy and mild intestinal metaplasia	-	+	+
Female	Petechial Gastropathy, Non-specific chronic gastritis	+	+	+
Female	Chronic gastropathy; Mild non-specific chronic gastritis	+	+	+
Female	Active chronic gastritis with moderate atrophy and moderate intestinal metaplasia; Chronic active gastritis with mild atrophy	+	+	+
Female	Nodular Gastropathy; Chronic active gastritis, chronic non-specific gastritis	+	+	+
Male	Antral superficial gastropathy; Chronic non-specific gastritis	+	+	+
Female	Intestinal metaplasia; Chronic gastritis with mild atrophy and mild intestinal metaplasia	+	+	+
Female	Antral Nodular Gastropathy; Active chronic gastritis with mild atrophy, Non-specific chronic gastritis	+	+	+
Female	Erosive duodenitis, antral superficial gastropathy; Active chronic gastritis with moderate atrophy	+	+	+
Female	Esophagitis type A Los Angeles, erosive gastropathy antral; Active chronic gastritis with mild atrophy	+	+	+
Female	Erosive gastropathy; Mild non-specific chronic gastritis	+	+	+
Female	Normal; Chronic non-specific gastritis	-	-	-
Female	Chronic gastropathy; Chronic active gastropathy with mild atrophy	+	+	+
Female	Chronic gastropathy; Chronic non-specific gastritis	+	+	+
Male	Chronic gastritis with mild atrophy and mild intestinal metaplasia	+	+	+

Histology with Giemsa stain classified 71/101 patients (70.3%) as *H. pylori* positive. Between these patients, 92 had a histologic diagnosis associated with gastrointestinal disease, and the remaining 9 tested normal for histology. Sensitivity for this test was 98.6% (95% C.I.: 92.5 to 100.0) and specificity 89.7% (95% C.I.: 72.7 to 97.8); PPV was 96.0% (95% C.I.: 89.0 to 98.6) and NPV was 96.3% (95% C.I.: 78.7 to 99.5).

Using a nested-qPCR approach, we identified infection of *H. pylori* in 71/102 patients (69.6%), with a sen-

sitivity to detect the presence of the bacterium in stools of 100% (95% C.I.: 94.9–100.0), and specificity of 83.9% (95% C.I.: 66.3 to 94.6); Finally, PPV and NPV were 93.4% (95% C.I.: 86.4 to 96.9) and 100.0% (95% C.I.: 84.0–100.0), respectively. A 2.5% agarose gel electrophoresis stained with ethidium bromide with the amplification of five positive samples for *H. pylori* is showed in Figure 1. Complete results are shown in Tables III–V. Comparative results between sensitivity, specificity, PPV and NPV for each test are shown in Table VI.

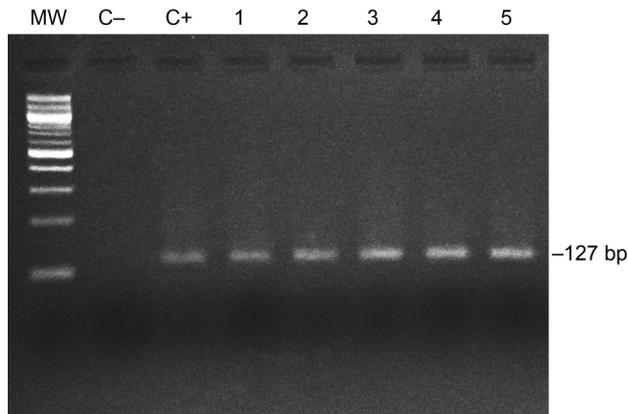


Fig. 1. 2.5% agarose gel electrophoresis stained with ethidium bromide, showing the amplification of five fecal samples positives for *H. pylori*.

C-: Negative control; C+: Positive control (*H. pylori* strain 26695); MW: Molecular weight 100 bp.

### Discussion

In this work, we evaluated a combined method of nested-qPCR for detection of infection by *H. pylori* in the stools of 143 gastrointestinal symptomatic patients, and demonstrated that this technique is superior to RUT, the invasive test commonly used in clinical practice today.

One of the pioneering works in this area was conducted in 1994, in which stools of 24 patients diagnosed with *H. pylori* infection were analyzed by PCR. Half of the patients had gastric ulcers at the time of

Table III  
Results of RUT vs Histology/qPCR.

	Disease Positive	Disease Negative
RUT +	71	2
RUT -	35	26

$p < 0.0001$

Table IV  
Results of histology-Giemsa vs RUT/qPCR.

	Disease Positive	Disease Negative
Histology +	71	3
Histology -	1	26

$p < 0.0001$

Table V  
Results of qPCR vs RUT/histology.

	Disease Positive	Disease Negative
qPCR +	71	5
qPCR -	0	26

$p < 0.0001$

endoscopy, while the other 12 had only related dyspepsia. Unfortunately, this study was unable to show that PCR technique is helpful to diagnose infection by the pathogen in the stools. Nonetheless, by inoculating each of the samples with  $10^3$  bacteria/mg of feces they received a positive result for all samples, a successful advancement in this technique (Mishra *et al.*, 2008). In 1998, a new protocol to detect *H. pylori* by PCR was tested on 100 patients (63 diagnosed with *H. pylori* and 37 healthy for the pathogen). This technique identified 59 infected patients (sensitivity 93.7%), while all uninfected patients tested negative by PCR (specificity 100%) (Aktepe *et al.*, 2011). Moving forward, several studies have shown that this technique may be a useful clinical alternative for *H. pylori* detection in stool samples. In this regard, a study from India in 2008 used nested-PCR of feces to demonstrate the prevalence of infection in the population of that country, finding that of 245 patients evaluated 105 were detected as positive for infection, using a new model for clinical evaluation (Momtaz *et al.*, 2012). Alas, this study did not corroborate their findings with currently accepted techniques, such as RUT or histology. However, this same group later showed that this technique has a high sensitivity, finding 40/52 patients positive for *H. pylori* according by RUT and biopsy, with a sensitivity of 72.5% (Smith *et al.*, 2012).

Another study applied the stool PCR test to 300 patients, 271 of them positive for *H. pylori* by RUT, finding 167/300 positive by PCR (61.6%), using the *ureC* gene as a marker (Uno *et al.*, 2016). Liu and coworkers (2016) evaluated stool samples of 97 digestive symptomatic patients by PCR, with the *ureC* marker and compared their results with UBT. In this work, sensitivity was 42.6% and specificity was 100% (Liu *et al.*, 2016). The authors claim that despite the observed low sensitivity, this technique could be useful for diagnosis in children, especially in health centers that do not have pediatric endoscopes.

In 2014 Patel *et al.* presented a review suggesting that PCR could be superior to other diagnostic tests for detection of *H. pylori* infection, owing to higher sensitivity and specificity, especially with nested and semi-nested approaches (Patel *et al.*, 2014).

In this work, we found that our nested-qPCR is more effective than RUT and similar to histology by

Table VI  
Comparative evaluation of RUT, histology and nested qPCR for detection of *H. pylori*.

Parameter	RUT	Histology	qPCR
Sensitivity (%)	67.0%	98.6%	100%
Specificity (%)	92.9%	89.7%	83.9%
PPV (%)	97.3%	96.0%	93.4%
NPV (%)	42.6%	96.3%	100%

Giemsa stain in detecting the presence of infection by *H. pylori* in the patients with digestive symptoms, with a sensitivity of 100% and a specificity of 83.9%. PPV and NPV values were 93.4% (95% C.I.: 86.4 to 96.9) and 100.0% (95% C.I.: 84.0–100.0), respectively.

It is important to note that specificity is close to 84%, as our technique detected five fecal samples as positive for *H. pylori*, which were not detected by RUT or histology. We repeated the test on the samples in question three more times with different portions of the fecal samples, and in all of them the result was positive for *H. pylori*. We hypothesized that our technique had the potential to detect the presence of small quantities of nucleotides from *H. pylori* beyond the limit of detection of the compared techniques.

Moreover, the nested-qPCR method is non-invasive and the patient needs only to send a stool sample to the laboratory, eliminating the need to go to the hospital. Currently RUT, with its low sensitivity, is the standard test in the medical practice. Indeed, in our patients the sensitivity of RUT was only of 67.0%, a result likely associated with ulcer bleeding or the use of proton pump inhibitors, which can give RUT a false negative (Coelho and Coelho, 2014). This is not an issue for PCR based diagnostics.

The proposal that blood could affect the sensitivity of RUT, by the presence of albumin acting as a buffer on the pH indicator of the reaction, is controversial, because other studies report that blood has no effect on the test (Honar *et al.*, 2016).

Our results are encouraging because this technique could soon become a non-invasive method for detection of *H. pylori* in stools, providing the population with an inexpensive and sensitive method to observe presence of the bacterium.

We are waiting for the approval of our patent request N° 2016-01214 for this protocol in INAPI.

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## Molecular Characterization of the *cry* Gene profile of *Bacillus thuringiensis* Isolated from a Caribbean Region of Colombia

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### Abstract

In order to characterize native strains of *Bacillus thuringiensis* of the Colombian Caribbean with toxic effect against insect vectors, 28 samples of bacteria identified as *B. thuringiensis* were isolated from different soils and muds around the city of Valledupar. Using a biological test, five isolates of *B. thuringiensis* showed toxic effect against larvae of *Aedes aegypti*. PCR methods were used to detect *cry1*, *cry2*, *cry4B*, *cry10* and *cyt1* genes. *Cry1* and *cry2* genes were detected in 35.7% and 32.1% of the 28 isolates analyzed, respectively. Surprisingly, reduced lengths of *cry4B* gene segments were detected in 28.6% of *B. thuringiensis* samples. The presence of *cry10* or *cyt1* was not detected in any of the 28 samples of *B. thuringiensis*, despite the high sensitivity of the assays used. The results show that *B. thuringiensis* samples from the Colombian Caribbean have atypical characteristics compared to those of Latin America and elsewhere in the world, which is consistent with the idea that the geographic origin of *B. thuringiensis* samples is associated with their biological and genetic characteristics.

**Key words:** *Bacillus thuringiensis* – Colombian strains, *cry* genes, *Aedes aegypti* larvae, PCR methods, biological test

### Introduction

Current methods for controlling insect vectors of different infectious diseases are based on the use of chemical insecticides (Hemingway *et al.*, 2004; Macoris *et al.*, 2007). However, excessive use of these chemicals has caused damage to ecosystems, environmental pollution, toxicity in humans and animals and development of resistance in insect vectors (Soderlund and Knipple, 2003; Grisales *et al.*, 2013). These side effects have led to the search for new and more harmless control methods including biological methods, in which the bacterium *Bacillus thuringiensis* represents a very promising alternative (Boyce *et al.*, 2013; Shingote *et al.*, 2013; WHO 2012; 2016; Ingabire *et al.*, 2017).

In Central and South America, several infectious diseases which affect humans, animals and plants are transmitted by insect vectors from all orders (López-

Pazos *et al.*, 2009; Hernández-Fernández 2011; Pérez *et al.*, 2016; WHO 2016; Camacho-Millán *et al.*, 2017). Among them are Diptera such as mosquitoes of *Anopheles* and *Aedes* genus involved in the transmission of malaria and dengue, respectively, which cause great impact in human health (WHO 2016; Soares-da-Silva *et al.*, 2017). Among the insect vectors that cause great pests in agriculture in Latin America, are Lepidoptera such as *Tuta absoluta* which produces great loss in tomato production (Salazar and Araya, 2001; Hernández-Fernández, 2011) and Coleoptera such as *Premnotrypes vorax* and *Anthonomus grandis* that cause devastating pests for potato and cotton crops, respectively (López-Pazos *et al.*, 2009; Pérez *et al.*, 2016). For these reasons it is very important in Latin America to seek more effective, sustainable and ecological methods to improve the campaigns to control and eradicate the various vectors of communicable diseases present

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in our countries (Prabakaran and Hoti, 2008; WHO 2016; Soares-da-Silva *et al.*, 2017). One of the possible alternatives for biological control of these vectors is *B. thuringiensis*.

*B. thuringiensis* (*Bt*) is a Gram-positive, aerobic and facultatively anaerobic bacterium that during the sporulation phase synthesizes a set of proteins that produce parasporal crystals with toxic activity against insects. These crystals consist of several proteins, including the Cry and Cyt protein families (Santos *et al.*, 2012; Bravo *et al.*, 2013). Using a nomenclature based on the phylogenetic relationships of the amino acid sequences deduced from coding genes, four orders of identity were established (Crickmore *et al.*, 1998). Currently more than 700 *cry* gene sequences have been identified, classified into at least 70 groups, named *cry1*, *cry2*, *cry3* ... *cry70*, whose corresponding insect toxicity is not as specific and simple as previously thought (Bravo *et al.*, 2013; Soares-da-Silva *et al.*, 2017).

Today it is relatively clear that both the intensity and specificity of the toxic effect against a particular insect order depends on the set of toxic proteins expressed by the bacterium. For example, larvicidal activity against Diptera larvae has been associated with proteins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1 and Cyt2 (Cantón *et al.*, 2011; Santo *et al.*, 2012). The classical example of a bacterium with this protein pattern is *B. thuringiensis* var. *israelensis*, used all over the world as commercial preparations against Diptera larvae (Cantón *et al.*, 2011; Santos *et al.*, 2012). However, in addition to this set of proteins, in some cases other complementary proteins such as Cry1 and Cry2, have been detected that enhance the toxic effect against Diptera (Ben-Dov *et al.*, 1997; Pinto *et al.*, 2012). Most of the genes encoding these  $\delta$ -endotoxins are localized in plasmids, such as the megaplasmid pBtoxis of 127,923 bp that is present in the *B. thuringiensis* var. *israelensis* strain (Berry *et al.*, 2002). This plasmid encodes the six proteins toxic to Diptera mentioned above (Berry *et al.*, 2002; Cantón *et al.*, 2011; Santos *et al.*, 2012). However, the Cry1 and Cry2 proteins are encoded by other plasmids (Porcar and Caballero, 2000).

Studies of *B. thuringiensis* strains isolated from Latin America strongly suggest that there is a heterogeneous geographic distribution of *cry* genes associated with toxicity against different insect orders (Bravo *et al.*, 1998; Ibarra *et al.*, 2003; Uribe *et al.*, 2003; López-Pazos *et al.*, 2009; Santos *et al.*, 2012; Pérez *et al.*, 2016; Camacho-Millán *et al.*, 2017; Soares-da-Silva *et al.*, 2017). Colombian strains of *B. thuringiensis* have been isolated mainly from the central region, e.g. Medellín (Segura *et al.*, 2000; Ruiz *et al.*, 2004) and few studies on *B. thuringiensis* samples from Caribbean regions of Colombia have been reported (Uribe *et al.*, 2003; López-Pazos *et al.*, 2009). It is very interesting to note that *B. thurin-*

*giensis* samples isolated from tropical forests contained different *cry* gene sets and showed distinct toxicity to insect orders compared to those isolated from agricultural soils. The tropical forest samples showed *cry1*, *cry3* and *cry7* genes and were toxic to coleopteran species, while the samples from agricultural soils only had *cry1* genes and were toxic to Lepidoptera (Uribe *et al.*, 2003).

Based on the literature reports mentioned above that suggest association between geographic origin and *cry* gene variants of *B. thuringiensis*, the aim of the present study was to isolate and characterize new *B. thuringiensis* strains toxic against dipterans of importance in human health from a tropical region not previously studied.

## Experimental

### Materials and Methods

**Strains.** Twenty-eight isolates of *B. thuringiensis* were obtained from urban soil samples and mud from the water treatment plants of the city of Valledupar and stored in the laboratory of Parasitología Agroecología Milenio of the Universidad Popular del Cesar, Colombia (Table S1 in Supplementary Material). Two commercial strains of *B. thuringiensis* were used as positive controls in the toxicity tests; Turilav<sup>®</sup>, produced by Laverlam S.A., which is the strain *B. thuringiensis* var. *kurstaki*, and VectoBac<sup>®</sup> WDG, produced by Valent Biosciences Corporation, which is *B. thuringiensis* var. *israelensis*.

**Production of spores and crystals.** Isolates of *B. thuringiensis* containing spores and crystals conserved in filter paper were cultured as described (Santos *et al.*, 2012). Briefly, these samples were submerged in 2 ml of nutrient broth: 0.1% (w/v) meat extract, 0.2% (w/v) yeast extract, 0.5% (w/v) bacteriological peptone and 0.5% (w/v) NaCl, incubated at 28°C for 24 h. Then, 200 ml of culture medium N° 1 were added and the solution was incubated at 28°C for 96 h with gentle agitation. The suspensions of each isolate were washed 3 times with 1.5 M NaCl and 3 times with distilled water and centrifuged at 10000 g for 10 min; the spore-crystal complex was stored at -20°C.

**Bioassays.** The toxic effect of the isolates of *B. thuringiensis* on larvae of *A. aegypti* was determined following the methodology recommended by the World Health Organization (WHO 2005). Twenty five third or fourth instar larvae of the reference strain (Rockefeller) were transferred to transparent watch glasses with 150 ml capacity (Santos *et al.*, 2012). Determinations were performed in triplicate (Table S2 in supplementary material). The positive controls Turilav<sup>®</sup> and VectoBac<sup>®</sup> WDG were included in each assay. As negative control 100 larvae were placed in distilled water. Mortality was recorded after 48 h. Pupae were omitted in the analyses.

If the negative control had mortality > 20% the assay was eliminated. Bioassays were performed in laboratory conditions at a temperature of 28°C and relative humidity of 65%.

**Detection and sequencing of *cry* genes.** The *cry1* and *cry2* genes were analyzed based on the methods described by Ben-Dov *et al.* (1997), using the primers Un1(d) and Un1(r) for *cry1* and Un2(d) and Un(r) for *cry2*. The PCR conditions used for amplification of *cry1* were: DNA denaturation for 3 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension at 72°C for 10 min. Amplification of the *cry2* gene was similar to the *cry1* gene, only changing the number of cycles and annealing temperature to 40 cycles and 51°C, respectively. The primers and conditions used to detect *cry4B*, *cry10* and *cyt1* were based on Santos *et al.* (2012). The PCR protocol to amplify these genes was: a DNA denaturation step of 3 min at 94°C; 35 cycles for 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and a final extension of 10 min at 72°C.

DNA amplification was performed in a TECHNE TC 412 thermocycler. All reactions were performed in a volume of 20 µl. PCR products were visualized in 2.5% agarose gels with TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3), also including a 100 pb (base pair) DNA ladder (Invitrogen, Brazil).

Positive controls for *cry1*, *cry4B*, *cry10* and *cyt1* assays were performed using a DNA sample of *B. thuringiensis* var. *israelensis* generously donated by Mario Soberón, of the Instituto de Biotecnología, Universidad Nacional Autónoma de México.

PCR products of the *cry1*, *cry4B* and *cry11* segments were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and then sent for sequencing to the DNA Core Sequencing Facility, University of Illinois, EEUU, with the corresponding primers. The GenBank accession codes for the sequences

Bt1-*cry1* and Bt3-*cry1* are MG271933 and MG271934, respectively.

**Analysis of DNA sequences.** Colombian *B. thuringiensis cry1* gene segments were aligned with *B. thuringiensis cry1A* gene for the insecticidal crystal protein segment located between 2780 and 3080 bp (GenBank accession code D17518.1). This information is available in the database <http://www.ncbi.nlm.nih.gov/>. Alignments were made with the CLUSTALX program and edition of the sequences was performed with the Bioedit software (Hall, 1999). The Colombian *cry4B* segment, corresponding to Bt-UPC-22 (Bt22), was aligned with the *B. thuringiensis* serovar *israelensis cry4B* gene coding sequence (*cry4B*, GenBank accession: D00247.1).

**Bioassays.** The highest mortality (27–30%) at the concentration 0.1 mg/l was caused by *B. thuringiensis* isolates Bt-UPC-15, Bt-UPC-20 and Bt-UPC-25 (Table S2, Supplementary Material). Two other isolates, Bt-UPC-5 and Bt-UPC-6, caused 17% and 23% mortality, respectively, at the highest concentration tested (10 mg/l). These five isolates with toxic activity came from urban soils (Table SI, Supplementary Material).

## Results

**Detection of *cry1* gene segments in three Colombian *B. thuringiensis* samples.** The analysis of various isolates of *B. thuringiensis* to detect the *cry1* gene showed a single PCR amplification product in the expected range of 274–277 bp (Fig. 1, Bt-UPC samples 1, 3, 8, 16 and 17). The *cry1* was detected in 35.7% of the Bt-UPC samples analyzed (Table SII, Supplementary Material). However, the gene was not detected in any of the Bt-UPC samples that were toxic to mosquito larvae.

Two *cry1* gene segments from DNA samples Bt-UPC-1 and Bt-UPC-3 were sequenced (Fig. 2).

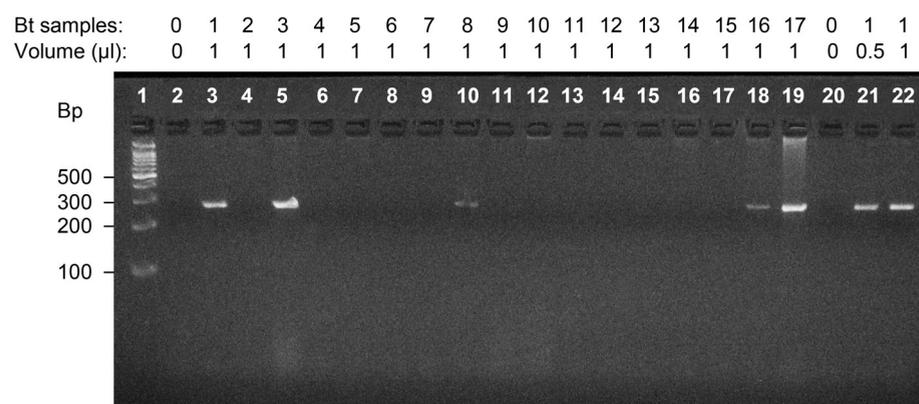


Fig. 1. Detection of the *cry1* gene in *Bacillus thuringiensis* DNA samples from a Caribbean region of Colombia.

Electrophoresis in 2.5% agarose gels, showing PCR amplification products for the *cry1* gene using the primers and protocols described by Ben-Dov *et al.* (1997). Molecular marker: 100 base pair (bp) DNA ladder (lane 1), without sample (lanes 2, 20). Lanes 3–19: *B. thuringiensis* samples (Bt-UPC-1 to Bt-UPC-17). Lanes 21, 22: different concentrations of Bt-UPC-1. The Bt DNA samples and the volume analyzed are indicated above the figure. Expected size 274–277 bp (See Material and Methods for more details).

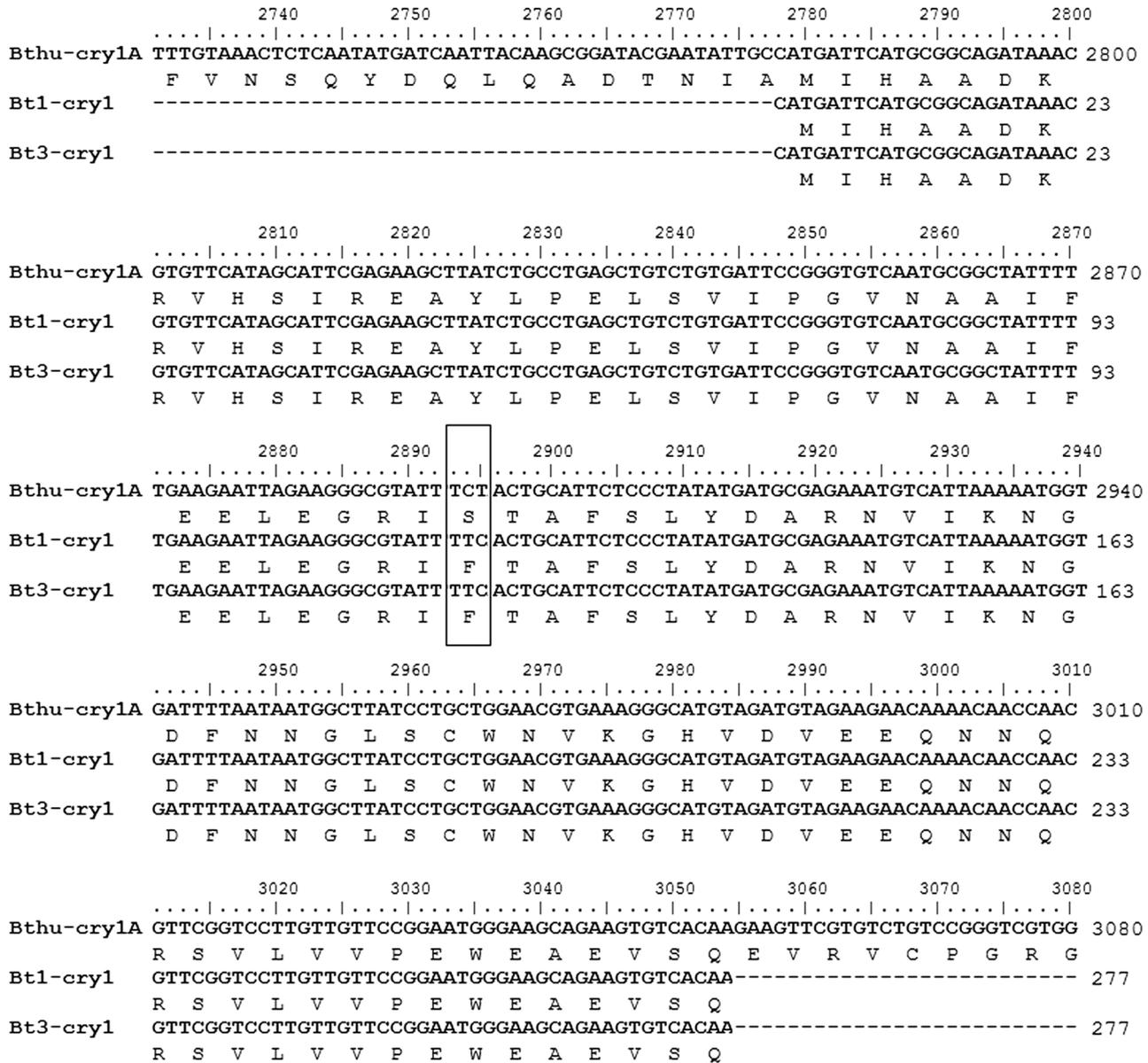


Fig. 2. Alignment of a *cry1* protein coding gene segment of two DNA samples from a Caribbean region of Colombia.

The corresponding deduced amino acid sequence is given below each nucleotide sequence. The first sequence corresponds to a segment of an open reading frame of the *B. thuringiensis* var. *kurstaki* HD-1 *cry1A* gene, from base pair 2731 to 3080 (Bthu-cry1A, Genbank code D17518.1). The second and third sequences are PCR products of *cry1* amplified from Colombian *B. thuringiensis* Bt-UPC-1 (Bt1-cry1, GenBank code MG271933) and Bt-UPC-3 (Bt3-cry1, GenBank code MG271934) DNA samples, respectively. The box indicates the two single nucleotide polymorphisms (SNP) between sequences, and the corresponding changes of Serine 965 to Phenylalanine in Bt sequences.

Importantly, the Bt1-cry1 and Bt3-cry1 sequences (GenBank accession numbers MG271933 and MG271934, respectively) are identical, and differ from the *cry1A* gene (GenBank accession number D17518.1) only in nucleotides 2894 and 2895 (indicated by a box into the figure). Interestingly, these nucleotide changes also produce a change in the aa (amino acid) encoded by the codon, from aa serine 965 to phenylalanine (S965F). The localization of the segment amplified by PCR with respect to the full length Cry1 amino acid sequence is shown in Fig. S1 in the Supplementary Material.

**Detection of a *cry2* gene segment in Colombian *B. thuringiensis* samples.** Using the method described

by Ben-Dov *et al.* (1997) we detected the described segment of about of 700 bp in 32.1% of Colombian *B. thuringiensis* samples (Table SII, Supplementary Material). For instance, in Figure 3 there is clearly a single band of 700 bp in Bt samples 8, 17 and 18, and a very faint band is also observed in Bt sample 16. Interestingly, in Bt samples 8, 16, 17, 18, 26 and 28 both *cry1* and *cry2* were detected (Table SII, Supplementary Material). However, in Bt samples 22, 23 and 27 the *cry2* segments were accompanied by *cry4B* gene segments (Table SII, Supplementary Material).

**Detection of *cry4B* gene segments in Colombian *B. thuringiensis* samples.** The detection of *cry4B*

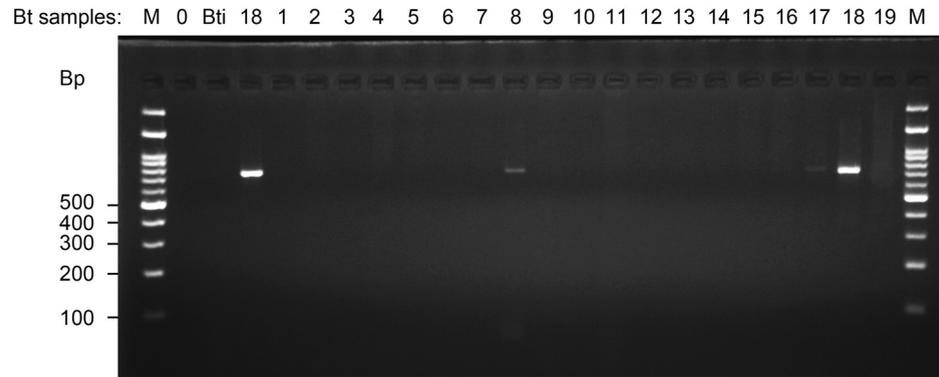


Fig. 3. Detection of the *cry2* gene in *Bacillus thuringiensis* DNA samples from a Caribbean region of Colombia.

Electrophoresis in 2.5% agarose gels, showing PCR amplification products for the *cry2* gene using the primers and protocols described by Ben-Dov *et al.* (1997). Molecular marker: 100 base pair (bp) DNA ladder (lane M), without sample (lane 0). DNA of *B. thuringiensis* var. *israelensis* (Bti), 0.34  $\mu$ g. Colombian *B. thuringiensis* samples Bt-UPC-18, Bt-UPC-1 to 19 (lanes 18, 1–19, respectively). The volume analyzed for each sample was 1  $\mu$ l. Expected size about 700 bp (See Material and Methods for more details).

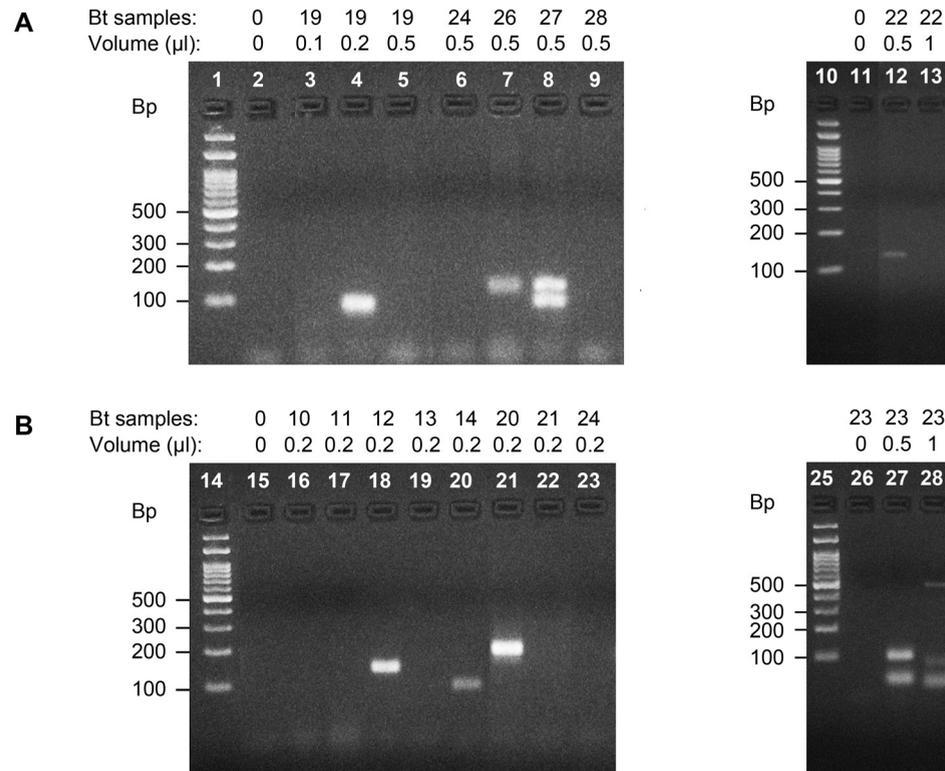


Fig. 4. Detection of the *cry4B* gene in different volumes of *Bacillus thuringiensis* DNA samples from a Caribbean region of Colombia.

The four electrophoreses were performed in 2.5% agarose gels, showing PCR amplification products for the *cry4B* gene using the primers and protocols described by Santos *et al.* (2012). Molecular marker: 100 base pair (bp) DNA ladder (lanes 1, 10, 14 and 25), without sample (lanes 2, 11, 15 and 26). Colombian *B. thuringiensis* samples (lanes 3–9, 12, 13, 16–23 and 27, 28, respectively). The corresponding Bt-UPC DNA samples and the volume analyzed are given above the figure.

was not easy, because it was necessary to try different volumes of each sample to observe any amplification product (Fig. 4). Thus in most Bt-UPC samples it was possible to obtain a PCR amplification product of 100 bp only with certain volumes (Lanes 4, 20 and 27), 150 bp (Lanes 7, 12 and 18) or 200 bp (Lane 21). At high DNA concentration in the PCR assay, a larger PCR product of about 500 bp was detected in some samples (Lane 28, Bt23 sample). To determine whether these

small length products really encoded segments of the *cry4B* gene, several attempts were made to obtain sufficient quantity of DNA of different-sized fragments to be sequenced. It was possible to obtain enough DNA for sequencing only from the Bt-UPC-22 sample. This PCR product of 150 bp was partially sequenced in the inverse sense; as shown (Fig. 5), a 97 bp segment was sequenced which aligned with segment 1707–1817 of the *cry4B* gene from *B. thuringiensis* serovar *israelensis* (accession

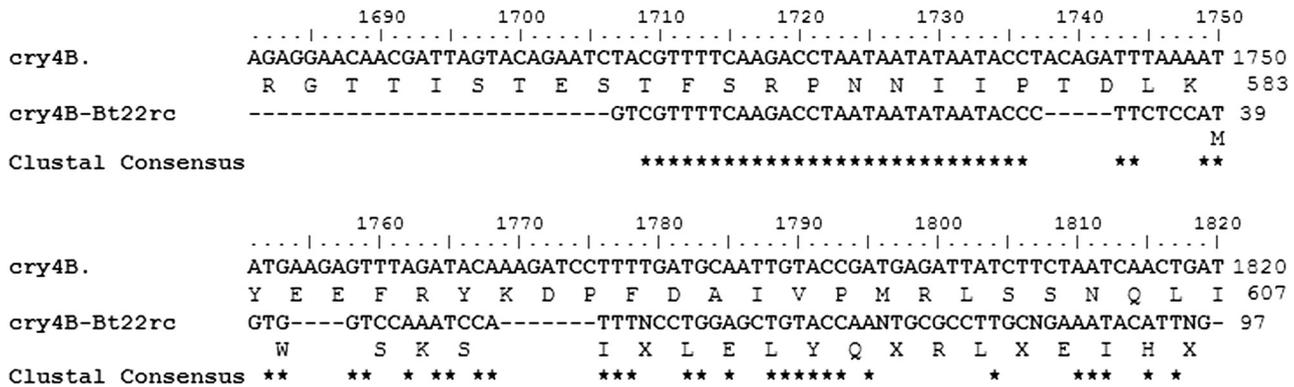


Fig. 5. Alignment of the *cry4B* segments from the Colombian *B. thuringiensis* Bt – UPC – 22 sample and from the *B. thuringiensis* serovar *israelensis* bacterium.

These nucleotide sequences correspond to a segment of 140 bp from *B. thuringiensis* serovar *israelensis* *cry4B* gene coding sequence (*cry4B*, GenBank code: D00247.1) and the 97 bp nucleotide segment sequenced from Colombian *B. thuringiensis* DNA sample Bt22 (*cry4B*-Bt22rc). Below each nucleotide sequence is the corresponding deduced amino acid segment.

code D00247.1). Three gaps and multiple nucleotide differences are shown between the sequences, corresponding to an identity of 50%. The localization of this *cry4B* segment amplified by PCR with respect to the full length *Cry4B* amino acid sequence is shown in Figure S1 (Supplementary Material).

***Cry10* and *cyt1* genes were not detected in any samples of the Colombian Caribbean.** Several attempts were made using diverse dilutions and PCR conditions to detect segments of genes *cry10* and *cyt1*, as shown in Figure S2, in which both assays clearly showed the expected fragments of 348 and 480 bp described for these gene segments, respectively, that were detected in the positive control with samples of *B. thuringiensis* var. *israelensis* (panels A and B, lanes 3–5). It must be mentioned that a very small amount of these *Bt* genes can be detected in the assay, even at dilutions higher than 1/1000, corresponding to a concentration lower than 17 ng/ $\mu$ l.

## Discussion

*B. thuringiensis* has been isolated from different ecosystems; the soil is one of the habitats in which it is most widely distributed (Arango *et al.*, 2002; Jara *et al.*, 2006; Pérez *et al.*, 2016; Camacho-Millán *et al.*, 2017; Soares-da-Silva *et al.*, 2017). This fact, coupled with the wide variety of climatic regions and high diversity of insects in Colombia, provides opportunities to isolate new strains with toxic activity against Diptera or other insect orders (Ibarra *et al.*, 2003). However, in spite of the importance of dipterans as etiological agents of infectious diseases, few studies have reported the molecular characterization of collections of *B. thuringiensis* in relation to the content of *cry* and *cyt* genes lethal to species of this order in Colombia (Segura *et al.*, 2000; Ibarra *et al.*, 2003; Ruiz *et al.*, 2004).

The toxicity of *B. thuringiensis* isolates against insect larvae is a complex phenomenon which depends upon several factors (Martínez and Caballero, 2002; Santos *et al.*, 2012; Soares-da-Silva *et al.*, 2017). These can be classified into bacterial, larval and environmental factors. Bacterial factors include the concentration of spores and crystals, presence and expression of toxic genes against insects (Martínez and Caballero, 2002; Santos *et al.*, 2012). Larval factors include the genus, species, strain and geographic origin of larvae, as well as the presence in their midgut of receptors which interact with the different toxic proteins expressed by the bacterium (Martínez and Caballero, 2002; Santos *et al.*, 2012; Soares-da-Silva *et al.*, 2017). Environmental factors include the temperature and pH of the water in which the larvae are infected with bacteria (Arunachalam *et al.*, 2010; Santos *et al.*, 2012). Thus it has been demonstrated that more acidic water yields higher toxicity of *B. thuringiensis* isolates against larvae (Martínez and Caballero, 2002; Santos *et al.*, 2012; Soares-da-Silva *et al.*, 2017). These authors suggest that one possible mechanism could be the stimulation of the ingestion of the bacteria by larvae. It appears that there is a range of optimal temperatures for high metabolism and ingestion of bacteria by larvae which is from 20–30°C (Arunachalam *et al.*, 2010), but this range could change depending on the strain or origin of the larvae (Santos *et al.*, 2012). In our study anti-larval activity was detected in 5 of the 28 *B. thuringiensis* isolates (17.9%), with mortality that ranged from 17% to 30% (Table SII, Supplementary Material). Considering the complex phenomenon of toxicity against larvae produced by toxins expressed by *B. thuringiensis*, we cannot rule out environmental factors in the low toxic effect observed in the present study.

*Cry1* and *cry2* genes have been associated mainly with toxic effects against Lepidoptera (Morse *et al.*, 2001; Zhao *et al.*, 2005; Mandal *et al.*, 2007; Camacho-

Millán *et al.*, 2017), but in association with other *cry* genes toxic effects have also been reported against Dipteran larvae (Ben-Dov *et al.*, 1997). In order to know whether *cry1* and *cry2* genes might be involved in toxic effects against *Aedes aegypti* larvae detected in Colombian Bt samples, the presence of these genes was investigated (Fig. 1–3). As mentioned above, the presence of *cry1* and *cry2* genes was not detected in any of the Bt-UPC samples that were toxic to mosquito larvae (Table SII). This result is concordant with results previously reported in Bt samples from Latin America, showing that *cry1* and *cry2* genes both together and separately are associated with toxicity against Lepidoptera and Coleoptera but not against larvae of Diptera (Uribe *et al.*, 2003; Lopez-Pazos *et al.*, 2009; Santos *et al.*, 2012; Camacho-Millán *et al.*, 2017; Pérez *et al.*, 2016).

It is noteworthy that the sequenced *cry1* gene segments of the two Colombian *B. thuringiensis* samples were almost identical to the respective *cry1A* homolog of the subspecies named *Bt kurstaki* HD-1 isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago) (Kondo *et al.*, 1987), differing from the database sequence only in two nucleotides which caused a non-silent mutation from serine to phenylalanine (Fig. 2).

Interestingly, detection of the *cry4B* gene was very difficult; in almost all samples we were only able to detect segments much shorter than expected. Segments of 100, 150 and 200 bp were found instead of 321 bp segments (Fig. 4). In some samples two fragments were detected. In order to confirm that at least some of these fragments indeed correspond to segments of the *cry4B* gene, we tried to sequence them. It was only possible to sequence a PCR product of 150 bp from the Bt22 sample, obtaining a sequence of 97 nucleotides (Fig. 5). This segment corresponds to the delta-endotoxin C region of the respective Cry4B protein. Our results show that indeed, at least the 150 bp PCR fragment corresponds to a segment of the *cry4B* gene (Fig. 5). Further studies will be necessary to determine the total length of these genes in these Colombian samples and confirm whether the other fragments also are *cry4B* gene segments.

Based on the reduced length of *cry4B* gene segments detected in our samples, one explanation for the null or low larvicidal effect against *A. aegypti* larvae could be that these *cry4B* segments are pseudogenes. Confirming or refuting this hypothesis will require further study.

### Conclusions

*B. thuringiensis* samples from the Colombian Caribbean area show atypical characteristics, which is consistent with the idea that the geographic origin of *B. thuringiensis* samples is associated with their biological and genetic characteristics.

Five of the twenty-eight isolates of *B. thuringiensis* showed a lethal effect against larvae of *A. aegypti*, the mosquito that transmits serious diseases such as dengue. *Cry1* and *cry2* genes were detected in 35.7% and 32.1% of the samples, respectively, but in none of the five Bt samples that were toxic against mosquito larvae.

Surprisingly, a reduced length of *cry4B* gene segments was detected in 28.6% of Bt samples, 100–200 bp instead of the expected 321 bp; neither of these segments was detected in the five Bt Colombian samples toxic to *A. aegypti* larvae. *Cry10* and *cyt1* were not detected in these Colombian *B. thuringiensis* samples.

Further studies will be required in order to identify other genes involved in the larvicidal effect observed in these Bt samples.

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## High-Throughput Sequencing Analysis of Endophytic Bacteria Diversity in Fruits of White and Red Pitayas from Three Different Origins

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### Abstract

Pitaya contains various types of polyphenols, flavonoid and vitamins which are beneficial for health and it is among the most important commercial tropical fruits worldwide. Endophytic bacteria might be beneficial for plant growth and yield. However, bacterial diversity in pitaya is poorly characterized. In this study, fruits of white and red pitayas from three different origins (Thailand, Vietnam and China) were chosen for endophytic bacteria diversity investigation by using Illumina HiSeq second-generation high-throughput sequencing technology. Large number of endophytic bacteria were detected and 22 phyla, 56 classes, 81 orders, 122 families and 159 genera were identified. Endophytic bacteria diversity was uneven among pitaya fruits from different origins and bacteria structure was different between white pitaya group and red pitaya group. Phylum Bacteroidetes, classes Bacteroidia and Coriobacteriia, orders Bacteroidales and Coriobacteriales, families Prevotellaceae, Bacteroidaceae, Ruminococcaceae, Paraprevotellaceae, Rikenellaceae, Alcaligenaceae and Coriobacteriaceae, genera *Prevotella*, *Bacteroides*, *Roseburia*, *Faecalibacterium* and *Sutterella* were statistically significant different species ( $P < 0.05$ ) between white and red pitayas. These findings might be useful for growth improvement, fruit preservation and processing of different pitaya species from different origins.

**Key words:** endophytic bacteria diversity, high-throughput sequencing, pitaya from three different origins

### Introduction

Pitayas (dragon fruits) are originally native to Latin America and the West Indies and are cultivated in tropical and subtropical regions all over the world. Pitaya belong to the genus *Hylocereus*, three major species of pitaya are *Hylocereus undatus* (white pitaya), *Hylocereus polyrhizus* (red pitaya) and *Hylocereus megalanthus* (yellow pitaya). The white and red pitayas are the most widely cultivated because of their economic values and health benefits (Ortiz and Takahashi, 2015; Suh *et al.*, 2014). Both white and red pitayas contain various types of polyphenols, flavonoid and vitamins which are beneficial for health (Esquivel *et al.*, 2007). The antidiabetic effect of white and red pitayas has been recently demonstrated. It is reported that the consumption of white pitaya attenuates insulin resistance and hepatic steatosis in diet-induced obese mice. Consumption of red pitaya could decrease total cholesterol, triglyceride and low-density lipoprotein cholesterol levels and increase the high-density lipoprotein cholesterol levels in type 2 dia-

betic subjects (Song *et al.*, 2016). In addition, red pitaya may serve as a therapy for attenuating some signs of high-carbohydrate and high-fat diet-induced metabolic syndrome (Ramli *et al.*, 2014). However, the active components in the flesh of white and red pitayas are different. Most of the betalain-related metabolites, the main contributors to antioxidant activity, were significantly higher in the flesh of red pitaya than in white pitaya (Ortiz and Takahashi, 2015; Suh *et al.*, 2014).

Endophytic bacteria live in almost all studied plant species. Plants provide nutrients for the growth of endophytes, meantime, many endophytes give beneficial feedback for their host by different ways, including growth promotion, pathogens suppression, contaminants remove, phosphate solubilization, nitrogen fixation, *etc.* It has been reported that endophytic bacteria and host plants are in an obligate mutualism through an evolutionary process (Assmus *et al.*, 1995; Hardoim *et al.*, 2008; Long *et al.*, 2008; Sessitsch *et al.*, 2002). Studies of endophytic bacteria diversity could improve knowledge about bacteria-plant interactions and this knowledge

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could be used for improving production and quality of the host plants (Berg, 2009; Compant *et al.*, 2005; Compant *et al.*, 2010). In the case of pitaya, the endophytic bacterial diversity is still poorly characterized.

Culture dependent methods were used to detect the composition of food associated microbiotas for decades, but these methods are difficult to reveal the real composition of microbiota due to some limitations, such as selective isolation, uncultivable state and outcompeted of low numbers of some microorganisms (Amann *et al.*, 1995; Ercolini *et al.*, 2001). Culture independent methods were developed then to overcome these problems partly. These methods could determine the composition and diversity of complex microbial communities with high speed and sensitivity (Giraffa and Neviani, 2001; Jany and Barbier, 2008). One of the most powerful methods is next generation sequencing (NGS) technology, and it has become more and more important in food quality and safety studies nowadays. NGS could produce millions of sequences in a single run and partly avoid some inherent biases in culture dependent methodology. Different microbial taxa, including uncultivable groups and some small groups which are difficult to detect by cultivated methods, could be identified based on sequence information (Ercolini, 2013; Mayo *et al.*, 2014). In addition, some cryptobiotic, dormant, moribund or latent bacteria, which might affect food quality and safety, could be identified using NGS (Davey, 2011; Mayo *et al.*, 2014). Studies of the microbiota of milk, fermented dairy products, plant, meat and fermented foods using NGS technologies had been reported more and more recently (Mayo *et al.*, 2014). Microaerophilic and anaerobic specific spoilage organisms in vacuum-packed ham had been characterized using culture-plating techniques and MiSeq NGS technologies also some microorganisms which facilitated changes in the pH value and organoleptic characteristics of the product were found (Piotrowska-Cyplik *et al.*, 2017). Comparative analysis of the metagenome mined from four diverse naturally fermented foods (bamboo shoot, milk, fish, soybean) were carried out to study the biases caused by different DNA extraction methods, Illumina MiSeq NGS showed the recovery of different bacteria varied by different DNA extraction methods (Keisam *et al.*, 2016). Assisted with traditional culture dependent and independent methods, NGS technologies have played key roles in studies on food quality and safety (Piotrowska-Cyplik *et al.*, 2017).

In this study, fruits of white and red pitayas from three different origins (Sing Buri, Thailand, Ho Chi Minh City, Vietnam and Jinghong, China) were chosen for endophytic bacteria diversity investigation by using Illumina HiSeq next-generation high-throughput sequencing technology. The aim of this work was to explore endophytic bacteria diversity in white and red

pitayas from different origins. Identification of endophytic bacterial composition and diversity in pitayas might be useful for improving their production and quality.

## Experimental

### Materials and Methods

**Sample collection.** Fresh fruits of white and red pitayas were collected from tropical and subtropical regions of Thailand, Vietnam and China. Ten white pitaya and ten red pitaya fresh mature fruits were randomly chosen from each site, 60 fruits were chosen in total. All the samples were stored at 4°C and processed within 12 h.

**Surface decontamination of the fruits.** Sixty fruits were washed by tap water and distilled water several times, then immersed three times in 70% ethanol (v/v) for 1 min. The flesh of surface decontaminated fruits was sampled in a laminar flow cabinet at room temperature.

**Genomic DNA extraction.** All ten flesh samples from the same site were pooled as one sample and mixed thoroughly. Finally, six samples (white pitayas from Thailand, Vietnam and China and red pitayas from Thailand, Vietnam and China) were generated for genomic DNA extraction. Genomic DNA was extracted by DNA quick plant system kit (tiangen, China) after maceration in liquid nitrogen following the manufacturer's instructions. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μl using sterile water.

**PCR amplification of 16S rDNA-V4 region.** PCR experiments were performed with Phusion® High-Fidelity PCR master mix with GC buffer (New England Biolabs) to ensure amplification efficiency and accuracy and run in an Eppendorf Gradient Thermocycler (Brinkman Instruments, Westbury, NY). With diluted genomic DNA as template, 16S rDNA-V4 region was amplified with specific primers 515F (5'-GTT TCG GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GCC AAT GGA CTA CHV GGG TWT CTA AT-3') with the barcode (Berry *et al.*, 2011; Klindworth *et al.*, 2013).

**Libraries construction and sequencing.** The PCR products were mixed with the same volume of 1× loading buffer (contained SYB green) and checked by electrophoresis on 2% agarose gel. Samples with bright main strip between 400–450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illu-

mina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated.

**Statistical analysis.** Paired-end reads obtained by sequencing were divided into six groups according to the unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged to generate raw tags (Magoc and Salzberg, 2011); quality filtering on the raw tags was performed to obtain the high-quality clean tags (Bokulich *et al.*, 2013; Caporaso *et al.*, 2010). Clean tags were compared with the reference database to detect and remove chimera sequences to generate effective Tags (Edgar *et al.*, 2011; Haas *et al.*, 2011). Sequences were analyzed by Uparse software and were assigned to the same OTUs with  $\geq 97\%$  similarity (Edgar, 2013). Representative sequence for each OTU was annotated by GreenGene database based on RDP classifier and multiple sequence alignment was performed by MUSCLE software (DeSantis *et al.*, 2006; Edgar, 2004; Wang *et al.*, 2007). Alpha diversity and beta diversity analysis, including chao1, shannon, simpson, ACE, good-coverage, rarefaction analysis, rank abundance analysis, principal component analysis (PCA), principal coordinate analysis (PCoA), unweighted pair-group method with arithmetic means (UPGMA), non-

metric multi-dimensional scaling (NMDS) analysis and T-test analysis were performed by QIIME and displayed with R software (Caporaso *et al.*, 2010).

**Nucleotide sequence accession number.** All the raw sequences after assembling and filtering were deposited in the NCBI SRA database under accession number SRP079944.

## Results

**OTU annotation and analysis.** After quality filtering and chimera sequences removal, 417,519 effective sequences of six groups were obtained in total (Table I). White pitayas from China (HLGW.3) containing more effective tags than from Thailand (HLGW.1) and Vietnam (HLGW.2), while red pitayas from three different origins (HLGR.1, Thailand; HLGR.2, Vietnam; HLGR.3, China) containing a similar amount of effective tags. Red pitayas contained 21.95% more effective tags on average than white pitayas. Nonetheless, white pitayas contained 43.8% more OTUs than red pitayas. OTUs from different origins varied from 313 to 603.

Top three microorganism populations from six samples were enumerated at phylum, class, order, family and genus level, respectively. The relative abundances of bacterial populations were different in each pitaya sample (Table II). Cyanobacteria, Proteobacteria and

Table I  
Sequence result from six samples.

Sample name	Sample origin	Raw Tags (#)	Clean Tags (#)	Effective Tags (#)	AvgLen (nt)	GC%	OTUs
HLGW.1	White Thailand	62449	62025	61785	253	53.93	485
HLGW.2	White Vietnam	61102	60691	60442	253	53.90	603
HLGW.3	White China	66586	66139	65885	253	53.82	576
HLGR.1	Red Thailand	76822	76347	76213	252	54.02	313
HLGR.2	Red Vietnam	78232	77729	77519	252	54.05	422
HLGR.3	Red China	76394	75811	75675	252	54.08	423

Table II  
Relative abundance of the top three bacteria at different levels of taxonomy.

Taxonomy levels	Top 1 (%)	Top 2 (%)	Top 3 (%)
<b>Phylum</b>	Cyanobacteria (64.6–72.7)	Proteobacteria (19.3–25.2)	Bacteroidetes (0.5–8.5)
<b>Class</b>	Alphaproteobacteria (18.2–24.5)	Bacteroidia (0.5–8.5)	Clostridia (1.2–4.5)
<b>Order</b>	Rickettsiales (18.0–24.2)	Bacteroidales (0.5–8.5)	Clostridiales (1.2–4.5)
<b>Family</b>	Mitochondria (18.0–24.2)	Prevotellaceae (0.2–4.0)	Bacteroidaceae (0.2–1.8)
<b>Genus</b>	Prevotella (0.2–3.9)	Bacteroides (0.1–1.6)	Acutodesmus (1.1–1.2)

Table III  
Alpha indices table.

Sample name	Shannon	Simpson	Chao1	ACE	Goods coverage
HLGW.1	2.275	0.556	484.583	490.011	0.999
HLGW.2	2.129	0.497	583.867	593.464	0.998
HLGW.3	2.231	0.533	550.647	569.752	0.998
HLGR.1	1.358	0.444	274.5	292.031	0.999
HLGR.2	1.584	0.462	414.337	429.591	0.998
HLGR.3	1.457	0.451	436.4	454.141	0.998

Bacteroidetes were found to be the most dominant phyla; *Alphaproteobacteria*, *Bacteroidia* and *Clostridia* were found to be the most dominant classes; *Rickettsiales*, *Bacteroidales* and *Clostridiales* were found to be the most dominant orders; *Mitochondria*, *Prevotellaceae* and *Bacteroidaceae* were found to be the most dominant families; *Prevotella*, *Bacteroides* and *Acutodesmus* were found to be the most dominant genera.

**Alpha diversity analysis.** Alpha diversity indices were calculated to analyze bacterial richness and diversity within communities (Table III). All of the alpha

indices of white pitaya group (HLGW) were higher than in red pitaya group (HLGR). Rarefaction analysis was carried out to determine the sequence coverage of six samples (Fig. 1). All of the rarefaction curves are almost approaching their asymptotes indicated the sequence amount is adequate. Rank abundance curves indicated the species richness and species evenness of each sample were different (Fig. 2). Venn graphs were constructed to show the number of shared and unique OTUs in different groups (Fig. 3). Each sample contained a certain number of unique OTUs and white

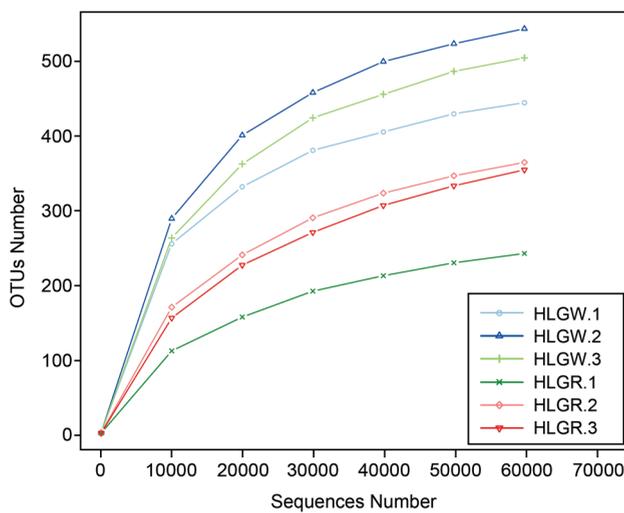


Fig. 1. Rarefaction curves of six pitaya samples. OTUs are shown at the 97% similarity.

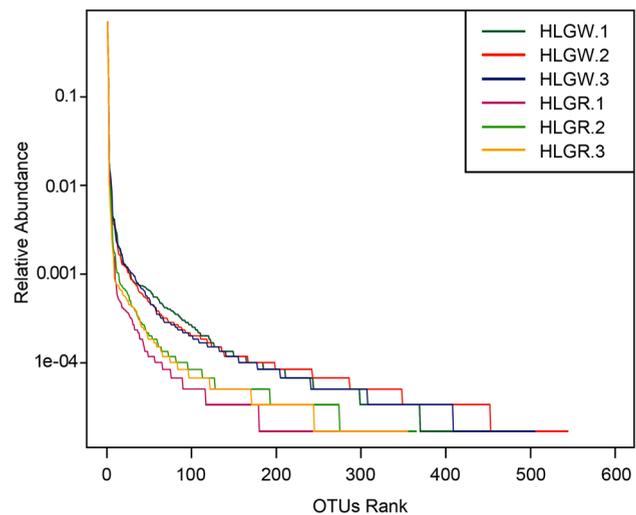


Fig. 2. Rank abundance curves of six pitaya samples.

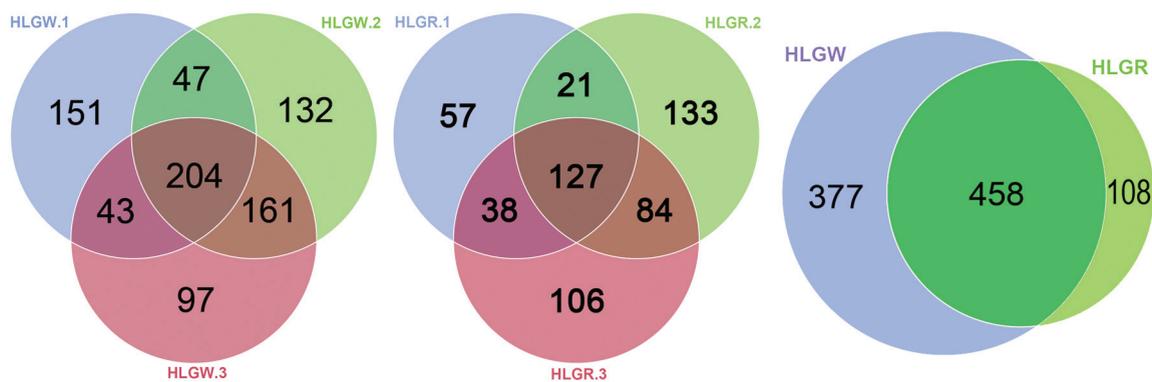


Fig. 3. Venn graphs of six pitaya samples.

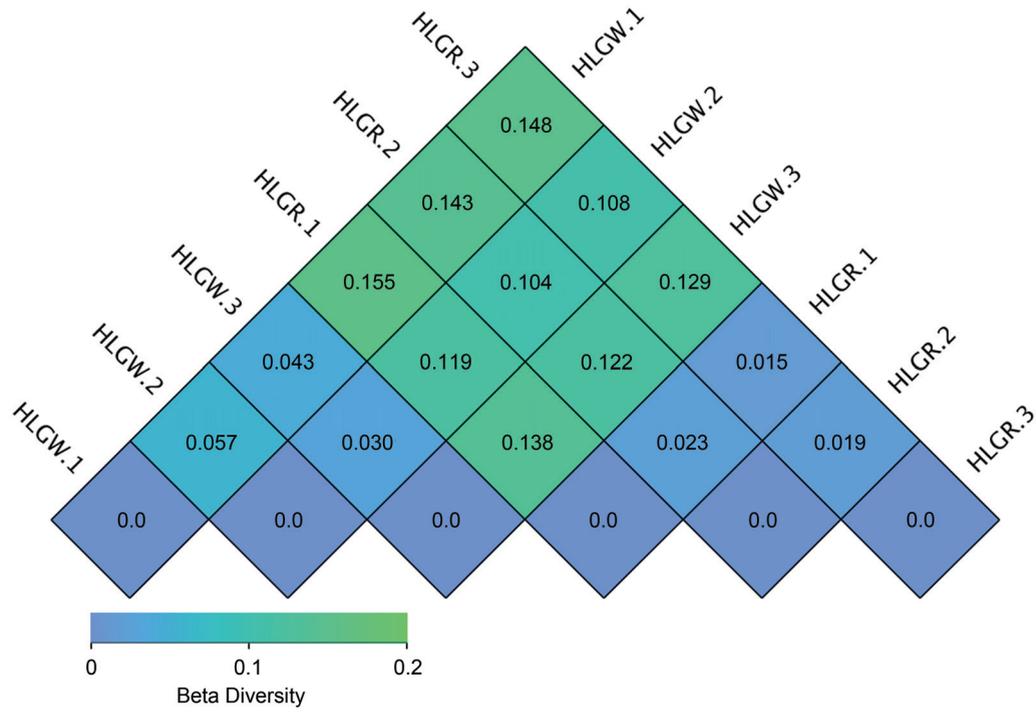


Fig. 4. Heat map of Beta diversity index.

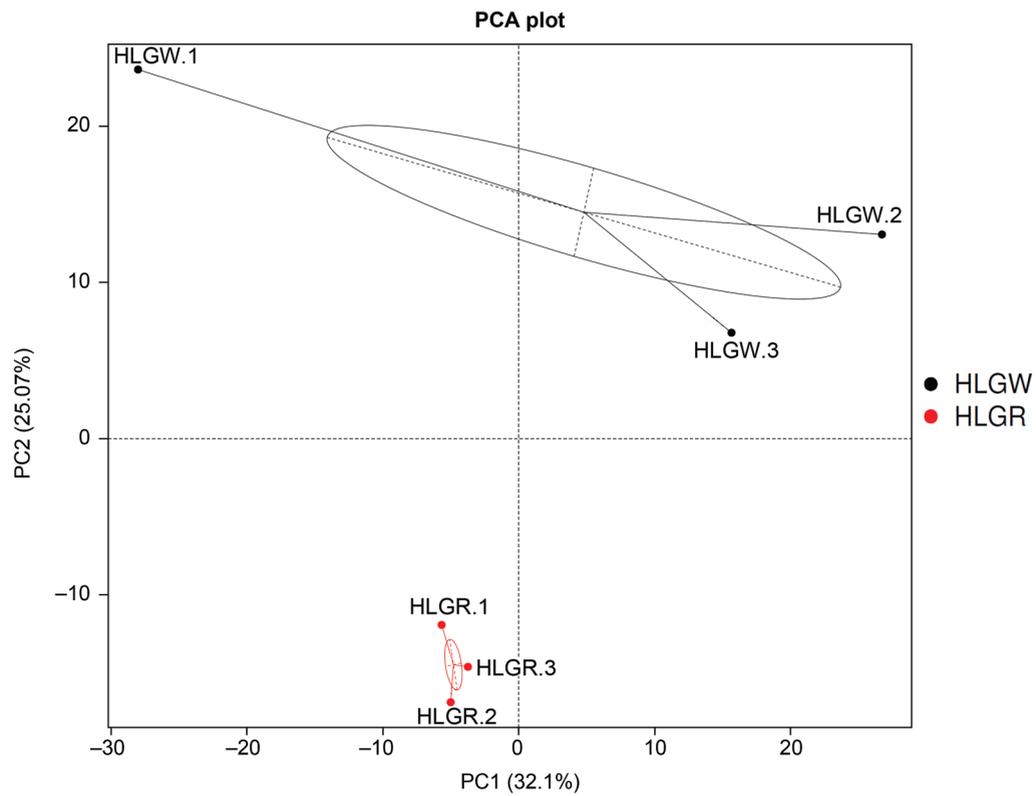


Fig. 5. Principal component analysis (PCA) plot.

pitaya group contained much more unique OTUs than the red pitaya group.

**Beta diversity analysis.** A heatmap of Beta diversity index was constructed (Fig. 4). Principal component analysis (PCA), principal coordinate analysis (PCoA),

non-Metric multi-dimensional scaling (NMDS) and unweighted pair-group method with arithmetic mean (UPGMA) were carried out to evaluate differences of samples in species complexity (Fig. 5–8). As a result, all analysis showed that the microbial communities

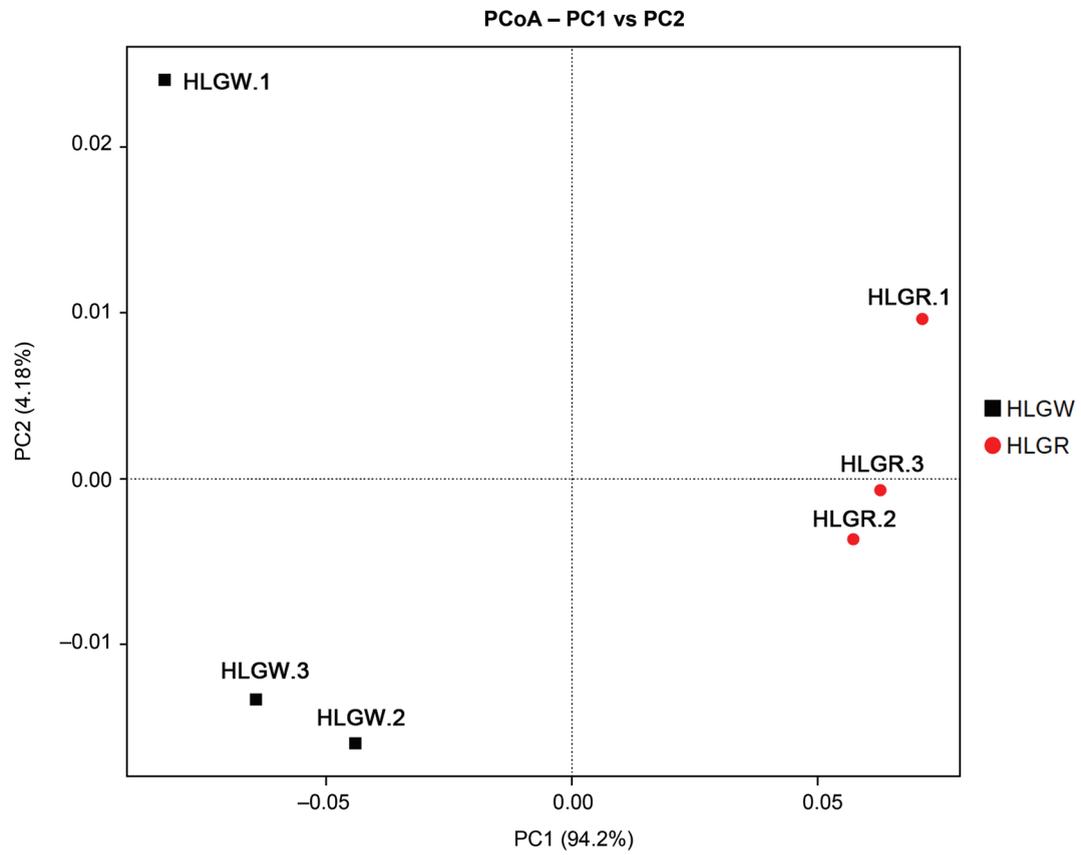


Fig. 6. Principal coordinate analysis (PCoA) plot.

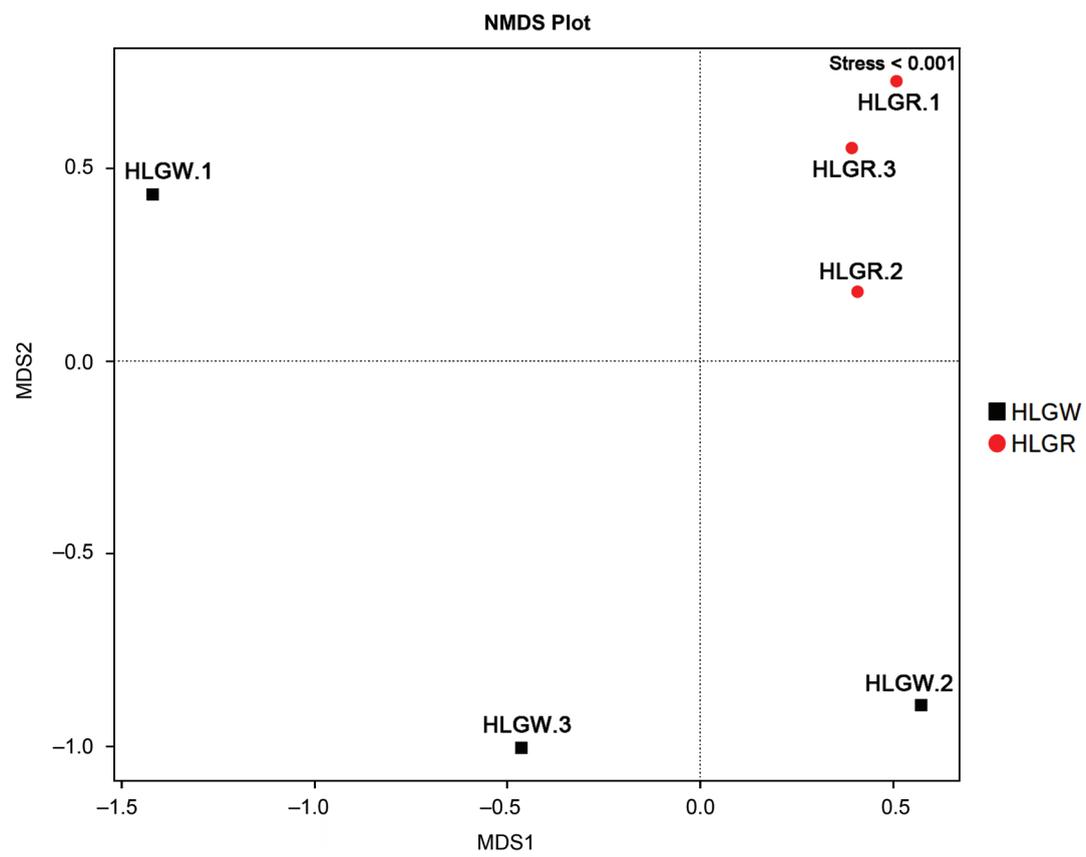


Fig. 7. Non-Metric multi-dimensional scaling (NMDS) plot.

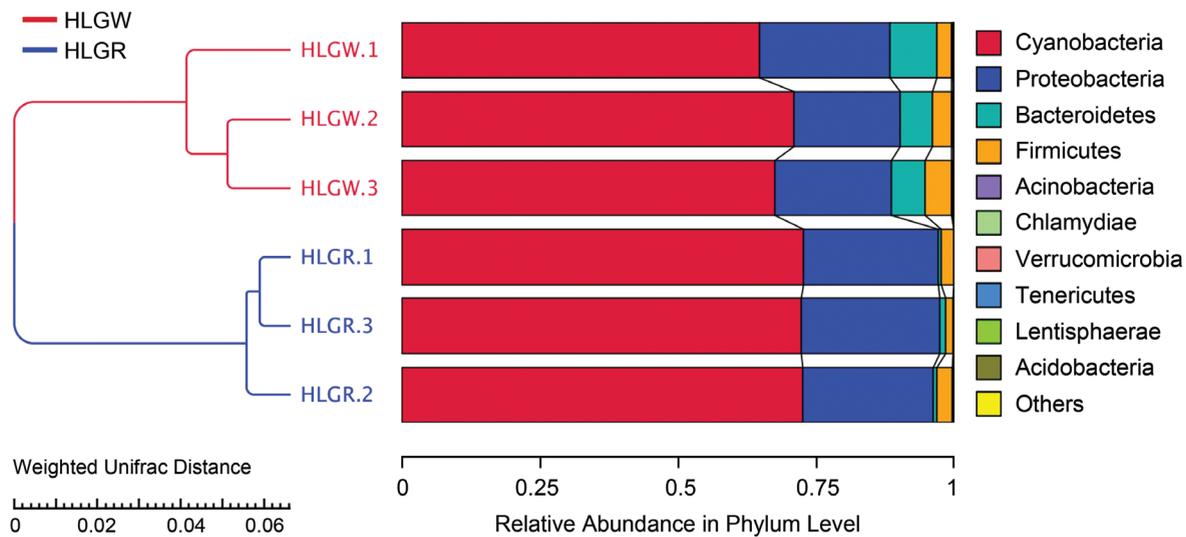


Fig. 8. Unweighted pair-group method with arithmetic mean (UPGMA) analysis.

were different between white and red pitaya groups. Samples from white pitaya and red pitaya were clustered together respectively. The bacterial community structure of each sample in red pitaya group showed higher similarity.

**T-test analysis.** T-test was carried out to find statistically significant different species ( $P < 0.05$ ) between white and red pitaya groups at different taxonomy levels. As a result, the differences in the relative abundance of phylum Bacteroidetes ( $p = 0.017$ ), classes *Bacteroidia* ( $p = 0.017$ ) and *Coriobacteria* ( $p = 0.023$ ), orders *Bacteroidales* ( $p = 0.017$ ) and *Coriobacteriales* ( $p = 0.023$ ), families *Prevotellaceae* ( $p = 0.001$ ), *Bacteroidaceae* ( $p = 0.006$ ), *Ruminococcaceae* ( $p = 0.026$ ), *Paraprevotellaceae* ( $p = 0.015$ ), *Rikenellaceae* ( $p = 0.043$ ), *Alcaligenaceae* ( $p = 0.023$ ) and *Coriobacteriaceae* ( $p = 0.023$ ), genera *Prevotella* ( $p = 0.001$ ), *Bacteroides* ( $p = 0.003$ ), *Roseburia* ( $p = 0.041$ ), *Faecalibacterium* ( $p = 0.034$ ) and *Sutterella* ( $p = 0.023$ ) showed statistically significant between white and red pitaya groups. All of these species in white pitaya group are much more than in red pitaya group.

### Discussion

In this study, we demonstrated the endophytic bacteria diversity in fruits of pitaya by using Illumina HiSeq second-generation high-throughput sequencing technology which can provide larger amount information than ever before. Large number of endophytic bacteria was found to colonize in pitaya flesh. A total of 417,519 effective sequences and 2822 OTUs with 97% similarity were obtained from six samples. Among them, 22 phyla, 56 classes, 81 orders, 122 families and 159 genera were identified.

Endophytic bacteria diversity was uneven among pitaya fruits from different origins. Rank abundance and venn graphs reflected the structure of each sample. In white pitaya group, samples from Vietnam and China shared more OTUs than other combinations. Samples from Vietnam contained most OTUs and samples from Thailand possessed most unique OTUs. Genera *Ruminococcus*, *RFN20*, *02d06*, *BF311* in samples from Thailand, genera *Escherichia*, *Bulleidia* and *Anaerostipes* in samples from Vietnam and genera *Oscillospira*, *Chlamydia* and *Succinivibrio* in samples from China showed higher relative frequencies respectively. In red pitaya group, samples from Vietnam and China shared more OTUs than other combinations. Samples from China contained most OTUs and samples from Vietnam possessed most unique OTUs. Genus *Clostridium* in samples from Thailand, genera *Bradyrhizobium* and *Mesorhizobium* in samples from Vietnam and genera *Halomonas* and *Lactobacillus* in samples from China showed higher relative frequencies, respectively.

Endophytic bacteria structure was different between the white pitaya group and red pitaya group. Although the white pitaya group contained a lower number of effective tags, it contained 43.8% OTUs more than the red pitaya group, which might be caused by different environmental factors in different pitaya fruits. All the alpha indices of the white pitaya group were higher than for the red pitaya group which indicates the bacterial richness and diversity were higher in the white pitaya group. Venn graph of the two groups also supported this conclusion. White pitaya group contains over three times more unique OTUs than the red pitaya group. To further investigate diversity differences between white and red pitaya, beta diversity analysis was carried out. The heatmap of beta diversity index clearly showed the difference between the two groups. PCA, PCoA, NMDS

and UPGMA analysis evidently demonstrated the bacterial diversity between white and pitaya groups was different. To explore the significant different species which might be used as biomarkers for white and red groups respectively, T-test was carried out. As a result, phylum *Bacteroidetes*, classes *Bacteroidia* and *Coriobacteria*, orders *Bacteroidales* and *Coriobacteriales*, families *Prevotellaceae*, *Bacteroidaceae*, *Ruminococcaceae*, *Paraprevotellaceae*, *Rikenellaceae*, *Alcaligenaceae* and *Coriobacteriaceae*, genera *Prevotella*, *Bacteroides*, *Roseburia*, *Faecalibacterium* and *Sutterella* were significantly much more in white pitaya group. Since the study showed the active components in flesh of white and red pitayas were different and the main contributors to antioxidant activity were significantly higher in red pitaya than in white pitaya (Suh *et al.*, 2014), the relationship between endophytic bacterial diversity and their living environment needs further study.

In conclusion, a large number of endophytic bacteria is found in fruits of white and red pitaya from Thailand, Vietnam and China. The bacterial diversity was different among pitayas from different origins and between white and red species. Further studies of the roles of these endophytic bacteria are needed. This study might be useful for growth improvement, fruits preservation and processing of different pitaya species from different origins.

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#### Conflict of Interest

The authors declare that they have no conflict of interest.

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## Natural Attenuation Potential of Polychlorinated Biphenyl-Polluted Marine Sediments

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### Abstract

The marine environment in Kuwait is polluted with various hazardous chemicals of industrial origin. These include petroleum hydrocarbons, halogenated compounds and heavy metals. Bioremediation with dedicated microorganisms can be effectively applied for reclamation of the polluted marine sediments. However, information on the autochthonous microbes and their ecophysiology is largely lacking. We analyzed sediments from Shuwaikh harbor to detect polychlorinated biphenyls (PCBs) and total petroleum hydrocarbons (TPHs). Then we adopted both culture-dependent and culture-independent (PCR-DGGE) approaches to identify bacterial inhabitants of the polluted marine sediments from Shuwaikh harbor. The chemical analysis revealed spatial variation among the sampling stations in terms of total amount of PCBs, TPHs and the PCB congener fingerprints. Moreover, in all analyzed sediments, the medium-chlorine PCB congeners were more abundant than the low-chlorine and high-chlorine counterparts. PCR-DGGE showed the presence of members of the Proteobacteria, Spirochaetes, Firmicutes and Bacteroidetes in the analyzed sediments. However, Chloroflexi-related bacteria dominated the detected bacterial community. We also enriched a biphenyl-utilizing mixed culture using the W2 station sediment as an inoculum in chemically defined medium using biphenyl as a sole carbon and energy source. The enriched mixed culture consisted mainly of the Firmicute *Paenibacillus* spp. Sequences of genes encoding putative aromatic ring-hydroxylating dioxygenases were detected in sediments from most sampling stations and the enriched mixed culture. The results suggest the potential of bioremediation as a means for natural attenuation of Shuwaikh harbor sediments polluted with PCBs and TPHs.

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**Key words:** aromatic ring oxygenases, bacterial communities, bioremediation, Chloroflexi, DGGE, polluted marine sediments

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### Introduction

The industrial, urban and agricultural development in Kuwait and other Arabian Gulf countries, following the discovery of oil, has burdened the marine environment in this region with many hazardous pollutants. Many different chemicals including heavy metals, petrochemicals and halogenated organic compounds have been introduced into coastal marine sediments (Al-Muzaini *et al.*, 1995; Beg *et al.*, 2001). Since the early 1980s, several studies have been conducted to assess the quality of coastal sediments in Kuwait. These investigations concluded that certain stretches of the coastline are polluted with high concentrations of toxic chemicals from municipal and industrial wastewater discharges (Shunbo and Literathy, 1984; Clark, 2001; Beolchini *et al.*, 2009). Most of the studies carried out in the Arabian Gulf region have focused on oil and related

chemicals as the source of pollution in the territorial water of the Gulf. Other persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) have received much less attention (Fowler *et al.*, 1993). The presence of PCBs in Kuwait's coastal marine sediments has been reported (Gevao *et al.*, 2012; 2006). In a study by Helaleh *et al.* (2012), samples from some marine biota (collected from local fish market in Kuwait) were analyzed for the occurrence of organochlorine pesticides and PCBs. The authors detected PCBs ( $\text{ng g}^{-1}$ ) in fish (2.53), squid (0.25), bivalves (0.24), shells (0.24), octopus (0.17) and shrimp (1.38).

PCBs were first manufactured commercially in 1929. The chemical structure of PCBs is based on a biphenyl core with 1 to 10 chlorine atoms introduced at different degrees of chlorination and substitution to produce 209 products called congeners (Furukawa and Fujihara, 2008). PCB congeners having identical number of

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chlorine atoms are referred to as homologues, whereas homologues with different chlorine positions are called isomers (Borja *et al.*, 2005). In the environment, PCBs generally exist as complex mixtures of different congeners such as Aroclor produced by Monsanto (USA) (Borja *et al.*, 2005). Due to their unique physicochemical properties, PCBs have been adopted for a variety of industrial applications such as lubricants, dielectric fluids and plasticizers (Gevao *et al.*, 2012). Because of their toxicity and persistence in the environment, PCBs were banned in most countries in the late 1970s (Ross, 2004). PCBs can be very hazardous to the health of human and other biota due to their bioaccumulation in different tissues (Passatore *et al.*, 2014).

Like other anthropogenic compounds, PCBs can enter the aquatic environment from different sources including: (i) direct deposition from the atmosphere, (ii) runoff from land, (iii) directly from industrial and wastewater treatment plant discharges, sewer branches, thermal and chlorinated effluents from power plants as well as shipping and dockyard activities in harbors (Bush and Kadlec, 1995). Among all the environmental media, sediments are recognized as significant reservoirs and sinks for a large variety of POPs such as PCBs. This is due to their hydrophobicity. They have a strong affinity for particulate matter and ultimately accumulate in bottom sediments (Bush and Kadlec, 1995; Zennegg *et al.*, 2007).

In Kuwait, only a few studies have addressed pollution of the marine environment with PCBs. However, the objective of those studies was to assess the occurrence, distribution and toxicity of PCBs without paying attention to the fate of these compounds in the environment. Biological transformation and degradation by microorganisms can play an important role in the elimination and/or detoxification of PCBs found in polluted ecosystems (Furukawa and Fujihara, 2008). Nonetheless, there is dearth of information concerning PCBs-degrading microbial communities in the Kuwait's marine environment. Moreover, the majority of the POPs biodegradation studies on the polluted ecosystems in Kuwait relied on the conventional enrichment and isolation techniques (culture-dependent procedures), which are laborious and usually underestimate the quantity and quality of the microbial communities inhabiting a polluted ecosystem (Dennis *et al.*, 2003; Van Hamme *et al.*, 2003).

The comprehensive understanding of the structure and function of microbial communities is a prerequisite for the development of an efficient bioremediation process. In this context, Dell'Anno *et al.* (2012) investigated the dynamics of bacterial abundance and biodiversity during bioremediation of hydrocarbon-contaminated marine harbor sediment. The authors reported that the incubation temperature was the principal determi-

nant of bacterial abundance, diversity and community structure. Furthermore, they found that hydrocarbon biodegradation efficiency was promoted as the bacterial richness and evenness increased. In a study on PCBs bioremediation, Wang and He (2013) studied the reductive dechlorination patterns and microorganisms involved in the dechlorination of Aroclor 1260 in sediment/soil microcosms and sediment-free enrichment cultures. They reported significant PCBs dechlorination activity with distinct patterns such as the N, H and T processes. They also identified *Dehalogenimonas* and *Dehalococcoides* spp. as major dechlorinators in the microcosms. Recently, Sydow *et al.* (2016) reported that a diesel-degrading bacterial consortium maintained its structural and functional integrity after short-term exposure to different hydrocarbon feeds.

In this study, bacterial diversity and abundance in PCBs-polluted sediments from Shuwaikh harbor in Kuwait were investigated. Spatial shift in community structure was also addressed. Moreover, biodegradation potential of the bacteria inhabiting the sediments was assessed *via* fingerprinting of the functional genes of aromatic ring oxygenases.

## Experimental

### Materials and Methods

#### Chemicals and molecular biology materials.

Chemicals and microbiological media were obtained from Fluka (Switzerland), Sigma-Aldrich (USA) and Promega (USA). Total community DNA was isolated from sediment samples with PowerSoil DNA Isolation Kit (MoBio, USA). Molecular biology enzymes and reagents were purchased from Qiagen (USA) and GE Healthcare (UK). The PCBs analytical standard (EC-4133) was purchased from Cambridge Isotope Laboratory (CIL, Andover, MA). The internal standard Mirex and the recovery standard EC 4058 were purchased from CIL.

#### Study area and collection of sediment samples.

Kuwait Bay is considered an ideal seaport in terms of its nature and geography. It encloses Shuwaikh harbor within an urban industrial area in the Al-Asimah Governorate (Capital Governorate) of Kuwait. Shuwaikh harbor lies on the south shore of Kuwait Bay at Latitude 29° 21' North and Longitude 47° 56' East. It is considered the main commercial port in the country. It is one of the busiest ports in the Middle East with 21 deep-water berths. For the PCR-DGGE experiments, sediment samples were collected at five different locations (W1, W2, W3, W4 and W5) from inside the Shuwaikh harbor using a sediment core sampler (Fig. 1). For the chemical analysis, a Van Veen Grab was used to collect

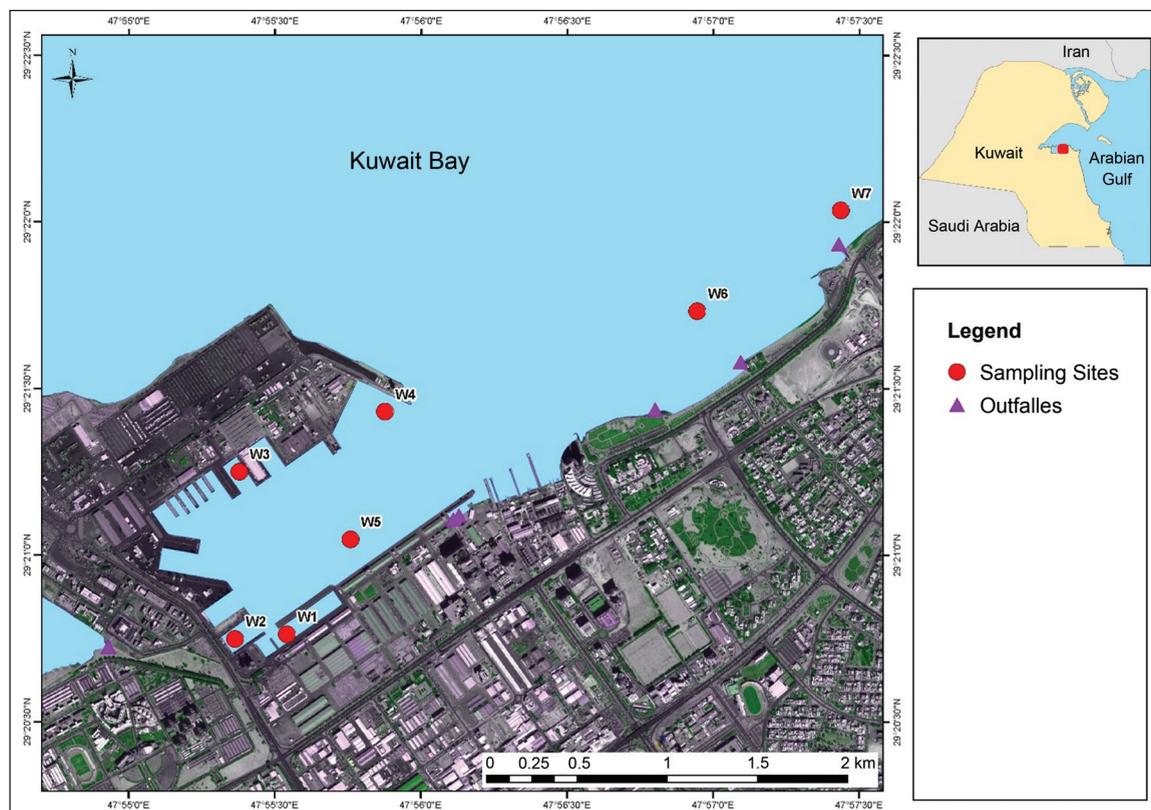


Fig. 1. A map showing Shuwaikh harbor and the 7 sampling sites (red points).

sediment samples from the W1-W5 sites in addition to two locations (W6 and W7) outside the harbor (Fig. 1). The cores were secured and returned back to the laboratory for immediate processing. The sediment cores were divided into layers or sections (5-cm long), which were kept in sterile glass Petri dishes. Each section was then homogenized into one composite sample using a sterile wooden spatula. Samples were transferred into 50-ml sterile tubes and marked according to the sampling location.

**Culture media and growth conditions.** Luria-Bertani (LB), trypticase soy broth (TSB) and trypticase soy agar (TSA) were prepared according to the instructions of the supplier and were used for growth and isolation of bacteria. Chemically defined medium (CDM) had the following composition per liter of deionized water:  $\text{KH}_2\text{PO}_4$ , 1.35 g;  $\text{K}_2\text{HPO}_4$ , 7.0 g;  $\text{NH}_4\text{Cl}$ , 0.54 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.044 g; vitamins (Cyanocobalamine 0.2 mg, pyridoxine-HCl 0.6 mg, thiamine-HCl 0.4 mg, nicotinic acid 0.4 mg, *p*-aminobenzoate 0.32 mg, biotin 0.04 mg, Ca-pantothenate 0.4 mg) and trace elements ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  2.0 mg,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  150  $\mu\text{g}$ ,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  85  $\mu\text{g}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  37  $\mu\text{g}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  200  $\mu\text{g}$ ,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  40  $\mu\text{g}$ ,  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  20  $\mu\text{g}$  and  $\text{H}_3\text{BO}_3$  20  $\mu\text{g}$ ). The carbon sources were added either as solid in case of biphenyl (1 g/l) or from acetone stock solutions for Aroclor 1242 (0.1 ml/100 ml of CDM, commercial PCBs mixture of

5 mg/100 ml acetone, Sigma-Aldrich) and 2,4-dichlorobiphenyl (0.1 ml/100 ml of CDM, commercially available as 2.5 mg/100 ml acetone stock, Sigma-Aldrich). All liquid culture media (100 ml) were routinely prepared in 250-ml Erlenmeyer flasks and incubated at 30°C in an orbital shaker (200 rpm). Agar plates were incubated at 30°C for 48 hours.

**Enrichment and isolation of biphenyl-degrading bacteria.** Two grams from the sediment samples W1, W2, W3, W4 and W5 (upper 5 cm of the core) were inoculated individually into CDM containing biphenyl as the sole carbon source. After 10 days of incubation, 10 ml aliquots from those initial enrichments were transferred to fresh medium followed by further incubation for 10 days. This sub-culturing step was repeated four times. Aliquots (1 ml) from the 4<sup>th</sup> enrichment (subculture) were serially diluted in sterile normal saline (0.9% NaCl) and samples (100  $\mu\text{l}$ ) from each dilution were spread on TSA plates. After incubation, single colonies (morphologically distinct) from the different plates were purified by subsequent streaking on TSA plates. The isolated single colonies were then grown in TSB (trypticase soy broth, 20 ml) medium for 48 h. The cells were harvested from culture samples (1 ml) by centrifugation (10,000 rpm, 5 min) and washed once with 10 ml of 0.1 M K-phosphate buffer (pH 6.8). The washed cells were suspended in phosphate buffer and inoculated into CDM containing biphenyl as a sole carbon source.

**Enrichment of PCB-degrading bacteria.** Portions (2 g) of the sediment samples collected from sites W1, W5 (uppermost layer of the core, 5 cm) and W6 were inoculated into CDM containing either Aroclor 1242 or 2,4-dichlorobiphenyl as a sole carbon source. After two weeks of incubation, 10-ml aliquots of those initial enrichments were transferred to fresh medium and further incubated for two weeks. This sub-culturing step was performed three times.

**Isolation of total community DNA from sediments and bacterial cultures.** Total genomic DNA of microbial communities inhabiting Shuwaikh harbor sediment samples was isolated with the PowerSoil DNA Isolation Kit. The initial step of extraction involved homogenizing 0.25 g of sediment samples using bead beater at 2500 bpm for 3 min. Then the rest of the extraction process was achieved following the manufacturer's protocol. The DNA from the unknown purified bacterial cultures was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA) following the manufacturer's protocol.

**Partial amplification of the 16S rRNA gene.** Partial 16S rRNA gene fragments were amplified using the universal bacterial primers 907R (5'-CCCCGTCAATTC-MTTTGAGTTT-3') and GM5F (5'-CCTACGGGAG-GCAGCAG-3') (Schafer and Muyzer, 2001) and the Pure Taq Ready-To-Go PCR Beads (Amersham Biosciences, UK). Aliquots (30 pmol) of both forward and reverse primers were added to the beads along with 1  $\mu$ l (25 ng DNA) of DNA and the final volume in the vials was brought up to 25  $\mu$ l with sterile water (Sigma, USA). The vials were incubated in a Thermocycler (Gene Amp PCR system 9700, Applied Biosystem, USA), where a standard PCR program was applied. The initial denaturing step took place at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Eventually, a final extension step was run at 72°C for 7 min.

**Amplification of genes encoding putative aromatic ring oxygenases.** Segments from genes encoding aromatic ring-hydroxylating oxygenases were amplified by PCR using DNA obtained from the sediment samples as a template. Total DNA was isolated from sediment samples collected from the locations W1, W2, W3, W4, W5, W6 and W7 with the PowerSoil DNA Isolation Kit. The forward primer 888 and the reverse primer 300R were used (Kitagawa *et al.*, 2001). The primer 888 consisted of the 40-bp GC-clamp sequence (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') and the sequence (5'-TGC ASS TWT CAC GGS TGG-3') and the primer 300R had the sequence (5'-CTC GAC TCC GAG CTT CCA GTT-3') conserved among aromatic ring-hydroxylating dioxygenases. The touchdown PCR procedure was used: 20 cycles at 94°C for 40 sec, 60°C for 40 sec (decreased

by 1°C every 2 cycles), 72°C for 40 sec and 10 cycles at 94°C for 40 sec, 50°C for 40 sec and 72°C for 40 sec. The expected 340-bp DNA fragments containing the 300-bp target plus 40-bp GC-clamp sequences were amplified from the extracted DNA (Kitagawa *et al.*, 2001).

**Denaturing gradient gel electrophoresis (DGGE).** DGGE was performed on Dcode Universal Mutation System (Bio-Rad, USA) according to the manufacturer's instructions where the denaturant's concentrations increase from the top of the gel toward the bottom. Three 6% polyacrylamide gel solutions with different denaturants concentrations (0%, 30% and 50%) were prepared. Samples (300–600 ng of the amplified DNA) were run at 50V and 60°C for 16 hours. The DGGE bands were excised for sequencing and were visualized using Dark Reader (Clare Chemical Reader, USA). Gels were post-stained for 20 min in 1  $\times$  TAE buffer supplied with SYBR Green (Invitrogen, USA). The DNA was allowed to passively diffuse from the gel pieces into the water at 4°C overnight. The resulting solution was used as a template to re-amplify the content of each band using the same PCR primers and programs mentioned previously. The DGGE data obtained were analyzed using Phoretix 1D analysis software. The DGGE profile was transformed to binary matrix depending on the presence (1) and absence (0) of the bands on the examined profile before being analyzed using hierarchical cluster analysis.

**DNA sequencing.** PCR products were purified using QIA Quick Purification Kit (Qiagen, USA) following the manufacturer's protocol to remove the excess Taq polymerase, primers and DNTPs that might interfere with the sequencing steps. BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) was used for labelling and amplifying the purified product. The PCR program applied included 1 cycle of denaturation at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 1 min, annealing at 50°C for 5 sec and extension at 60°C for 4 min. The final products were further purified using sodium acetate (pH 5.2) and absolute ethanol. Then 20  $\mu$ l of absolute ethanol were added to each sample. After the denaturation step, samples were kept on ice and loaded directly in the 3130xl genetic analyzer (Applied Biosystems, USA) and the results were obtained using Sequencing Analysis v5.2 Software (Applied Biosystems, USA). The obtained sequences were compared with others in the GenBank database using BLAST (Altschul *et al.*, 1997). The partial 16S rRNA gene sequences were deposited in the GenBank (accession numbers – see Supplementary Material).

**Analysis of PCBs and total petroleum hydrocarbons (TPHs).** For the chemical analysis, sediment samples from the seven sampling stations W1-W7 were collected with a Van Veen grab. Analysis of the PCBs was performed in the Central Analytical Laboratory-Kuwait

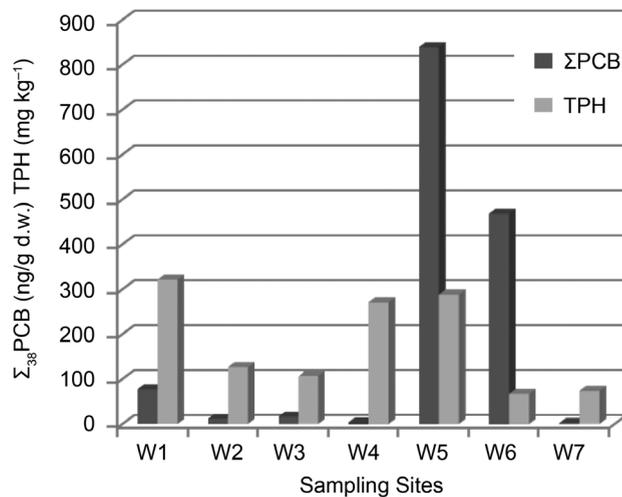


Fig. 2. Total PCBs concentrations (38 congeners) and TPHs measured for the 7 sites sampled at the chosen Shuwaikh harbor location, d.w. (dry weight).

Institute for Scientific Research (Kuwait) according to the methods described in Gevao *et al.* (2012). TPHs in sediment samples were measured by the Infrared Spectroscopic Method recommended by United States Environmental Protection Agency (USEPA, 1978). The calibration plot was used to calculate the concentration of hydrocarbons in sediments with the following equation:

$$\text{mg/kg} = I.V/a.w$$

where I = integral value from IR measurement, V = volume of the extract (100 ml), a = slope of the calibration curve, w = weight of the sediment sample (about 5 to 10 g).

## Results

**Pollution of Shuwaikh marine sediments with PCBs and TPHs.** Figure 2 shows that stations W5 and W6 had the highest measured PCB levels (W5: 59.1% of  $\Sigma_{38}$ PCB – all sites, W6: 33.0%). Taking a closer look at the distribution of congener concentrations for the locations across the different homologue series represented (those PCBs containing the same number of chlorine atoms), it can be seen that PCB-138 (2, 2', 3, 4, 4', 5'-hexachlorobiphenyl) was the dominant pollutant congener with a  $\Sigma$ PCB of 165.4 ng/g (Fig. 3, Table S1). Other congeners, which exceeded 100 ng/g were PCB-101, 110, 118, 153 and 209. However, it is important to note that PCB-138, PCB-101 and PCB-153 were only present in the two most contaminated sites (W5 and W6), whereas PCB-110 and PCB-118 were observed in all of the sampling sites (Fig. 3). The sum of Cl<sub>5</sub> and Cl<sub>6</sub> congeners (16 out of the 38 measured congeners, 42%) accounted for more than two-thirds of all the detected PCB pollutants. No Cl<sub>9</sub> congeners were observed above the measurement detection limit. In terms of the PCB recovery data (Table S2), the average site recovery (for all congeners) was 84.5% (range of 55.7% [W2] to 101.4% [W7]). Coincidentally, the average congener recovery for all sites was also 84.5% (range of 67.1% [PCB-52] to 104.3% [PCB-180]). Total TPHs measured at the location (summed over seven sampling sites) was 1255.9 mg/kg with an average of 179.4 mg/kg. The lowest levels were observed at the two sites most distant from the harbor (Fig. 2).

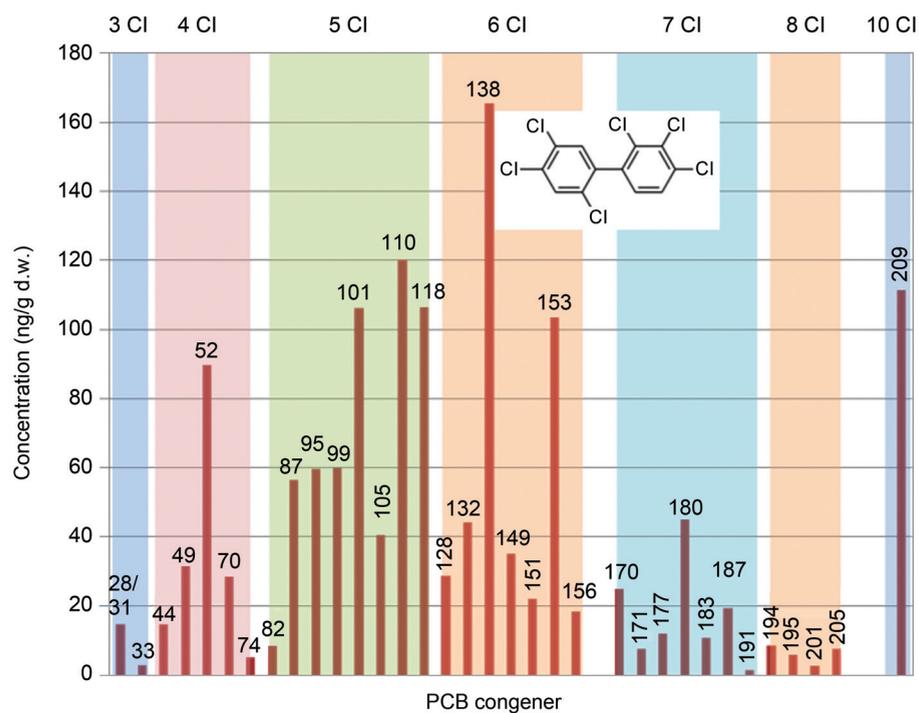


Fig. 3. Distribution of PCBs congeners in Shuwaikh harbor sediments (summed across all sampling sites), d.w. (dry weight).

**Enrichment of biphenyl-degrading bacteria.** Sediment samples from different sites were inoculated into CDM containing biphenyl as a sole carbon source to enrich biphenyl-utilizing bacteria. Culture turbidity due to bacterial growth was sustained only in the enrichment containing sediment sample collected from the W2 station. The W2 biphenyl enrichment culture (whole culture) appeared yellow in color. This was evident even after the third sub-culturing. When samples from the W2 biphenyl enrichment (fourth subculture) were serially diluted and spread on TSA plates, it was possible to differentiate three morphologically distinct colonies. After purification, none of those three strains could grow individually on biphenyl as a sole carbon source in CDM. Enrichments with Aroclor 1242 or 2,4-dichlorobiphenyl as carbon sources did not reveal any bacterial growth even after repeated sub-culturing. Moreover, the W2 biphenyl enrichment culture did not grow on either Aroclor 1242 or 2,4-dichlorobiphenyl as a sole carbon source even after prolonged incubation.

**Natural bacterial communities dwelling in Shuwaikh harbor sediments.** The bacterial community inhabiting different depths (sections) of the sediment cores was investigated by comparing the DGGE band pattern obtained from various lamina recovered from each core (Table I). In terms of the number of bands, the

DGGE profile showed almost the same pattern among the different sampling sites. Generally, the uppermost layer in the cores revealed a larger number of bands. The five sampling sites showed no remarkable variation in the average number of bands. In terms of total band numbers, site W4 revealed the highest, while W3 had the lowest number.

The DNA extracted from different sections of each core was pooled and the DGGE band patterns for such pooled samples were compared with their counterparts from other sites. The results (Fig. 4) revealed that sites W1 and W5 shared a similar band pattern. Only one additional band (band c) was found in site W1 and band a was found in site W5. Furthermore, sites W1 and W5 clusters were very close to each other (Fig. 4). Sites W2 and W3 clusters were also quite close to sites W1 and W5, whereas site W4 was the most divergent in terms of DGGE band pattern. In total, 32 bands were excised and sequenced. Table II summarizes the identity of only 18 bands that showed good sequences. The Genbank match search for the sequenced bands showed the dominance of bacteria belonging to Chloroflexi members (58.3%). In addition, sequences related to members of Spirochaetaceae, Proteobacteria (both  $\gamma$  and  $\delta$ ), Firmicutes and Bacteroidetes were detected. Site W1 was dominated by sequences affiliated to *Dehalogenimonas*

Table I  
Number of DGGE bands obtained from the various layers of the sediment cores.

Layer (section) number*	Site W1	Site W2	Site W3	Site W4	Site W5
1	12	14	8	19	12
2	12	11	8	15	12
3	10	9	4	6	8
4	9	8	9	12	12
5	3	7		10	3
6	5	12		13	9
7	9			8	
8				13	
9				7	
10				4	
11				8	
12				8	
13				9	
14				9	
15				8	
16				3	
17				3	
Total	60	61	29	155	56
Average	8.57	10.16	7.25	9.11	9.33
(Min-Max)	(3-12)	(7-14)	(4-9)	(3-19)	(3-12)
SD	3.4	2.6	2.2	4.2	3.5

\* Layer 1 is at the top of the sediment core

Table II  
Phylogenetic affiliation of partial 16S rRNA gene sequences retrieved from Shuwaikh harbor sediments.

DGGE Band Code	Sample Name	Total Base Pair	Phylum/ Subdivision	Base Compared	Nearest GenBank Match	Similarity (%)	Accession no.
1m	<i>Dehalococcoides</i> sp. SPS1F2	485	Chloroflexi	430/495	<i>Dehalococcoides</i> sp. BHI80-15	87	AJ431246
1c	<i>Vibrio</i> sp. SPS1F3	513	$\gamma$ -Proteobacteria	391/438	<i>Vibrio campbellii</i> ATCC baa-1116 chromosome 1	89	CP006605
1g	<i>Dehalogenimonas</i> sp. W1F4	502	Chloroflexi	337/391	<i>Dehalogenimonas alkenigignens</i> SBP1	68	JQ994267
2m	<i>Dehalococcoides</i> sp. W2F9	506	Chloroflexi	347/399	<i>Dehalococcoides</i> sp. BHI80-52	87	AJ431247
3b	<i>Dictyoglomus</i> sp. W3F13	492	Dictyoglomi	334/405	<i>Dictyoglomus thermophilum</i> strain H-12	82	NR_074876
3k	<i>Chloroflexi</i> sp. W3R15	488	Chloroflexi	399/479	Chloroflexi bacterium SCJCAAA240-M02	83	HQ675620
3d	Spirochaetaceae W3F-17	507	Spirochaetes	475/498	Spirochaetaceae bacterium Tc33_10	95	AB518752
4a	<i>Desulfatibacillum</i> sp. W4R18	500	$\delta$ -Proteobacteria	415/486	<i>Desulfatibacillum alkenivorans</i> AK-01	85	NR_074962
4f	<i>Halothermothrix</i> sp. W4R-19	489	Firmicutes	321/407	<i>Halothermothrix orenii</i> strain H 168	79	NR_074915
4h	<i>Thalassiosira</i> sp. W4F24	479	Chloroplast	470/480	<i>Thalassiosira gravida</i> isolate C140 chloroplast	97	FJ002211
4g	<i>Lutimonas</i> sp. W4R24	499	Bacteroidetes	473/500	<i>Lutimonas</i> sp. MOLA 107	95	AM990881
5g	<i>Dehalogenimonas</i> sp. W5F27	479	Chloroflexi	404/480	<i>Dehalogenimonas</i> sp. SBP1	84	JQ994267
5m	<i>Dehalococcoides</i> sp. W5F28	477	Chloroflexi	411/465	<i>Dehalococcoides</i> sp. BHI80-52	88	AJ431247
5h	<i>Thalassiosira</i> sp. W5F30	501	Chloroplast	479/489	<i>Thalassiosira gravida</i> isolate C140 chloroplast	98	FJ002211
5a	<i>Desulfofaba</i> sp. W5R31	509	$\delta$ -Proteobacteria	400/473	<i>Desulfofaba gelida</i> strain PSv29	85	NR_O28730
2k	<i>Chloroflexi</i> sp. W2S	467	Chloroflexi	456/467	Uncultured Chloroflexi bacterium clone D28	98	GQ249570.1
4k	<i>Chloroflexi</i> sp. W4F23	486	Chloroflexi	383/462	Chloroflexi bacterium SCJCAAA240-C11	83	HQ675557
4m	<i>Dehalococcoides</i> sp. W4F21	491	Chloroflexi	434/498	<i>Dehalococcoides</i> sp. BHI80-15	87	AJ431246

sp., *Dehalococcoides* sp. and *Vibrio* sp., where each comprised 33% of the obtained sequences. *Vibrio* sp. was found only in site W1. Site W2 contained 50% each of Chloroflexi-related bacteria and *Dehalococcoides* sp. Site W3 contained 33% of Spirochaetes-related sequences and 34% of *Dictyoglomus* sp., both of which were not found in the other four sites. Site W3 did not contain *Dehalococcoides*-related sequences, which were present in all the other sites. Site W4 harbored larger diversity of bacteria than the rest of the sites based on the sequencing results. Apart from *Dehalococcoides* sp., site W4 contained three unique species that were not found in the rest of the sites. These were *Lutimonas* sp., *Halothermothrix* sp. and *Desulfatibacillum* sp. Site W5 with

four different kinds of bacteria, had the second most diverse bacterial community. It contained 40% of the *Dehalococcoides* sp. and 20% of *Desulfofaba* sp. Chloroflexi bacteria were found in three of the five sites and were the second most abundant organisms found in the investigated area. *Thalassiosira* sp. was the third most abundant organism and was found in two sites. *Dehalococcoides* sp. dominated sequences obtained from sites 1, 2, 4 and 5, while Chloroflexi members were found in sites 2, 3 and 4.

**Structure of the biphenyl-utilizing mixed culture.** Sediment samples (uppermost part) from site W2 were enriched in batch cultures supplied with biphenyl as a sole carbon source. The enriched bacteria were

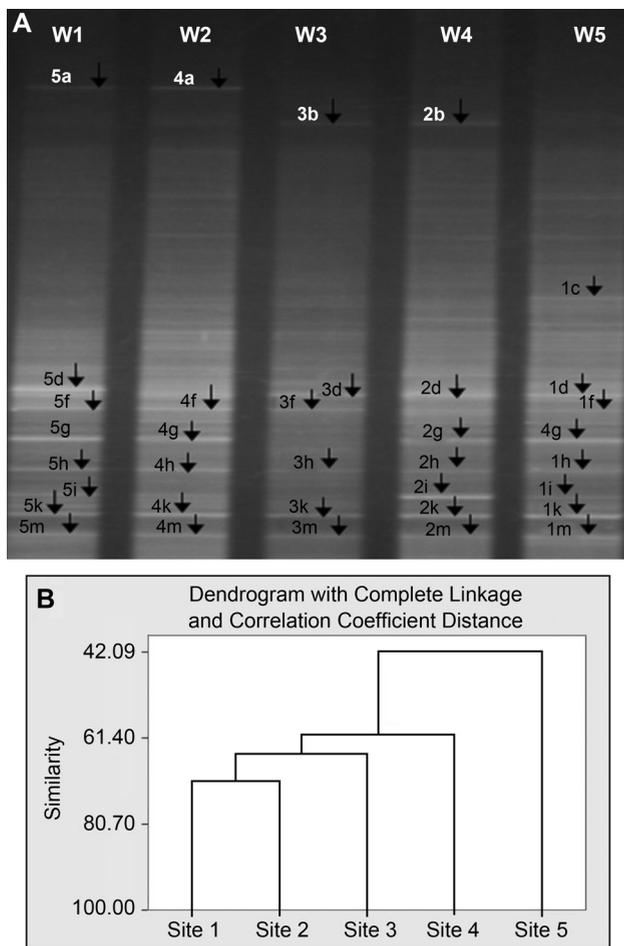


Fig. 4. (A) DGGE profile based on DNA pooled from all the sediment core layers for the 5 sampling sites W1-W5 at Shuwaikh harbor. (B) Cluster analysis performed using binary matrix (0, 1) of DGGE showing variation between bacterial populations inhabiting the five sampling locations W1-W5 (site1 – site 5).

characterized by DGGE. The total number of bands recovered from the mixed culture was 18 as compared to the average number of bands of 14 obtained from the natural sediment bacterial community of site W2. Sequences of bacteria affiliated to Firmicutes (~82%) and Proteobacteria (9%) were detected. *Paenibacillus* sp.-related sequences dominated the sequenced bands.

**Aromatic ring-hydroxylating dioxygenases in Shuwaikh harbor sediments and the biphenyl-degrading mixed culture.** Table III lists the sequences retrieved from the sediment samples along with their closest matches. The genes detected encode proteins similar to phenylpropionate dioxygenase and related aromatic ring-hydroxylating dioxygenases. In addition, protein sequences similar to the large subunit of aromatic ring oxygenases were detected. The biphenyl-utilizing mixed culture contained one sequence that is related to hydrocarbon degradation (the large subunit of aromatic ring oxygenases) (Table III). Cluster analysis showed that sites W2 and W3 shared similar band pattern, while sites W4, W5 and W7 clustered together (Fig. 5).

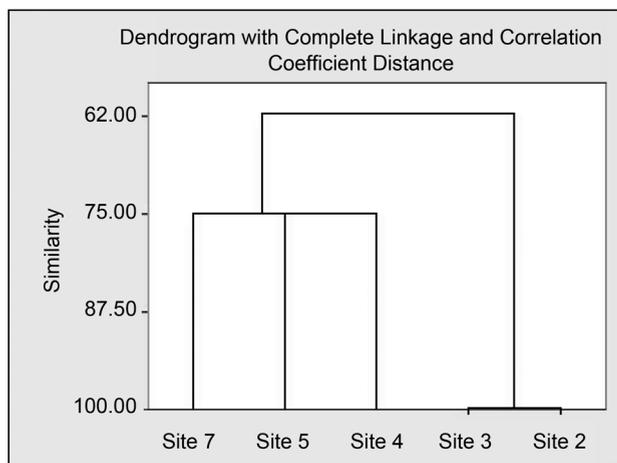


Fig. 5. Cluster analysis of sequences related to aromatic ring-hydroxylating dioxygenases retrieved from different sampling sites at Shuwaikh harbor (sites 2, 3, 4, 5 and 7 designate sites W2, W3, W4, W5 and W7, respectively).

## Discussion

**Pollution of Shuwaikh harbor sediments with PCBs and TPHs.** It was evident from the results that Shuwaikh harbor sediments are polluted with PCBs and TPHs. The presence PCBs in Shuwaikh harbor sediments has not been reported before. However, there are a few reports on PCBs pollution in Kuwait coastal sediments (Gevao *et al.*, 2012) and marine biota (Helaleh *et al.*, 2012). There was a spatial variation among the sampling sites in terms of total PCBs amounts and congener distribution. This could be due to different pollution sources contaminating the different sampling stations. Other factors may be involved such as microbial biodegradation, the pollution history, *in situ* abiotic degradation, diffusion through the sediment column, lateral movement and resuspension of the sediments, and resuspension of PCBs into the water column (Li *et al.*, 2009). Pollution of the Kuwait's coast with petroleum hydrocarbons has been frequently reported (Ahmed *et al.*, 1998; Mahmoud *et al.*, 2009; Michel, 2011). Oil spills, accidental or deliberate, and oil transport and processing operations are major sources of pollution of the Arabian Gulf with petroleum hydrocarbons. The decreasing trend of pollution in the stations from inside to the outside of the harbor is consistent with previous reports (Beg *et al.*, 2001).

**The biphenyl-utilizing mixed culture.** A biphenyl-utilizing culture was enriched from sampling site W2 sediments. This indicates the presence of biphenyl-, and probably PCBs-, degrading bacteria in the W2 sediment. The yellow color observed in the biphenyl whole culture could be due to the meta-cleavage of the aromatic ring of 2,3-dihydroxybiphenyl, which produces 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (Kolar *et al.*, 2007).

Table III  
Gene sequences encoding putative aromatic ring hydroxylases/oxygenases recovered from sediments of the Shuwaikh harbor and a biphenyl-utilizing mixed culture enriched from the sediment of the W2 sampling site.

DGGE Band Code	Sample Name	Number of Bases	Microbial Source	Nearest GenBank Match	Base Compared	Similarity (%)	Accession no
3b	R32	194	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	44/63	70	CAM58106
2a	R211	193	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	40/64	63	CAM58106
5a	R512	193	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	43/64	67	CAM58106
4d	R413	200	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	35/63	56	CAM58106
3a	R311	201	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	44/65	68	CAM58106
3d	R313	203	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	43/66	65	CAM58106
4a	R431	198	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases (large subunit)	42/65	65	CAM58106
2d	R2111	203	Uncultured marine microorganism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenase (large subunit)	38/62	61	CAM58106
–	RB1*	189	<i>Luminiphilus sylvensis</i>	Large subunit aromatic oxygenase	34/56	61	WP_009018933
–	RB2*	192	<i>Luminiphilus sylvensis</i>	Large subunit aromatic oxygenase	41/63	65	WP_009018933
–	FB3*	225	<i>Pseudomonas putida</i> DOT-T1E	Large subunit aromatic oxygenase	33/50	66	YP_006532342

\* Sequences obtained from biphenyl-degrading mixed culture

The W2 culture was shown to be a bacterial consortium as confirmed by the presence of three different bacteria on TSA plates. Kolar *et al.* (2007) reported the isolation of biphenyl-degrading mixed culture from marine sediments. It was not possible to enrich PCBs-degrading cultures from the tested sediments. This could be due to toxicity of the substrate and/or growth conditions that did not allow the growth of PCBs degraders. Alternatively, it is possible that the adopted culture medium and growth conditions were not conducive for the establishment of relevant consortia that can cooperatively degrade the PCB substrate *via* synergistic interactions, provision of necessary growth factors, or elimination of toxic products (McGenity *et al.*, 2012; Mikesková *et al.*, 2012; Van Hamme *et al.*, 2003).

The biphenyl-utilizing mixed culture was dominated by *Paenibacillus naphthalenovorans*-affiliated sequences. This limited taxonomic diversity in the biphenyl-degrading culture W2 is consistent with the results reported by Kolar *et al.* (2007). These authors found that in a biphe-

nyl-degrading mixed culture, six of the seven isolates were affiliated to the genus *Rhodococcus*. Other bacteria that were found in the biphenyl W2 culture represent the minor fraction and are related to *Pseudomonas* and *Azoarcus* spp. Some members of the genera *Paenibacillus*, *Pseudomonas* and *Azoarcus* are known to degrade aromatic compounds under aerobic conditions such as polyaromatic hydrocarbons (PAHs), biphenyl, and PCBs (Daane *et al.*, 2002; Sakai *et al.*, 2005; Ismail and Gescher, 2012; Koubek *et al.*, 2013; Nam *et al.*, 2014). Accordingly, the detection of sequences related to these bacteria in the aerobically grown biphenyl-utilizing mixed culture is not surprising.

**Natural bacterial communities inhabiting Shuwaikh harbor sediments.** The observed variations in number of DGGE bands among the sections of the same sediment core or cores from the different sampling sites might reflect changes in bacterial community structure. The latter could be due to fluctuations in oxygen tension, types and concentrations of the

pollutants (*e.g.*, PCBs congeners), organic carbon content, availability of nutrients, electron acceptors and electron donors, as well as resuspension and sedimentation processes (Black *et al.*, 2017; Correa *et al.*, 2010; Zhang *et al.*, 2015). Decrease in the number of bands with increasing the core depth might reflect the anoxic conditions in the deep layers, which restrict bacterial growth and reproduction.

In general, there was a little spatial variation among the sampling sites in terms of the number of the DGGE bands. Considering the phylum level, the sequenced DGGE bands were affiliated to Chloroflexi, Spirochaetes, Proteobacteria, Firmicutes and Bacteroidetes. Bacteria belonging to these groups have been identified before in sediments polluted with PCBs (and other hydrocarbons) and in enrichment cultures containing PCBs or TPHs. For instance, Zanaroli *et al.* (2012) applied DGGE and T-RFLP to characterize the microbial community enriched in PCBs-containing slurry microcosms from marine sediments. The authors identified members of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\epsilon$ -divisions of Proteobacteria, Firmicutes and Chloroflexi. Moreover, members of Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes and Bacteroidetes were identified in sediment-free PCB-dechlorinating cultures (Wang and He, 2013).

At the genus level, the observed dominance of *Dehalococcoides*-related sequences and other Chloroflexi members is consistent with the chemical analysis, which revealed PCBs pollution of the tested sediments. These bacteria are known key players in the reductive dechlorination of various PCB congeners. To date, all identified PCB-dechlorinating bacteria belong to *Dehalococcoides* spp. and the phylogenetically related Chloroflexi bacteria like DF-1 and o-17 (Bedard, 2008; Zanaroli *et al.*, 2012). Wang and He (2013) identified *Dehalococcoides* spp., *Dehalobacter* spp. and *Dehalogenimonas* spp. in sediment-free cultures dechlorinating Aroclor 1260. Also recently, LaRoe *et al.* (2014) isolated *Dehalococcoides mccartyi* strain JNA in a pure culture dechlorinating Aroclor 1260. The literature, however, does not contain any reports on PCB-dechlorinating bacteria in marine sediments from Kuwait.

Members of the different Proteobacteria subdivisions have been identified by other investigators in hydrocarbon-polluted soil (Correa *et al.*, 2010) and marine sediments even in the Arabian Gulf water (Al-Awadhi *et al.*, 2013; Kolar *et al.*, 2007). Other dominant genera that were detected in the current study include *Dictyoglomus* sp. and a Spirochaetaceae bacterium, which were unique to sampling site W3. *Dictyoglomus thermophilum* is an anaerobic, chemoorganotrophic thermophilic bacterium isolated from a hot spring in Japan (Coil *et al.* 2014). Spirochaetes were identified by Wang and He (2013) in sediment-free cultures dechlorinating Aroclor 1260.

The community of site W4 was characterized by two sequences that were missing in the other sites. *Desulfatibacillum alkenivorans* AK-01 is a sulfate-reducing alkane-degrading bacterium isolated from estuarine sediment (Callaghan *et al.*, 2012). Accordingly, it might be involved in the sulfate reduction-coupled anaerobic biodegradation of aliphatic hydrocarbons in the sediment at site W4. *Halothermothrix orenii* is related to Clostridia. It is a strictly anaerobic thermophilic bacterium isolated from sediment of a Tunisian salt lake (Mavromatis *et al.*, 2009). *Desulfofaba*-related sequence was found only in site W5 sediments. These bacteria are sulfate reducers and were isolated from marine sediments (Knoblauch *et al.*, 1999).

**Aromatic ring-hydroxylating oxygenases in Shuwaikh sediments.** DGGE revealed the presence of sequences related to aromatic ring hydroxylation proteins in the sediments and biphenyl mixed culture. Aromatic ring-hydroxylating dioxygenases are key enzymes in the aerobic degradation of aromatic compounds by many microorganisms (Ismail and Gescher, 2012). Hence, the detection of oxygenases-encoding genes in the sediments and the biphenyl-utilizing culture suggests that they might be involved in the degradation of PCBs, biphenyl and other hydrocarbon pollutants in the Shuwaikh sediment. In accordance with these results, Correa *et al.* (2010) reported higher levels of biphenyl dioxygenases in soil microcosms exposed to Aroclor 1242 and individual PCB congeners. Studies on aromatic ring oxygenases in Kuwait's marine sediments are largely lacking.

**Natural attenuation potential in polluted Shuwaikh sediments.** We have provided some lines of indirect evidence suggesting that Shuwaikh sediments polluted with PCBs and TPHs might exhibit natural attenuation *via* biodegradation/biotransformation. First, sequences affiliated to bacteria that are known as hydrocarbon and PCBs degraders were detected in the sediment and the enriched biphenyl-degrading mixed culture. Second, genes, which encode putative aromatic ring-hydroxylating oxygenases were detected in the sediments and the biphenyl-degrading mixed culture. Third, the observed decrease in the amounts of the high-chlorine PCB congeners (7–10 Cl) as compared to the medium-chlorine ones (4–6 Cl) could be due to microbial reductive dechlorination (Adler *et al.*, 1993; Liang *et al.*, 2014). Moreover, the decreased amount of the low-chlorine PCBs (1–3 Cl) resulting from anaerobic dechlorination is probably because of co-metabolic degradation, which is mediated by oxygenases of the aerobic biphenyl-degrading bacteria (Liang *et al.*, 2014). These are only indirect indicators and further investigations are still needed to unambiguously reveal the microbial biodegradation of organic pollutants in Shuwaikh harbor sediments *in situ*. Future research may

include laboratory microcosms, monitoring temporal changes in pollutants levels and microbial community structure and detection of signature metabolites and key catabolic genes. For better understanding of the ecophysiology and systems biology of the natural microbial communities of the sediments, metaproteomics, metatranscriptomics and metabolomics can be of great value in this context. Furthermore, the application of metagenomics combined with the recent next generation sequencing techniques can help overcome the limitations of DGGE and provide deeper insight into the functional microbial communities.

### Conclusion

Marine sediments of the Shuwaikh harbor in Kuwait are polluted with PCBs and TPHs. These polluted sediments are inhabited by diverse hydrocarbon-degrading bacteria having potential to cope with the toxicity of the pollutants and probably utilize them as carbon sources (TPHs and PCBs) and/or electron acceptors (PCBs).

### Supplementary Materials

Supplementary materials contain tables S1 and S2. Supplementary materials accompanies the paper on Polish Journal of Microbiology website.

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## Isolation and Characteristics of Biotechnologically Important Antagonistic Thermophilic Bacteria from Rhizosphere of *Haloxylon salicornicum*

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### Abstract

Rhizobacteria are an active part of microbial population in the rhizosphere of plants. In this study, twenty rhizobacteria were isolated from the rhizosphere of a perennial grass, *Haloxylon salicornicum*, found in Cholistan desert, an arid landmass near Bahawalpur Pakistan, in one set of experimental conditions. Colony characteristics, biochemical and molecular analyses of these isolates were performed. All isolates were bacilli, gram positive with off-white colonies and exhibited typical bacilli colony morphology. None of the isolates was gelatinase, urease, indole, H<sub>2</sub>S and catalase producer. Eleven isolates were amylase producers and 8 isolates were acid producers. All isolates fermented glucose, 3 fermented lactose and 19 fermented fructose. Molecular data revealed that out of twenty isolates, 14 isolates showed 91–99% identity with *Brevibacillus borstelensis*, 4 with *Bacillus subtilis* (97–98%) and 2 with *Bacillus licheniformis* (94–99%) through BLAST analysis. All identified bacterial isolates cladded with their respective groups in the phylogenetic tree. Many (11–15 out of 20) of the isolates were more effective in inhibiting growth of the tested bacterial strains as compared to the positive control (Ampicillin 50 µg/disc). We conclude that bacilli are the predominant form populating rhizosphere of this desert grass. Among the isolated bacteria *Brevibacillus borstelensis*, *Bacillus subtilis* and *Bacillus licheniformis* are the most predominant species.

**Key words:** *Haloxylon salicornicum*, rhizobacteria, 16S rRNA for phylogenetic analysis, rhizosphere on Cholistan desert

### Introduction

Rhizobacteria can promote plant growth directly or indirectly and are found in rhizosphere in association with plant roots. A great number of bacteria have been reported for their plant growth promoting abilities such as *Bacillus*, *Azotobacter*, *Pseudomonas*, *Klebsiella*, *Alcaligenes*, *Arthrobacter*, *Serratia*, *Burkholderia*, *Azospirillum*, *Enterobacter* and *Rhizobium* (Kumar *et al.*, 2012). Rhizosphere has been defined as the soil volume that is directly influenced by presence of roots of living plants. The rhizosphere thus supports an active microbial population (Ahmad *et al.*, 2008). A number of studies have revealed that due to the presence of rhizodeposits and root exudates, the soil environment of root system is a favorable place of microbial abundance and activity (Hartmann *et al.*, 2008; Smalla *et al.*, 2006). Rhizobacteria are of great interest for their

applications as biofertilizers or pesticides in agriculture and for phytoremediation. The plant growth promoting rhizobacteria (PGPR) can enhance plant growth and yield, and reduce chances of pathogen infection and biotic or abiotic plant stresses (Lugtenberg and Kamilova, 2009; van Loon and Bakker, 2006). In a number of cases, the required effects of PGPR are not attained in the field probably due to insufficient rhizosphere or plant colonization required for revealing beneficial effects (Lugtenberg *et al.*, 2001), and the lack of thorough understanding of the mechanisms responsible to promote plant growth.

*Haloxylon salicornicum* (vernacular name: *Lana* or *Khar*) belongs to Chenopodiaceae family and is found in Cholistan desert of Pakistan as a common shrub (Shafi *et al.*, 2002). It is much branched, erect leafless and woody at base perennial shrub. Its stem and branches are pale yellow, flowers and fruits are

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not found, and joints produce into two short triangular points in the form of leaves and are woolly within. The plant is used as fodder for domestic animals and is mostly grazed by camels. It has high salt contents and therefore, better to retrieve the desert soil. The extract of this plant is used for washing clothes (Arshad *et al.*, 2002). The plant has medicinal importance and is taken as tea by women who are experiencing problems during pregnancy and to relieve dysmenorrhea (Saleem *et al.*, 2013). Because of its unique characteristics, this plant is expected to host biotechnologically important microbes in its rhizosphere.

Ribosomal RNA sequences especially 16S rRNA are the most important targets for bacterial identification to study evolution, ecology and to determine phylogenetic relationships among various taxa. These sequences also serve for the exploration of bacterial diversity present in an environment and quantification of relative abundance of taxa of different ranks (Hugenholtz *et al.*, 1998). The 16S rRNA gene is suitable for bacterial identification because it allows studying phylogenetic relationships among distant taxa. The gene of 16S rRNA is functionally indispensable part of the core gene set (Daubin *et al.*, 2003) that promotes its importance for studying phylogenetic relationships. It is subject to variations, especially in the variable regions, and allows enough diversification for bacterial classification. The suitable PCR primers have been enabled due to the presence of conserved regions in 16S rRNA gene to study different taxa at various taxonomic levels that range from individual bacterial strains to whole phyla (Head *et al.*, 1998).

The current study was carried out to isolate some thermophilic bacterial strains from the rhizospheric soil of *H. salicornicum* of Cholistan desert. Morphological and biochemical properties of the isolated bacteria are reported. The 16S rRNA gene was amplified to identify these bacterial isolates and to study their phylogenetic relationships with other strains.

## Experimental

### Material and Methods

**Study area.** Cholistan desert spreads over an area of 26,000 km<sup>2</sup> and is situated in Southern Punjab of Pakistan. It is located between 27°42' and 29°45' N latitude and 69°52' and 75°24' E longitude (Wariss *et al.*, 2013). It is sandy and hot desert with mean annual rainfall of 100–150 mm. The rain usually falls during the period of monsoon, spring and winter seasons. The temperature reaches above 50°C in summer (Jamil *et al.*, 2013). The inconsistent rainfall promotes only spiny, leafless, small and grazed shrubs in addition to some trees. The soils of

this desert are mostly alkaline, saline, gypsiferous and often dunned (Akbar *et al.*, 1996).

**Collection of rhizospheric soil.** Soil samples (5–10 g) from the rhizosphere of *H. salicornicum* growing in Cholistan were collected at various places in extreme summer (June and July), winter (December and January) and rainy seasons (August and September), mixed to get random samples. Samples were collected in sterile sealed plastic bags on dry ice using ethanol flamed spatula, preserved in properly labeled sterile plastic jars and stored in a dark and cool place. Some soil samples were also stored at 4°C and processed within a week for biochemical and molecular analyses.

**Growth of cultivable bacteria in sterile normal saline/Ringer solution.** Rhizospheric microbial suspension was made from the soil sample (1 g) including pieces of plant roots, about 3.5 cm in length, in 30 ml sterile ringer solution in a sterile cotton plugged 250 ml flask. After continuous swirling on shaker a homogeneous suspension was obtained that was used as stock to prepare serial dilutions.

The original suspension was serially diluted to get 6 dilutions ranging from 10<sup>-1</sup> to 10<sup>-6</sup>. For bacterial growth, 100 µl of soil suspension (10<sup>-3</sup> to 10<sup>-6</sup> dilutions) was taken and poured/spread on LB agar plates. This step was followed by incubation of the plates at 30°C for 16–24 hrs. Several colonies were observed and colonies seen to be inhibiting the growth of another bacterial colony and having clear zone around (data not shown) were considered as antagonistic to each other and were isolated.

To get purified isolates, individual bacterial colonies grown on agar plates were picked and transferred to fresh LB agar plates. Repeated streaking and transfer was attempted to obtain pure strains. Following isolation, pure cultivable bacteria were stored as LB glycerol stocks (LB: glycerol, 50:50) at -70°C till further analysis.

For analyses, overnight LB broth cultures of bacterial isolates were grown using LB glycerol stocks as seed cultures.

**Determination of antibacterial activity of the isolates.** Disc diffusion method was used to evaluate the antibacterial potential of the isolates. Broth cultures of the isolates were grown for 16 hours at 50°C and 40 µl of the culture's supernatant was applied on the filter paper discs (5 mm diameter) in installments of 10 µl. Discs were tested against 4 Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Shigella sonnei*) and 2 Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*).

**Physical and biochemical analysis of cultivable bacteria.** Colony and staining characteristics like gram staining, motility and cell morphology *etc.* of bacterial isolates were observed under the microscope. Biochemical analysis included tests for the production of amylase, acid, urease, gelatinase, catalase, protease,

H<sub>2</sub>S and antibiotic production. Analysis was further extended to MR, VP, motility, and glucose, lactose and fructose fermentation tests. All analyses were carried out using standard reagents and following optimized procedures (Cappuccino and Sherman, 1999).

**Extraction of genomic DNA from bacterial isolates.** LB broth cultures of purified bacterial isolates were used for DNA isolation using standard protocol consisting of four stages including disruption, lysis, removal of proteins and contaminants, and finally recovery of DNA. In this study, SDS based method was employed (Vivantis, Kit. Catalogue No. GF-BA-100 preps) as suggested by the manufacturer.

**Amplification of 16S rRNA gene.** For the amplification of 16S rRNA gene of bacterial isolates, PCR conditions were optimized and reactions were carried out in a thermocycler (MY GENE<sub>tm</sub> Model MG-96+). Universal forward and reverse primers specifically designed and reported for bacterial 16S rRNA genes in earlier studies (Porteous and Armstrong, 1993) were used for PCR amplification. Forward and reverse primers used in this study are shown below.

Sense primer: 5'-AACACATGCAAGTGGAAAC-3'  
Antisense primer: 5'-ACGGGCGGTGTGTACAAG-3'

Expected size of PCR amplicons using *E. coli* DNA was calculated to be 1357 bp. Each tube was loaded with 50 µl final volume of a solution containing 1 × PCR buffer, 1.5 mM MgCl<sub>2</sub>, 100 µM dNTPs mix, 0.3 µl of each primer (10 pmol), 1.25U of *Taq* DNA polymerase and an appropriate amount of chromosomal DNA. PCR amplifications were carried out as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles each of 94°C for 30 s, 50°C for 60 s and 72°C for 60 s with a final extension step of 72°C for 10 min.

**RFLP analysis of 16S rRNA amplicons using four cutter restriction enzymes.** Five types of 4 base cutter restriction enzymes, *RsaI*, *TaqI*, *HpaII*, *HinfI*, and *HhaI*, were used in this study for RFLP of 16S rRNA gene PCR amplicons. The documented gels were labeled using 1 and 0 to indicate the presence/absence of band on gel. Data was transferred to excel sheet for preparation of dendrograms by PAST-3 software. Dendrograms helped to identify the pattern of similarity and dissimilarity within the group and among the other groups of rhizospheric soils bacteria.

**Phylogenetic analysis of 16S rRNA genes.** PCR amplified 16S rRNA genes of the isolates were selected based on dendrograms analyses. At least 2–3 members of each group were randomly selected and sequenced (Macrogen, South Korea). The sequences of 16S rRNA genes were assembled and analyzed using BLAST (Altschul *et al.*, 1990). Along with our twenty sequences for 16S rRNA, twelve sequences of the most nearest relatives were also retrieved from GenBank for the comparison study. All the sequences were aligned

using ClustalX and imported into the Bioedit program for manual alignment. Neighbor joining phylogenetic tree for 16S rRNA gene was constructed and the evolutionary analyses were conducted using MEGA7 (Kumar *et al.*, 2016) with 100 bootstrap replicates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. The final dataset consisted a total of 682 positions.

## Results

**Determination of the antibacterial activity.** Discs prepared using cultures of the isolated bacteria exhibited antibacterial activity against all tested microorganisms. Ampicillin was used as standard and tested at the concentration of 50 µg/disc. It is obvious from the results (Table I) that some isolates (HSHM1105, HSHM1109, HSHM1111, HSHM1118, HSHM1207, HSHM1212-HSHM1215, HSHM1219) were more effective against *E. coli* as compared to ampicillin. Isolates HSHM1103-1105, HSHM1117, HSHM1120, HSHM1202, HSHM1206, HSHM1208, HSHM1212, HSHM1214-1216, HSHM1219 were found to possess higher antibacterial potential against *P. aeruginosa* than the standard. Similarly, all isolates except HSHM1109, HSHM1110 and HSHM1216 exhibited an antagonistic behavior against *S. typhi* higher than the standard. However, against *S. sonnei* 12 isolates (HSHM1111, HSHM1117, HSHM1118, HSHM1120, HSHM1201, HSHM1202, HSHM1206-1208, HSHM1213-1215) exhibited antibacterial activity higher than ampicillin. Multiple isolates were examined to be more active against *S. aureus* (HSHM1118, HSHM1120, HSHM1201, HSHM1207, HSHM1208, HSHM1212-1216, HSHM1219) and *B. subtilis* (HSHM1103, HSHM1104, HSHM1111, HSHM1118, HSHM1120, HSHM1202, HSHM1206-1208, HSHM1212, HSHM1214-1216, HSHM1219) as compared to ampicillin.

**Phenotypic characterization of bacterial isolates.** Total 20 rhizobacteria were isolated on agar plates from the rhizosphere of *H. salicornicum*. The bacterial isolates showed various phenotypic properties including different morphological traits, fermentable carbohydrates profiles and enzyme production patterns (Table II). In all cases, colonies were off-white, round and irregular after 15 and 24 hours, respectively. No chromogenesis and odor was observed in any bacterial isolate. All isolates were found to be gram-positive bacteria that belonged to bacilli class that is an extremely

Table I  
Antibacterial activity of selected bacteria isolated from rhizosphere of *Haloxylon salicornicum* (HS)\*.

Sr.	Tested isolate	Zone of inhibition (mm) against					
		Gram-negative bacteria			Gram-positive bacteria		
		<i>E. coli</i> Mean ± SD	<i>P. aeruginosa</i> Mean ± SD	<i>S. typhi</i> Mean ± SD	<i>S. sonnei</i> Mean ± SD	<i>S. aureus</i> Mean ± SD	<i>B. subtilis</i> Mean ± SD
1	HSHM1201	11.33 ± 0.57	13.00 ± 0.00	<b>18.66 ± 1.125</b>	<b>18.33 ± 1.00</b>	<b>20.00 ± 1.00</b>	10.00 ± 0.00
2	HSHM1202	13.33 ± 0.57	<b>19.00 ± 0.00</b>	<b>17.00 ± 0.00</b>	<b>21.00 ± 1.00</b>	12.33 ± 0.57	<b>16.66 ± 0.57</b>
3	HSHM1103	11.33 ± 0.57	<b>16.00 ± 0.00</b>	<b>15.00 ± 0.00</b>	8.66 ± 0.57	9.33 ± 0.57	<b>14.00 ± 1.00</b>
4	HSHM1104	11.00 ± 0.00	<b>16.66 ± 0.57</b>	<b>15.00 ± 1.00</b>	8.63 ± 0.57	9.33 ± 0.57	<b>14.00 ± 0.00</b>
5	HSHM1105	<b>17.33 ± 1.13</b>	<b>17.33 ± 1.13</b>	<b>18.66 ± 0.57</b>	11.00 ± 1.00	12.00 ± 0.00	12.33 ± 0.57
6	HSHM1206	12.00 ± 1.00	<b>22.33 ± 0.57</b>	<b>22.00 ± 0.00</b>	<b>21.00 ± 1.00</b>	12.00 ± 0.00	<b>16.66 ± 0.57</b>
7	HSHM1207	<b>20.00 ± 0.00</b>	9.00 ± 0.00	<b>17.33 ± 0.57</b>	<b>22.33 ± 0.57</b>	<b>18.33 ± 1.13</b>	<b>21.33 ± 1.13</b>
8	HSHM1208	12.33 ± 0.57	<b>21.33 ± 0.57</b>	<b>23.66 ± 0.57</b>	<b>14.00 ± 0.00</b>	<b>20.00 ± 0.00</b>	<b>15.00 ± 1.00</b>
9	HSHM1109	<b>20.00 ± 0.00</b>	8.00 ± 0.00	8.33 ± 0.57	9.00 ± 1.00	9.00 ± 1.00	13.00 ± 0.00
10	HSHM1110	<b>15.66 ± 0.57</b>	8.66 ± 0.57	8.00 ± 0.00	9.00 ± 0.00	9.33 ± 0.57	13.33 ± 0.57
11	HSHM1111	<b>15.00 ± 0.00</b>	9.33 ± 0.57	<b>15.33 ± 0.57</b>	<b>18.33 ± 0.57</b>	8.00 ± 0.00	<b>15.33 ± 1.13</b>
12	HSHM1212	<b>17.33 ± 0.57</b>	<b>25.00 ± 0.00</b>	<b>16.66 ± 0.57</b>	12.33 ± 0.57	<b>21.00 ± 0.00</b>	<b>17.33 ± 0.57</b>
13	HSHM1213	<b>20.00 ± 0.00</b>	11.00 ± 1.00	<b>15.33 ± 0.57</b>	<b>18.66 ± 0.57</b>	<b>18.33 ± 0.57</b>	12.33 ± 0.57
14	HSHM1214	<b>20.66 ± 0.57</b>	<b>22.33 ± 0.57</b>	<b>23.00 ± 0.00</b>	<b>20.00 ± 0.00</b>	<b>20.00 ± 1.00</b>	<b>21.00 ± 1.00</b>
15	HSHM1215	<b>20.33 ± 1.13</b>	<b>22.33 ± 0.57</b>	<b>23.33 ± 1.13</b>	<b>22.00 ± 1.00</b>	<b>18.33 ± 0.57</b>	<b>21.00 ± 0.00</b>
16	HSHM1216	13.33 ± 1.13	15.33 ± 0.57	10.33 ± 0.57	9.00 ± 0.00	<b>20.00 ± 1.00</b>	<b>20.00 ± 0.00</b>
17	HSHM1117	13.33 ± 0.57	<b>17.00 ± 1.00</b>	13.66 ± 0.57	<b>19.33 ± 0.57</b>	8.00 ± 0.00	13.00 ± 0.00
18	HSHM1118	<b>25.00 ± 1.00</b>	13.33 ± 0.57	<b>15.00 ± 1.00</b>	<b>18.66 ± 0.57</b>	<b>17.33 ± 1.13</b>	<b>15.00 ± 0.00</b>
19	HSHM1219	<b>17.33 ± 0.57</b>	<b>22.00 ± 0.00</b>	<b>15.00 ± 1.00</b>	12.33 ± 0.57	<b>15.33 ± 1.13</b>	<b>17.00 ± 0.00</b>
20	HSHM1120	11.00 ± 1.00	<b>18.33 ± 0.57</b>	13.33 ± 0.57	<b>18.66 ± 0.57</b>	<b>18.00 ± 0.00</b>	<b>22.33 ± 1.13</b>
21	Ampicillin	13.22 ± 0.58	13.15 ± 0.65	13.11 ± 1.22	12.99 ± 0.59	13.01 ± 1.23	13.45 ± 0.89
		11	12	15	12	11	14

diverse group of bacteria. Production of urease, gelatinase, indole and hydrogen sulfide was not seen in these isolates. Catalase production and glucose fermentation were observed in all selected bacterial isolates.

**Analysis of 16S rRNA sequences of isolates.** The selected isolates showed varying percentage identity with different bacteria through nucleotide BLAST search of partial sequences (~ 1100 bp) of the 16S rRNA. Although, it has been suggested that RFLP can help differentiate bacterial strains up to species level (Dec *et al.*, 2016), in the present study, phylogenetic trees developed on the basis of RFLP and base sequences exhibited some differences. This could be attributed to the number of restriction enzymes used for RFLP analyses.

Nearest relatives of selected bacteria isolated from rhizosphere of *H. salicornicum* are given (Table III) along with their percentage identity. Out of twenty nearest relative isolates, 14 isolates showed 91–99% identity with *Brevibacillus borstelensis*, 4 with *Bacillus subtilis* (97–98%) and 2 isolates with *Bacillus licheniformis* (94–99%). The partial nucleotide 16S rRNA gene sequences of all bacterial isolates were deposited in GenBank have been allotted accession numbers (Table III).

A neighbor-joining (NJ) phylogenetic tree was generated through aligning 16S rRNA sequences of the bacterial isolates examined in this study and their nearest relatives taken from GenBank, NCBI (Fig. 1) to study their evolutionary relationships and cladding pattern. Three main groups of bacteria were observed in this phylogeny on the basis of which bacteria fell in different clades. *HS1219*, *HS1215*, *HS1214*, *HS1208*, *HS1202*, *HS1119*, *HS1118*, *HS1110*, *HS1201*, *HS1120*, *HS1103*, *HS1207*, *HS1105* and *HS1109* grouped with *B. borstelensis*, showing their homology with other *B. borstelensis* species. Only two isolates, that is, *HS1206* and *HS1213* were found in the clade of *B. licheniformis* with ESR26 and PF4H\_1 strains of *B. licheniformis*. Four isolates were identified as *Bacillus subtilis* species *HS1111*, *HS1212*, *HS1104* and *HS1216*) but the interesting observation in this clade was the unusual branching of *HS1111* that was not cladded with other species of *B. subtilis*. The nearest relative of *HS1111* (accession number: DQ420172) was also found as a separate branch above *HS1111* that was showing evolutionary divergence of both sequences from other *B. subtilis* species. Phenotypic characteristics showed that *HS1111* is

Table II  
Phenotypic characteristics of selected bacteria isolated from rhizosphere of *Haloxylon salicornicum* (HS)\*.

Sr. No.	Tested Parameter	Result	Samples % (n)	Sample ID
<b>(a) Morphological characteristics</b>				
1	Color	Off-white	100 (20)	All
2	Margin	Branching	10 (2)	HS1103, HS1111
		Non-branching	90 (18)	HS1104, HS1105, HS1109, HS1110, HS1118, HS1119, HS1120, HS1201, HS1202, HS1206, HS1207, HS1208, HS1212, HS1213, HS1214, HS1215, HS1216, HS1219
3	Elevation	+ve	10 (2)	HS1103, HS1111
		-ve	90 (18)	HS1104, HS1105, HS1109, HS1110, HS1118, HS1119, HS1120, HS1201, HS1202, HS1206, HS1207, HS1208, HS1212, HS1213, HS1214, HS1215, HS1216, HS1219
4	Configuration after 15 hrs	Round	100 (20)	All
5	Configuration after 24 hrs	Irregular	100 (20)	All
6	Chromogenesis	-ve	100 (20)	All
7	Opacity	+ve	10 (2)	HS1103, HS1111
		-ve	90 (18)	HS1104, HS1105, HS1109, HS1110, HS1118, HS1119, HS1120, HS1201, HS1202, HS1206, HS1207, HS1208, HS1212, HS1213, HS1214, HS1215, HS1216, HS1219
8	Odor	No odor	100 (20)	All
9	Gram Staining	+ve	100 (20)	All
10	Cell Morphology	Bacilli	100 (20)	All
<b>(b) Biochemical characteristics</b>				
1	Amylase production	+ve	55 (11)	HS1201, HS1202, HS1206, HS1212, HS1214, HS1216, HS1103, HS1104, HS1111, HS1119, HS1118
		-ve	45 (9)	HS1105, HS1109, HS1110, HS1120, HS1207, HS1208, HS1213, HS1215, HS1219
2	Acid production	+ve	45 (8)	HS1105, HS1109, HS1110, HS1120, HS1208, HS1213, HS1215, HS1219
		-ve	55 (12)	HS1103, HS1104, HS1111, HS1118, HS1119, HS1201, HS1202, HS1206, HS1207, HS1212, HS1214, HS1216
3	Urease production	-ve	100 (20)	All
4	Gelatinase production	-ve	100 (20)	All
5	Motility test	+ve	15 (3)	HS1118, HS1208, HS1215
		-ve	85 (17)	HS1103, HS1104, HS1105, HS1109, HS1110, HS1111, HS1119, HS1120, HS1201, HS1202, HS1206, HS1207, HS1212, HS1213, HS1214, HS1216, HS1219
6	MR	-ve	100 (20)	All
7	VP	-ve	100 (20)	All
8	Indol production	-ve	100 (20)	All
9	H <sub>2</sub> S production	-ve	100 (20)	All
10	Catalase production	+ve	100 (20)	All
11	Glucose fermentation	+ve	100 (20)	All
12	Lactose fermentation	+ve	15 (3)	HS1208, HS1213, HS1215
		-ve	85 (17)	HS1103, HS1104, HS1105, HS1109, HS1110, HS1111, HS1118, HS1119, HS1120, HS1201, HS1202, HS1206, HS1207, HS1212, HS1214, HS1216, HS1219
13	Fructose fermentation	+ve	95 (19)	HS1103, HS1104, HS1105, HS1109, HS1110, HS1111, HS1118, HS1119, HS1120, HS1201, HS1202, HS1206, HS1207, HS1208, HS1213, HS1214, HS1215, HS1216, HS1219
		-ve	5 (1)	HS1212
14	Litmus (Milk)	-ve	100 (20)	All

Total number of isolates = 20, n = number of samples, +ve = Gram-positive, -ve = Gram-negative,

\* Names of the isolates have been abbreviated as HS for convenience. Names of the in-lab stocks start with HSHM.

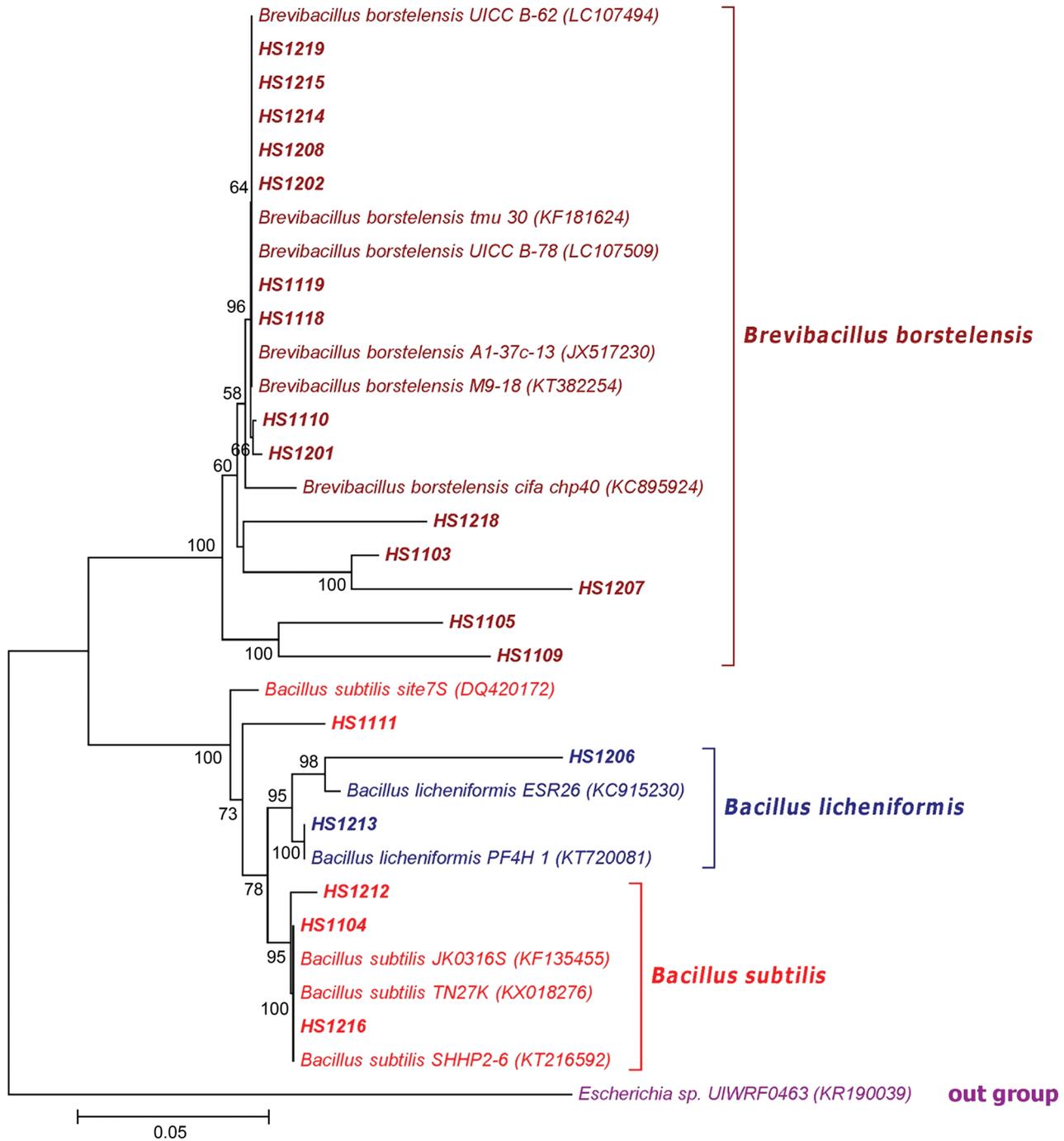


Fig. 1. Neighbour-joining phylogenetic tree of the selected bacterial isolates.

The tree was rooted with *Escherichia* spp. as an out-group. The scale bar represents the sequence divergence. Bootstrap values (100 replicates) are shown at the nodes. The optimal tree with the sum of branch length = 0.65614117 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

different from other *B. subtilis* species in terms of margin, elevation and opacity as it had branching filaments with positive values for colony elevation and opacity.

**RFLP and phylogenetic analysis.** RFLP analysis of the 20 selected bacterial isolates using 16S rRNA products identified 5 distinct patterns. Representative patterns are shown in Fig. 2. These patterns were found to be consistent and absolutely reproducible. Among 222 16S rRNA

restriction fragments recorded, *RsaI* and *HinfI* each gave 20 shared fragments (18% of total), 16 fragments were unique among accessions (7.2%) and remaining 166 fragments were phylogenetically informative (74.7%). A dendrogram was constructed based on RFLP analysis of 16S rRNA gene of bacteria isolated from rhizosphere of *H. salicornicum* (Fig. 3). The RFLP dendrogram showed two main clades (labeled with letters A and B).

Table III  
Nearest relatives of selected isolates of bacteria.

Isolate	Allotted Accession Number	Nearest relative	Accession Number	Identity (%)
HS1103	KX426601	<i>Brevibacillus borstelensis</i> M9-18	KT382254.1	93
HS1104	KX426602	<i>Bacillus subtilis</i> JK0316S	KF135455	98
HS1105	KX426603	<i>Brevibacillus borstelensis</i> A1-37c-13	JX517230.1	93
HS1109	KX426604	<i>Brevibacillus borstelensis</i> cifa_chp40	KC895924.1	96
HS1110	KX426605	<i>Brevibacillus borstelensis</i> M9-18	KT382254.1	97
HS1111	KX426606	<i>Bacillus subtilis</i> site7S	DQ420172.1	97
HS1118	KX426607	<i>Brevibacillus borstelensis</i> M9-18	KT382254.1	99
HS1119	KX426608	<i>Brevibacillus borstelensis</i> UICC B-78	LC107509.1	99
HS1120	KX426609	<i>Brevibacillus borstelensis</i> tmu30	KF181624.1	95
HS1201	KX426610	<i>Brevibacillus borstelensis</i> M9-18	KT382254.1	96
HS1202	KX426611	<i>Brevibacillus borstelensis</i> UICC B-78	LC107509.1	98
HS1206	KX426612	<i>Bacillus lecheniformis</i> ESR26	KC915230.1	94
HS1207	KX426613	<i>Brevibacillus borstelensis</i> A1-37c-13	JX517230.1	91
HS1208	KX426614	<i>Brevibacillus borstelensis</i> M9-18	KT382254.1	98
HS1212	KX426615	<i>Bacillus subtilis</i> TN27K	KX018276.1	98
HS1213	KX426616	<i>Bacillus lecheniformis</i> PF4H_1	KT720081.1	99
HS1214	KX426617	<i>Brevibacillus borstelensis</i> M9-18	KT382254	98
HS1215	KX426618	<i>Brevibacillus borstelensis</i> UICC B-62	LC107494.1	97
HS1216	KX426619	<i>Bacillus subtilis</i> SHHP2-6	KT216592	98
HS1219	KX426620	<i>Brevibacillus borstelensis</i> M9-18	KT382254.1	99

Clade B has only five isolates, that is, HS1109, HS1119, HS1202, HS1208 (all have identity with *B. borstelensis*) and HS1213 (that has identity with *B. lecheniformis*). Clade A was further divided into 2 subclades (labeled with a1 and a2). Only three isolates, that is, HS1214,

HS1215 (had identity with *B. borstelensis*) and HS1216 (identity with *B. subtilis*) fell into subclades a2. Species of the same genera did not appear together in RFLP dendrograms revealing that identification of bacterial isolates on the basis of RFLP is not a reliable technique.

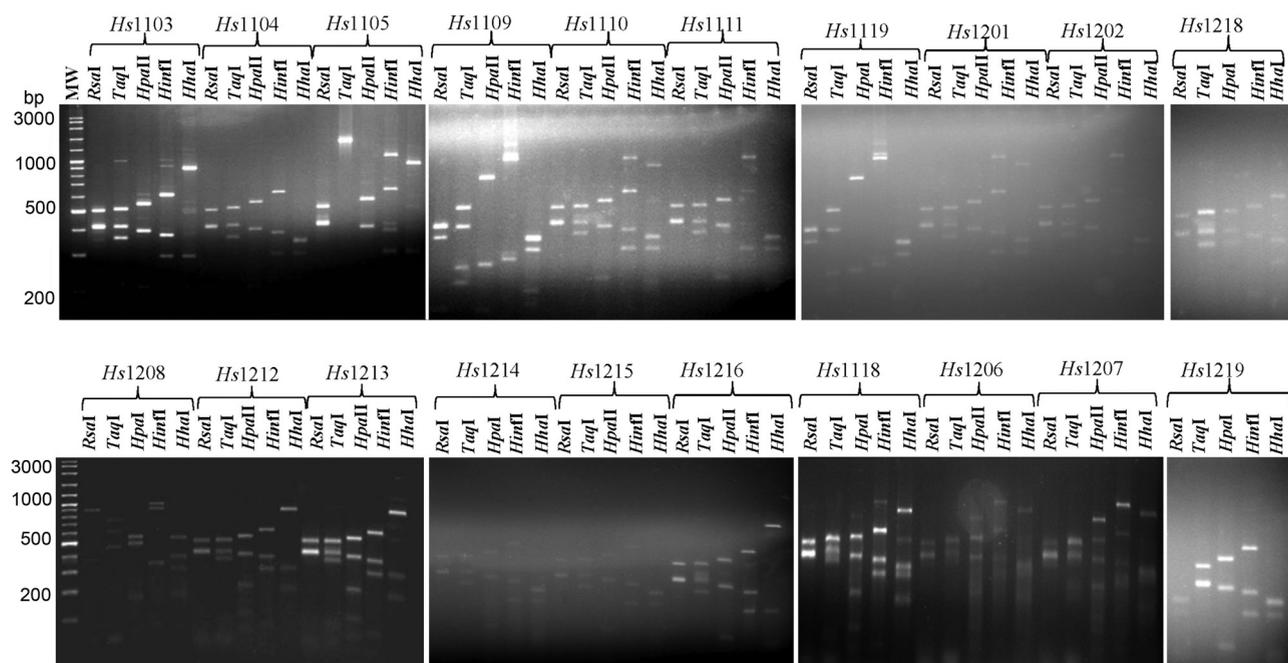


Fig. 2. RFLP analysis of the selected bacterial isolates using 5 different restriction endonucleases.

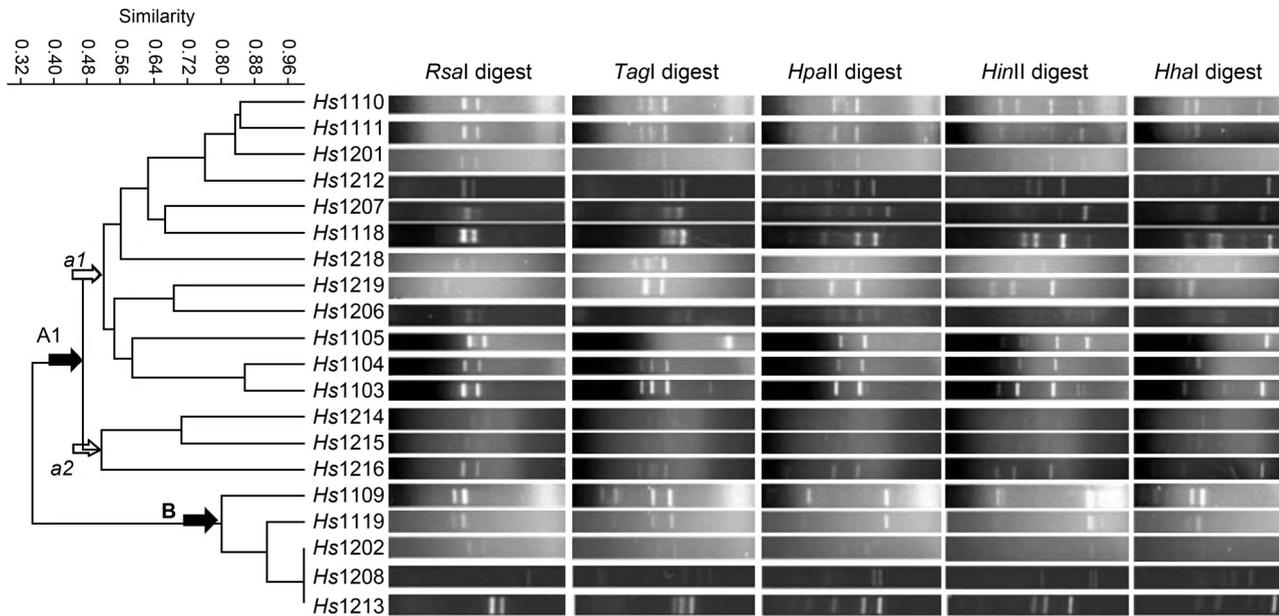


Fig. 3. Dendrogram constructed based on RFLP analysis of 16S rRNA gene of bacteria isolated from rhizosphere of *H. salicornicum*.

### Discussion

A number of plant-associated rhizobacteria are known for their role in promotion of plant growth and to increase plant resistance against different diseases and abiotic stresses. Cholistan desert has not been explored for its rhizobacterial diversity associated with its plants. Therefore, the present study represents the very first study of its kind that has been performed to isolate and identify rhizobacteria from a representative plant *H. salicornicum* from Cholistan desert, a desert having unique soil with high salinity and basic pH (Hameed *et al.*, 2011). Based on biochemical characterization these isolates were found to be catalase positive and glucose fermentive; 19 were found positive for fructose fermentation and 11 were positive for amylase production. Amylase producers are more important industrially because bacterial enzymes are obtained in high yield and they possess high thermostability. A thermostable  $\alpha$ -amylase from *B. licheniformis* has also been reported (Morgan and Priest, 1981).

Bacterial classification is accomplished when morphological and biochemical methods are used along with molecular methods of analysis. The amplified fragment length polymorphism (AFLP) technique of DNA fingerprinting has been shown to be useful for the identification of different bacterial species (Duim *et al.*, 2001) but it is expensive and laborious. Another technique, whole-genome DNA-DNA hybridization allows identification of bacterial species but is not routinely used (Stackebrandt and Goebel, 1994). Community structures, metabolic function, composition and ecological roles are the characteristics of different microbial communities. The 16S rRNA gene has been employed

to investigate environmental microbial diversity that does not require isolation and cultivation of bacteria to offer phylogenetic taxonomic classification. Because of heterogeneity of 16S phylogenetic marker among operations of same genome or lack of its resolution at species level, the use of this technique is often criticized but the technique is still believed to be a standard for bacterial identification (Pontes *et al.*, 2007). The 16S rRNA based identification of bacteria has therefore come up as a potential and useful alternative.

In the present studies, 14 *Brevibacillus* and 6 *Bacillus* (4 *B. subtilis* and 2 *B. licheniformis*) species were identified from rhizosphere of *H. salicornicum*. The isolate HS1111 depicted a divergence through different branching pattern in the phylogenetic tree because it didn't clade with other strains of *B. subtilis* (Fig. 1). The importance of *Bacillus* species in plant growth promotion is widely accepted and a large number including *B. subtilis* have been reported for their bio-control because of the production of various antibiotics. Plant growth promotion is also supported through phytohormones production, phosphate solubilization and release of ammonia from nitrogenous organic matter (Hayat *et al.*, 2010). *B. subtilis* and *B. licheniformis* are also reported for producing two important plant hormones, indole-3-acetic acid and indole-3-butyric acid (Lim and Kim, 2009). It has also been observed that the colonization of *B. subtilis* at root hairs of plants is accompanied with morphological changes of these root hairs (Huang *et al.*, 2011). The PGPR strain of *B. licheniformis* has been found capable of survival under drought stress conditions and the PGPR treated plants, therefore, continue to accumulate plant growth when exposed to drought stress (Lim and Kim, 2013). In other studies, it

is also revealed that *B. licheniformis* has great resistance against abiotic stress including drought stress (Cheng *et al.*, 2007; Klopper *et al.*, 2007; Sziderics *et al.*, 2007).

*Bacillus* species are aerobic endospore forming plant growth promoting rhizobacteria which are known to produce many valuable enzymes including proteases, amylases, laccase and lipases. In addition, the *Bacillus* species have potential to degrade complex carbohydrates including cellulose, xylulose and oligosaccharides like arabinogalactan, stachyose and raffinose (Ahmad *et al.*, 2008; Amoa-Awua and Jakobsen, 1995; Larsen *et al.*, 2014; Ouoba *et al.*, 2003a; Ouoba *et al.*, 2003b; Ouoba *et al.*, 2003c; Ouoba *et al.*, 2007; Reiss *et al.*, 2011). Many *Bacillus* species have been identified to have antimicrobial potential. Antimicrobial potential of thermophilic bacterial species including *Brevibacillus borstelensis* and *B. licheniformis* has been documented against gram positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*) and gram negative bacteria including *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia* (Muhammad *et al.*, 2009). *B. subtilis* is capable of producing an amocumacin-A like secondary metabolite that has antimicrobial potential against *Candida albicans*, *Ustilago maydis*, *Cryptococcus neoformans*, *S. aureus* and *E. coli* (Esikova *et al.*, 2002; Shankarrao *et al.*, 2014). *B. borstelensis* and *B. licheniformis* induce systemic resistance in plants and inhibit many phytopathogens (Klopper *et al.*, 2004; Sharma *et al.*, 2014). Contrary to these positive effects, few studies have reported the pathogenic properties of *B. licheniformis* against humans (Salkinoja-Salonen *et al.*, 1999). Moreover, *Brevibacillus* species have potential of degrading many toxic chemicals like Toluidine Blue dye and carbendazim, a known fungicide (Alhassani *et al.*, 2007; Arya and Sharma, 2014; Mallick *et al.*, 2014).

In conclusion, the morphological, biochemical and the molecular approaches described above present the first ever study carried out under given experimental conditions to identify and characterize 20 rhizobacteria from a representative plant of Cholistan desert. The phylogenetic significance of the presence of all bacilli from three species in the rhizosphere of one desert plant remains to be determined. Work is in progress on the molecular analysis of rhizobacteria from other plants of Cholistan desert in this regard. These bacteria on further characterization can be explored for the production of valuable molecules and can be manipulated to induce tolerance efficiently against both biotic and abiotic stress in economically important plants.

**Availability of data and materials.** Ribosomal DNA sequences are presented in the GenBank data as per accession numbers presented in Table II.

#### Conflict of interests

The authors declare that they have no financial or non-financial competing interests.

#### Author's contributions

FHN was the PI and HEC grant recipient. M Ashraf, JR and MSC were the collaborators who contributed through sample identification, collection and analyses. M Aslam, RR and HM performed the bench work. SE, FHN, M Ashraf, M Aslam and JR were involved in the data analyses and manuscript writing. M Aslam and GM prepared the dendrograms and conducted the related analysis.

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## A Low-Tech Bioreactor System for the Enrichment and Production of Ureolytic Microbes

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### Abstract

Ureolysis-driven microbially induced carbonate precipitation (MICP) has recently received attention for its potential biotechnological applications. However, information on the enrichment and production of ureolytic microbes by using bioreactor systems is limited. Here, we report a low-tech down-flow hanging sponge (DHS) bioreactor system for the enrichment and production of ureolytic microbes. Using this bioreactor system and a yeast extract-based medium containing 0.17 M urea, ureolytic microbes with high potential urease activity (> 10 μmol urea hydrolyzed per min per ml of enrichment culture) were repeatedly enriched under non-sterile conditions. In addition, the ureolytic enrichment obtained in this study showed *in vitro* calcium carbonate precipitation. Fluorescence *in situ* hybridization analysis showed the existence of bacteria of the phylum *Firmicutes* in the bioreactor system. Our data demonstrate that this DHS bioreactor system is a useful system for the enrichment and production of ureolytic microbes for MICP applications.

**Key words:** DHS bioreactor system, enrichment culture, microbially induced carbonate precipitation (MICP), ureolytic microbes

### Introduction

Microbially induced carbonate precipitation (MICP) occurs as a by-product of microbial activities in the environment (*e.g.*, ureolysis, photosynthesis, denitrification, ammonification, sulfate reduction, and methane oxidation; Zhu and Dittrich, 2016). In recent years, MICP processes have received attention for their potential biotechnological applications, including remediation of heavy metals and radionuclides, carbon dioxide sequestration, and biocementation (Anbu *et al.*, 2016; Zhu and Dittrich, 2016). In ureolysis-driven calcium carbonate (CaCO<sub>3</sub>) precipitation, urease (urea [CO(NH<sub>2</sub>)<sub>2</sub>] amidohydrolase; EC 3.5.1.5), a nickel-containing metallo-enzyme, catalyzes the hydrolysis of urea to ammonium ion (NH<sub>4</sub><sup>+</sup>) and carbonate ion (CO<sub>3</sub><sup>2-</sup>; eq. 1). Following the production of CO<sub>3</sub><sup>2-</sup>, CaCO<sub>3</sub> can be precipitated in the presence of calcium ion (Ca<sup>2+</sup>; eq. 2).



Urease activity is widely distributed in the environment, and urease is produced by a wide range of microbes (Anbu *et al.*, 2016; Mobley and Hausinger, 1989). *Sporosarcina pasteurii* (previously known as *Bacillus pasteurii*) has been extensively used for ureolysis-driven MICP applications because this bacterium is non-pathogenic, has high urease activity, and can tolerate the highly alkaline pH required for effective carbonate precipitation (Anbu *et al.*, 2016). Several research groups have successfully isolated microbes with the capacity for ureolysis-driven MICP (Achal *et al.*, 2012; Dhami *et al.*, 2013; Hammes *et al.*, 2003; Kang *et al.*, 2016; Li *et al.*, 2013; Vahabi *et al.*, 2013; Wei *et al.*, 2015). However, fundamental knowledge on the development of bioreactor systems for the enrichment and production of ureolytic microbes is quite limited.

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The major cost associated with ureolysis-driven MICP applications is the production of ureolytic microbes (Cheng and Cord-Ruwisch, 2013; Whiffin, 2004). The cost of sterilizing the cultivation medium is in the range of US\$0.46–0.66 per l of medium, which is comparatively higher than the cost of producing a yeast extract-based medium for culture of *S. pasteurii* (Whiffin, 2004). Therefore, to minimize the cost associated with MICP applications, the production of microbes with high urease activity without sterilization is a significant challenge. Whiffin (2004) demonstrated that the level of urease activity in a *S. pasteurii* culture was not adversely affected by the presence of a significant amount (*e.g.*, 50% [v/v]) of wastewater treatment sludge contaminants in the inoculum. In addition, Chen and Cord-Ruwisch (2013) reproducibly enriched and continuously produced highly ureolytic microbes from activated sludge by using a non-sterile chemostat under selective conditions (high pH and high concentration of urea). These results indicated that, under certain conditions, ureolytic microbes can be enriched and produced without sterilization.

Here, we report the enrichment and production of ureolytic microbes from a soil sample by using a down-flow hanging sponge (DHS) bioreactor system. This bioreactor system was originally developed as a low-tech biofilm-type sewage treatment technology (Agrawal *et al.*, 1997; Uemura and Harada, 2010). The distinctive feature of a DHS bioreactor system is the use of polyurethane sponges, which provide increased surface areas for retaining greater microbial biomass. In addition, the sponge cubes are not submerged in medium but are hanging freely in the air. Thus, adequate oxygen supply for robust growth of aerobic microbes is possible under non-turbulent conditions. Furthermore, in terms of operation, this simple, low-tech bioreactor system can be operated and maintained without highly skilled personnel (Tandukar *et al.*, 2007). The aim of this study was to evaluate the applicability of a DHS bioreactor system for the enrichment and production of ureolytic microbes under laboratory conditions. In addition, we also tested whether the enrichment and production of ureolytic microbes is feasible under non-sterile conditions.

## Experimental

### Materials and Methods

**Bioreactor and enrichment conditions.** A schematic diagram of the DHS bioreactor system used in this study is shown in Fig. 1. The bioreactor system was composed of an acrylic column with 10 polyurethane sponge cubes (32 mm × 32 mm × 32 mm; poros-

ity=98%). A surface soil sample (from ~0–10 cm depth; 100 g) was collected from the schoolyard of the National Institute of Technology at Wakayama College (33°50'00"N, 135°10'34"E), and was used as the inoculum for the bioreactor. The sponge cubes were soaked in the soil sample diluted in 500 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7.5]), and then, the sponge cubes were inserted into cylindrical plastic frames (32 mm diameter, 32 mm tall, 2 mm thick) connected vertically using plastic clamping bands. The total volume of the sponge cubes in the plastic frames was 197 cm<sup>3</sup>. The bioreactor operation period (130 days) was divided into four phases based on the yeast extract concentrations in the supplied medium and the presence/absence of an external air supply. The composition of the yeast extract-based medium (pH 8.0) was as follows: 0.17 M urea, 1 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 10 μM NiCl<sub>2</sub>, and yeast extract at 1 g/l in phases 1 and 2 (day 0–57), 5 g/l in phase 3 (day 58–99), and 10 g/l in phase 4 (day 100–130; Becton-Dickinson and Company, NJ, USA). The urea and NiCl<sub>2</sub> concentrations were set based on the reports by Cheng and Cord-Ruwisch (2013) and Gat *et al.* (2016). The medium was stored in a refrigerator at 3 ± 2°C and

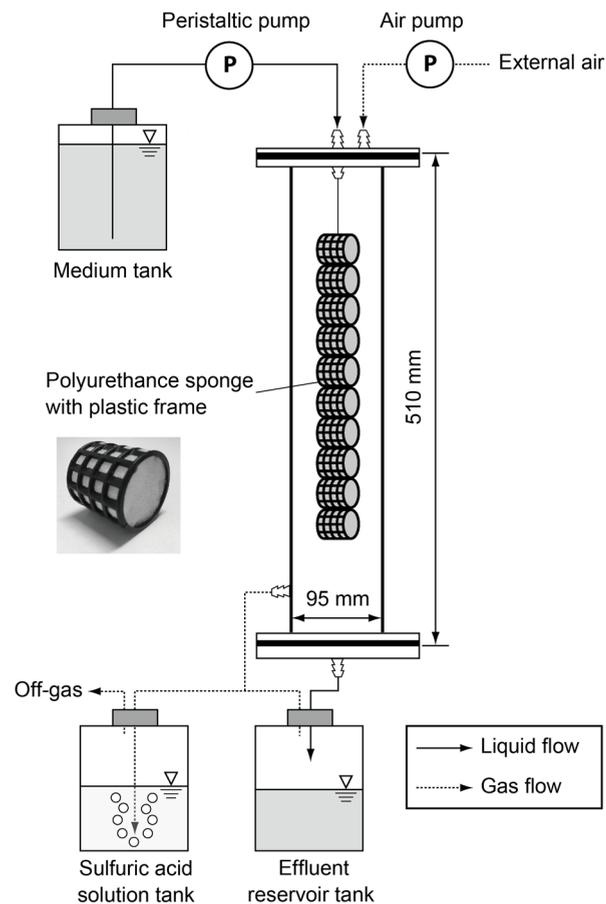


Fig. 1. Schematic diagram of the down-flow hanging sponge bioreactor system used in this study.

was continuously supplied to the bioreactor with a peristaltic pump (MP-2000; Eyela, Tokyo, Japan) and Viton tubing (Cole-Parmer, Vernon Hills, IL, USA). The theoretical hydraulic retention time, which was calculated based on the total void volume of the sponge cubes, was set at 5.5 h. Beginning on day 24, external air was continuously supplied from the upper part of the bioreactor *via* an air pump with a discharge air-flow rate of 8.0 l/min (C-5BN; Techno Takatsuki Co., Ltd., Osaka, Japan). To prevent significant atmospheric diffusion of ammonia species produced by ureolysis, the bioreactor was a closed system, and effluent air was passed through a 2N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) solution. Effluent medium was collected in an effluent reservoir tank and was manually removed before the tank was filled. The bioreactor system was operated in a dark, temperature-controlled room at 25 ± 2°C.

**Analytical methods for bioreactor effluent and enrichment.** The pH and oxidation-reduction potential (ORP) values of freshly collected bioreactor effluent samples were measured using a pH meter (LAQUAtwin B-712; HORIBA, Ltd., Kyoto, Japan) and an ORP meter (ULTRAPEN PT3; Myron L Company, CA, USA), respectively.

Enrichment samples squeezed from even-numbered sponge cubes (counting from the top) were used to determine urease activity and biomass concentration and for fluorescence *in situ* hybridization (FISH) analysis, as described below. After every sampling, the sponge cubes were re-inserted into the cylindrical plastic frames to recover the ureolytic enrichments.

Potential urease activity was determined using a conductivity method as reported by Cheng and Cord-Ruwisch (2013), with slight modification. In brief, the relative conductivity changes in 10 ml of urea solution (1.5 M final concentration) containing 1 ml of enrichment culture at 25°C was measured using a conductivity meter (LAQUAtwin B-771; HORIBA) at 3–15 min intervals. The rate increase in conductivity was converted to potential urease activity as described previously (Cheng and Cord-Ruwisch, 2013; Whiffin, 2004). One unit (U) of potential urease activity was defined as the enzyme activity that hydrolyzes 1.0 μmol of urea per minute at 25°C. All assays were performed in triplicate.

Biomass concentrations were recorded as mg of dry weight per ml of volume. The dry weight of the biomass was determined after drying samples to a constant weight at 105°C. Subsampled enrichment cultures used for the dry weight determination were washed twice with sterilized distilled water before drying. All assays were performed in triplicate.

**16S rRNA-targeted FISH analysis.** 16S rRNA-targeted FISH was performed according to a previously described method (Snaidr *et al.*, 1997), with some modifications. Samples were fixed by adding 16% par-

aformaldehyde (PFA) solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to obtain a final concentration of 4% PFA and incubation overnight at 4°C, and then stored in a 1:1 mix of PBS and 99.5% ethanol at –20°C until analysis. The PFA-fixed samples were sonicated six times on ice for 15 s each at output setting 1 with an ultrasonic disrupter (UD-211; TOMY SEIKO Co., Ltd., Tokyo, Japan). The sonicated samples were embedded in 0.1% MetaPhor agarose (Cambrex Bio Science Rockland, Inc., ME, USA) in 8-well glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan) and treated with 10 mg/ml lysozyme (from egg whites; Wako Pure Chemical Industries, Ltd.) in TE buffer (100 mM Tris-HCl and 50 mM EDTA [pH 8.0]) at 37°C for 60 min. To dehydrate the samples, the glass slides were immersed in 50%, 80%, and 99.5% ethanol for 2 min each, and then air-dried at 46°C. The 16S rRNA-targeted oligonucleotide probes used in this study were 5'-Cy3-labeled EUB338mix (an equimolar mixture of probes EUB338 [5'-GCT GCC TCC CGT AGG AGT-3'], EUB338-I [5'-GCA GCC TCC CGT AGG AGT-3'], EUB338-II [5'-GCA GCC ACC CGT AGG TGT-3'], and EUB338-III [5'-GCT GCC ACC CGT AGG TGT-3']; Amann *et al.*, 1990; Daims *et al.*, 1999) for most bacteria and 5'-6-FAM-labeled LGC354mix (an equimolar mixture of probes LGC354A [5'-TGG AAG ATT CCC TAC TGC-3'], LGC354B [5'-CGG AAG ATT CCC TAC TGC-3'], and LGC354C [5'-CCG AAG ATT CCC TAC TGC-3']; Meier *et al.*, 1999) for the phylum *Firmicutes*. The hybridization was performed in a hybridization buffer containing 20 mM Tris-HCl (pH 7.4), 0.9 M NaCl, 0.01% sodium dodecyl sulfate (SDS), 0.5 μM oligonucleotide probe, and either 20% formamide (for EUB338mix probe) or 35% formamide (for LGC354mix) for 3 h at 46°C in the dark. To remove excess probe, the glass slides were washed for 15 min in a washing buffer containing 20 mM Tris-HCl (pH 7.4), 0.01% SDS, and either 225 mM NaCl (for EUB338mix) or 80 mM NaCl (for LGC354mix) at 48°C. The glass slides were finally counterstained with ProLong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, MA, USA) for quantification of total cells. An epi-fluorescence microscope (ECLIPSE E600; Nikon, Tokyo, Japan) equipped with a digital camera (DXM1200; Nikon) was used for microscopic observation. To determine the percentage of FISH-positive cells, 10 different microscopic fields with at least 1 × 10<sup>2</sup> DAPI-stained cells per field were examined for each sample.

**CaCO<sub>3</sub> precipitation test.** DHS bioreactor enrichment collected on day 105 was subjected to a CaCO<sub>3</sub> precipitation test. To precipitate CaCO<sub>3</sub>, 2 ml of the bioreactor enrichment, with average potential urease activity of 27.0 U/ml of culture, was aliquoted into three 50-ml polypropylene tubes containing 40 ml of

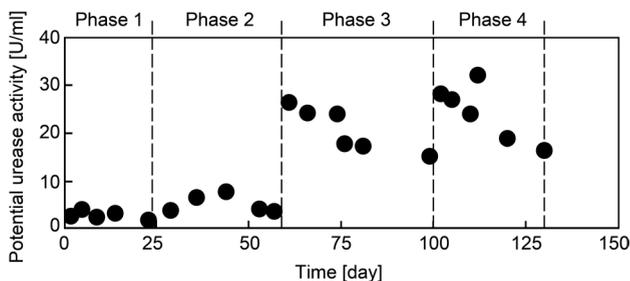


Fig. 2. Time course changes in the potential urease activity of down-flow hanging sponge bioreactor enrichments.

The standard errors of the mean of potential urease activity values ( $n=3$ ) were smaller than the size of the filed circles; therefore, they are not shown.

filter-sterilized  $\text{CaCl}_2$ -urea solution (1.0 M urea and 1.0 M  $\text{CaCl}_2$ ). Under the experimental conditions, the  $\text{CaCl}_2$  in the solution should be completely dissolved according to eq. (3):



The tubes were incubated at 25°C for 24 h with shaking (at 120 rpm). Negative controls, without the enrichment, ( $n=3$ ) were used to detect any non-biological  $\text{CaCO}_3$  precipitation. After the incubation period, precipitates were filtered with Whatman filter paper No. 1 (GE Healthcare UK Ltd., Buckinghamshire, UK) and washed with distilled-deionized water. Then, the filters were dried at 105°C for 24 h and were weighed. The weight of precipitates ( $W_a$ ) was determined from eq. (4):

$$W_a = W_b - W_c \quad (4)$$

where,  $W_b$  is the weight of the filter paper with precipitates and  $W_c$  is the weight of the filter paper without precipitates.

To reveal the mineralogy and surface morphology of the precipitates, a representative sample from the  $\text{CaCO}_3$  precipitation test was subjected to X-ray diffraction (XRD) and scanning electron microscope (SEM) analyses. The XRD analysis was performed on a SmartLab (Rigaku, Tokyo, Japan) with Cu K $\alpha$  radiation operated at 40 kV and 30 mA. Prior to the XRD analysis, the collected precipitates were powdered with a mortar and pestle. The XRD data were collected on the

scale of diffraction angle ( $2\theta$ ) = 5°–90°. The XRD analysis was performed by the Industrial Technology Center of Wakayama Prefecture (Wakayama, Japan). The SEM analysis was performed with a JSM-6510 (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 5 kV. The collected precipitates used for the SEM analysis were sputter coated with platinum prior to the analysis.

## Results and Discussion

**Enrichment of ureolytic microbes with a DHS bioreactor system.** A previous study by Cheng and Cord-Ruwisch (2013) demonstrated that a non-sterile chemostat system has great potential for reproducibly enriching and continuously producing ureolytic microbes with approximately 60 U of urease activity per ml of culture. In the present study, a high concentration (0.17 M) of urea in the supplied yeast extract-based medium was used as a selective factor for the enrichment and production of ureolytic microbes under non-sterile laboratory conditions (Cheng and Cord-Ruwisch, 2013).

To confirm the enrichment and production of ureolytic microbes in the DHS bioreactor system, the potential urease activity of enrichment samples retrieved from the sponge cubes was determined. Although no obvious potential urease activity (*i.e.*,  $\geq 0.1$  U/ml of inoculum) was detected in the soil slurry inoculum (data not shown), relatively high potential urease activity was repeatedly detected in the enrichments (Fig. 2). The higher pH values of the bioreactor effluents compared to that of the supplied medium (*i.e.*, pH 8.0; Fig. 3) were likely due to ammonia production by ureolysis.

In the initial phase 1 (day 0–23), the yeast extract concentration in the supplied medium was set at 1 g/l. The average potential urease activity observed in this phase was 2.9 U/ml (Fig. 2). Since the bioreactor system used in this study was a closed system, we expected that oxygen could not be effectively supplied to any aerobic ureolytic microbes that colonized the sponge cubes. In

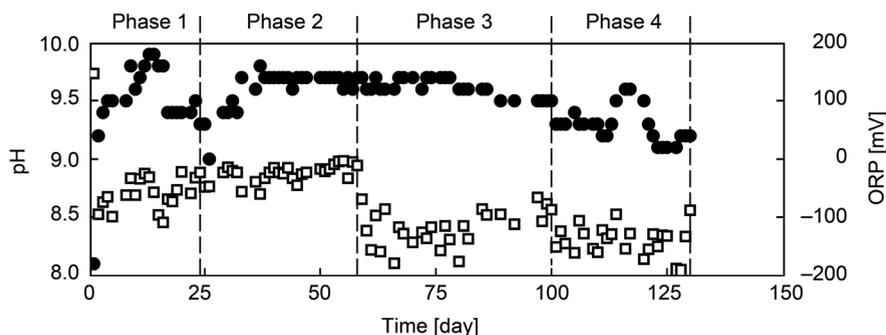


Fig. 3. Time-course changes in the pH (●) and oxidation-reduction potential (ORP; □) values of the effluent from the down-flow hanging sponge bioreactor.

fact, the negative ORP values of the bioreactor effluents (Fig. 3) suggested depletion of the dissolved oxygen in the supplied medium as the medium flowed down into the sponge cubes. Therefore, from day 24 to 130 (*i.e.*, phases 2–4), external air was continuously supplied to the bioreactor to enhance the growth of aerobic ureolytic microbes. It should be noticed that the negative ORP values of the bioreactor effluents were also confirmed, even in the presence of an external air supply (Fig. 3). This phenomenon may be explained by enhanced oxygen consumption by the increasing aerobic biomass in the sponge cubes. During phase 2 (day 24–57), the operational conditions of the bioreactor system were same as those during phase 1, except for the external air supply. The average potential urease activity observed during phase 2 was 5.2 U/ml (Fig. 2). However, the potential urease activity values in phase 2 did not exceed 10 U/ml, which could be required for biocementation applications (Whiffin, 2004). Since yeast extract was thought to be the growth-limiting substrate for the ureolytic microbes in the bioreactor system, the yeast extract concentrations in the supplied medium were increased to 5 and 10 g/l in phases 3 (day 58–99) and 4 (day 100–130), respectively. In phases 3 and 4, the average potential ureolytic activity values were increased to 20.8 and 24.4 U/ml (Fig. 2), respectively. These average values were similar to those of the highly ureolytic *S. pasteurii* (17–34 U/ml of culture; Harkes *et al.*, 2010), and this level of ureolytic activity could be sufficient for biocementation applications.

A positive correlation (coefficient of determination  $R^2=0.604$ ) between the potential urease activity values and the biomass concentrations of the DHS bioreactor enrichment (Fig. 4) suggested that the biomass concentration of the enrichment significantly affected the potential urease activity values. In addition, based on the correlation shown in Fig. 4, an average potential urease activity of 1.5 U/mg of biomass was calculated for the bioreactor enrichment. The types of ureolytic microbes present in the inoculum could have significantly affected the potential urease activity per dry weight of biomass, and the average potential urease activity of the DHS bioreactor enrichment was roughly 8 and 10 times lower than that of *S. pasteurii* under normal growth conditions (Whiffin, 2004), and the ureolytic chemostat enrichment reported by Cheng and Cord-Ruwisch (2013), respectively. Here, it should be noted that the biomass concentrations observed in phases 3 (day 58–99) and 4 (day 100–130; Fig. 4) were 1.5–4.0 times higher than the maximum average biomass concentration observed in the ureolytic chemostat system (Cheng and Cord-Ruwisch, 2013). The high biomass-retention capacity of DHS bioreactor systems has been discussed elsewhere (Onodera *et al.*, 2013; Tandukar *et al.*, 2007). Taken together, it seems that the

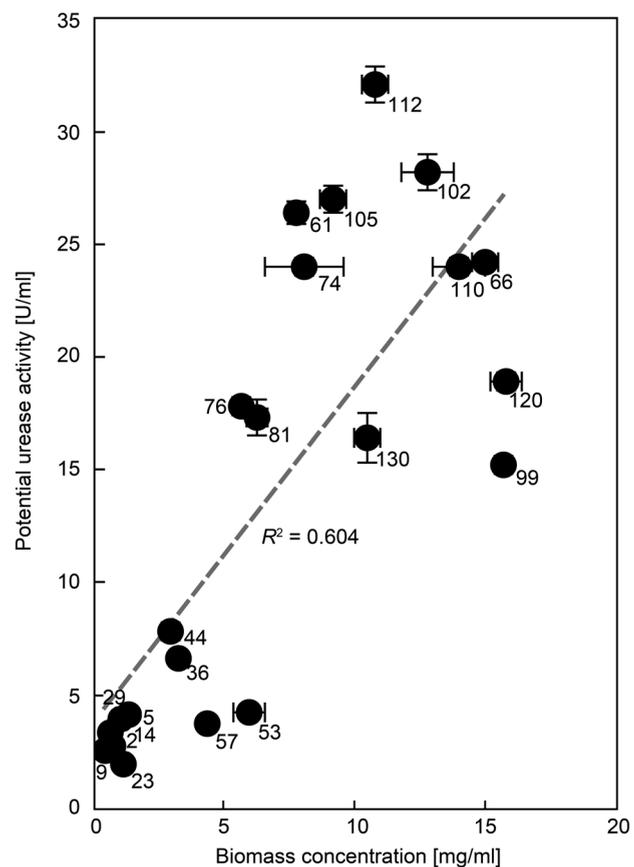


Fig. 4. Scatter plot of potential urease activities and biomass concentrations observed in the down-flow hanging sponge bioreactor enrichments.

The numbers associated with the filled circles are the sampling times for the bioreactor enrichments. The error bars are the standard error of the mean ( $n=3$ ), and filled circle symbols without error bars have standard errors that are smaller than the size of the circle.

high biomass retention capacity allowed the successful establishment of ureolytic enrichments that could be applicable for biocementation applications.

**Detection of the active microbial components by FISH.** The genera *Bacillus* and *Sporosarcina* within the phylum *Firmicutes* are well-known bacterial genera with the capacity for ureolysis-driven MICP (Anbu *et al.*, 2016; Zhu and Dittrich, 2016). In addition, bacteria in these genera have been repeatedly isolated from various environments under ureolytic conditions (Dhami *et al.*, 2013; Hammes *et al.*, 2003; Kang *et al.*, 2016; Li *et al.*, 2013; Vahabi *et al.*, 2013; Wei *et al.*, 2015). Therefore, we performed a FISH analysis using the universal bacterial probe EUB338mix and the *Firmicutes* specific LGC354mix probe to obtain preliminary information on the active microbial components in the DHS bioreactor system. Our FISH analysis revealed that the existence of bacteria in the phylum *Firmicutes* in the bioreactor system (Fig. 5). Since microbial cultivation in a bioreactor selects a simplified microbial community that is optimally adapted to the experimental conditions, these LGC354mix-detected *Firmicutes*

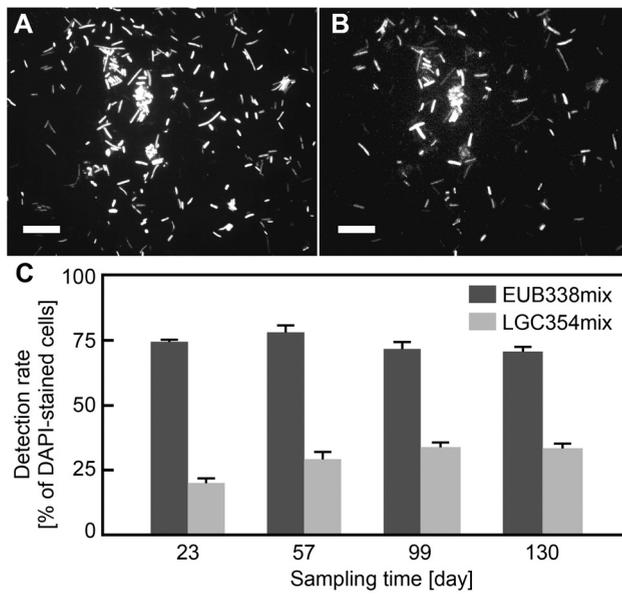


Fig. 5. Fluorescence *in situ* hybridization analysis.

(A) and (B) Representative photomicrographs of a down-flow hanging sponge bioreactor enrichment collected on day 130 after *in situ* hybridization with the phylum *Firmicutes*-targeted LGC354mix probe. Photomicrographs of DAPI-stained cells (A) and the LGC354mix-stained cells (B) in an identical field. Scale bars = 10  $\mu\text{m}$  [in (A) and (B)]. (C) Detection rates (% DAPI-stained cells) of EUB338mix and LGC354mix probe-targeted microbial populations in down-flow hanging sponge bioreactor enrichments. The error bars in (C) are the standard error of the mean from 10 different microscopic fields.

populations might be associated with ureolysis. Considering previous enrichment results (Cheng and Cord-Ruwisch, 2013; Gat *et al.*, 2016), it seems that the high concentration (0.17 M) of urea in the supplied medium functioned as a selective factor for the enrichment of bacteria in the phylum *Firmicutes*. It is a well known fact that the composition of cultivation medium affects the microbial community structure of enrichment cultures (*e.g.*, Gat *et al.*, 2016).

**CaCO<sub>3</sub> precipitation by DHS bioreactor enrichment.** In the CaCO<sub>3</sub> precipitation test using the DHS bioreactor enrichment collected on day 105, the average amount of precipitates ( $\pm$  standard error of the mean,  $n=3$ ) obtained by the CaCl<sub>2</sub>-urea solutions plus the enrichment was  $92 \pm 7$  mg/ml of CaCl<sub>2</sub>-urea solution. This amount was close to the theoretical value (100 mg/ml of CaCl<sub>2</sub>-urea solution), which was calculated according to the sum of eqs. (1), (2), and (3). In contrast, the negative controls (CaCl<sub>2</sub>-urea solution without the enrichment;  $n=3$ ) did not show much precipitate (*i.e.*,  $\geq 1$  mg/ml of CaCl<sub>2</sub>-urea solution). These results indicated that the precipitates were produced by the enrichment. XRD analysis of a representative sample from the CaCO<sub>3</sub> precipitation test showed that the dominant precipitate was calcite (Fig. 6A), which is one of the most common CaCO<sub>3</sub> polymorphs induced by

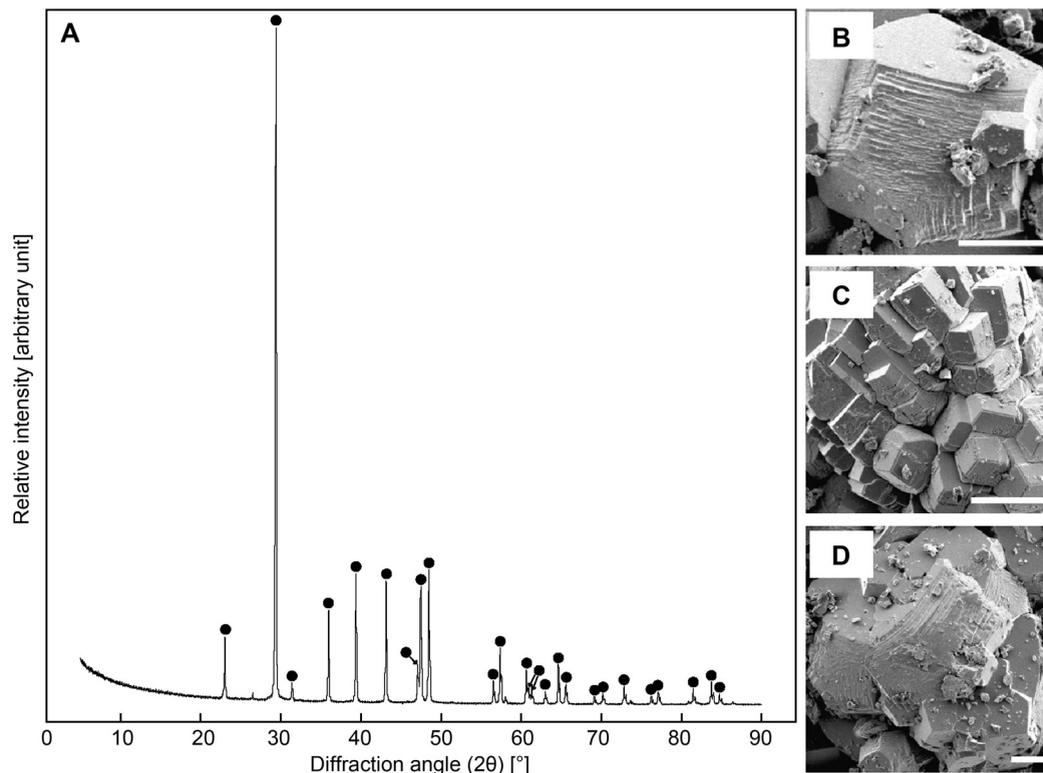


Fig. 6. Mineralogy and surface morphology of precipitates obtained by a down-flow hanging sponge bioreactor enrichment collected on day 105.

(A) X-ray diffraction pattern of the induced precipitates. The peaks marked with filled circles arose from calcite. (B)-(D) Representative scanning electron microscopy images of the induced precipitates. (B) Dodecahedron-like crystals. (C) A cluster of hexahedral-like crystals. (D) A cluster of irregular-shaped crystals. Scale bar = 10  $\mu\text{m}$  [in (B)-(D)].

ureolytic bacteria (Wei *et al.*, 2015). The SEM analysis demonstrated the existence of dodecahedron-like crystals (Fig. 6B) and clusters of hexahedral-like (Fig. 6C) and irregular-shaped crystals (Fig. 6D) in the sample. It has been reported that not only microbial cells, but also extracellular polymeric substances (the major component of biofilm matrix) can function as nucleation sites for CaCO<sub>3</sub> precipitation (Dupraz *et al.*, 2009; Zhu and Dittrich, 2016). In addition, it seems that microbes with biofilm-forming capacity are capable of occupying ecological niches in the DHS bioreactor system because of the bioreactor's characteristics. Therefore, the effective CaCO<sub>3</sub> precipitation observed in this study might be attributed to the possible biofilm-forming capacity of the ureolytic microbes in the bioreactor enrichment.

### Conclusion

Our data demonstrate that the low-tech DHS bioreactor system described in this study has potential for the enrichment and production of ureolytic microbes under non-sterile conditions. In addition, the ureolytic enrichment obtained from the bioreactor system showed effective CaCO<sub>3</sub> precipitation capacity *in vitro*. The results presented in this study provide profound insights into the development of large-scale systems for the enrichment and production of ureolytic microbes for industrial MICP applications.

### Acknowledgments

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## MALDI-TOF MS Detection of Endophytic Bacteria Associated with Great Nettle (*Urtica dioica* L.), Grown in Algeria

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### Abstract

Any plant with a vascular system has a specific endophytic microflora. The identification of bacteria is essential in plant pathology. Although identification methods are effective, they are costly and time consuming. The purpose of this work is to isolate and to identify the different bacteria from the internal tissues of *Urtica dioica* L. and to study their diversity. This last is based on the different parts of the plant (stems, leaves and roots) and the harvest regions (Dellys and Tlemcen). The identification of bacteria is done by biochemical tests and confirmed by MALDI-TOF MS. Seven genus and eleven species were isolated from the Great Nettle. They belong to the genera *Bacillus*, *Escherichia*, *Pantoea*, *Enterobacter*, *Staphylococcus*, *Enterococcus* and *Paenibacillus*. The majority of these bacteria were isolated from Tlemcen which makes this region the richest in endophytic bacteria compared to that harvested from Dellys. The results show also that the leaves are the most diversified in endophytic bacteria. *Bacillus pumilus*-ME is the common species of the three parts of the plant harvested in both regions. From this work, it emerges that the Great Nettle can be settled by various endophytic bacteria which are differently distributed within the same plant harvested in different regions.

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Key words: *Bacillus pumilus*-ME, diversity, endophytic bacteria, MALDI-TOF MS, *Urtica dioica* L.

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### Introduction

The internal tissues of plants can be a niche for various types of endophytic microorganisms (bacteria and fungi) (Rosenblueth and Martinez-Romero, 2006, Goryluk *et al.*, 2009). Endophytic bacteria are very ubiquitous in plants and can be isolated from the stems, leaves, roots, fruits, tubers and nodules of leguminous plants (Kobayashi and Palumbo, 2000).

Some endophytes are very beneficial, even necessary for the growth of their host plants (Dudeja *et al.*, 2012, Jasim *et al.*, 2014). They prevent some pathogenic organisms from colonizing plants and can also act as biological control agents against insects (Laib, 2014). However, it is probable that beneficial endophytes can become pathogenic under certain stress conditions when the plant no longer controls them (Arnold, 2007).

The traditional methods of bacterial identification are very effective but have the disadvantage of being laborious and time-consuming (Tshikhudo *et al.*, 2013).

Matrix-Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is an identification tool that is easy to use, fast, accurate and cost-effective (Gravet and Gessier, 2013, Sauget *et al.*, 2017). Due to the lack of robust information tools and effective databases, this technique did not appear in public and private laboratories until 2008 (Tshikhudo *et al.*, 2013). It was reserved exclusively for biochemical or research laboratories (Gravet and Gessier, 2013). This technique is based on the generation of mass spectra from whole cells and their comparison with reference spectra after ionization (Sauget *et al.*, 2017).

Belonging to the Urticaceae family, *U. dioica* L. is a plant used for food and medicinal purposes. It is also on the list of medicinal plants selected by the French Pharmacopoeia (Draghi, 2005). The aim of this work was to identify the endophytic bacteria isolated from the stems, leaves and roots of the Great Nettle harvested at Dellys and Tlemcen by biochemical tests and by MALDI-TOF MS.

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## Experimental

### Material and Methods

**Description of the study area.** The different parts of *U. dioica* L. (stems, leaves and roots) were harvested during the month of February 2016 in two different regions of northern Algeria. The first is Dellys, a coastal town located at 115 km from Algiers. The second is Tlemcen, located in the north-west of Algeria, 520 km west of Algiers.

**Isolation of endophytic bacteria.** The plant freshly harvested under aseptic conditions and showing no pathological symptoms was sent directly to the laboratory within a period not exceeding 24 h in view of the microbiological studies.

In order to remove the microorganisms present on the cortex, the whole plant (stems, leaves and roots) was washed with tap water, then underwent a series of disinfection with 95% ethanol for 30 s, with sodium

hypochlorite 10% and 75% ethanol for 2 min., then rinsed 3 times with sterile distilled water to remove traces of the disinfectant (Evans *et al.*, 2003, Rubini *et al.*, 2005). The superficial tissues were scoured using a scalpel and then crushed using a sterile forceps. A volume of 100 µl was deposited and then spread on the surface of a Petri dish containing nutrient agar (Jasim *et al.*, 2014). At the same time, a Petri dish containing a drop of sterile distilled water from the last washing of the plant served as a control. The whole was incubated at 37°C for 24 h. The operation was repeated three times for each of the different parts of *U. dioica* L.

The isolated bacteria were coded by two letters and one number. The first letter derived from the harvest area, and the second from the part of the plant. The number indicates the order in which the bacteria appear.

**Macroscopic and biochemical identification.** After incubation, the different bacteria associated with the Great Nettle underwent a successive series of transplanting until adequate purification and isolation of the colo-

Table I  
Macroscopic and microscopic appearance of isolated bacteria.

Codes	Macroscopic appearance					Microscopic appearance				
	Form	Area	Elevation	Size	Chromogenesis	Contour	Opacity	Fresh state	Spores	Gram
TS1 TR2 DS2 DL2 DR3	Large round colonies	Rough	Flat	< 4 mm	Yellowish	Irregular	Opaque	Long bacilli in a chain motionless	+	+
TS2 TR1 DS3 DL3 DR3	Medium colonies, rounds	Smooth	Flat	< 2 mm	Beige	Irregular	Opaque	Small bacilli in a chain motionless	+	+
TL6 TS3 TR3 DS1 DL1 DR1	Large colonies	Smooth	Flat	3 mm	Whitish	Irregular	Opaque	Small bacilli in a chain	+	+
TL1 DL4	Small, round and well-insulated colonies	Brilliant	Bomb	> 1 mm	Whitish	Regular	Translucent	Colibacilles, mobile	-	-
TL2	Round	Dried	Slightly bulging	2 mm	Whitish	Regular	Opaque	Cocci very mobile	-	+
TL3	Round colonies	Smooth	Flat	> 1 mm	Whitish	Regular	Opaque	Cocci mobile	-	-
TL4	Small, round colonies Convex	Smooth Brilliant	Bomb	> 1 mm	Whitish	Regular	Opaque	Cocci in cluster	-	+
TL5	Small colonies	Smooth	Flat	> 1 mm	Transparent	Regular	Translucent	Small mobile bacilli	-	-
TR4	Large colonies	Rough	Flat	> 1 mm	Whitish	Irregular	Opaque	Small bacilli	-	+
DR2	Large colonies	Smooth	Flat	< 2 mm	Whitish	Irregular	Opaque	Small, very mobile bacilli	+	+
TR5	Large colonies	Rough	Flat	> 1 mm	Whitish	Irregular	Opaque	Bacillus	-	+

TS = Tlemcen stem, DS = Dellys stem, TR = Tlemcen root, DR = Dellys roots, TL = Tlemcen leaves, DL = Dellys leave

Table II  
Biochemical study of isolated bacteria.

Codes	Catalase	Oxidase	Acetoin	Indole	Citrate	Urease	Nitrate	Motility	Mannitol	H <sub>2</sub> S	ONPG	TDA	Glucose	Lactose
TS1 TR2 DS2 DL2 DR3	+	-	+	+	-	-	+	-	-	-	-	-	+	-
TS2 TR1 DS3 DL3 DR3	+	+	-	-	+	-	-	+	+	-	+	/	+	-
TL6 TS3 TR3 DS1 DL1 DR1	+	+	+	-	+	-	-	+	+	-	+	-	+	-
DR2	+	+	+	-	-	-	+	+	-	-	-	+	+	-
TL1 DL4	+	-	-	+	-	-	+	+	+	-	+	-	+	+
TR4	+	-	+	-	-	+	-	+	+	-	+	/	+	+
TL3	+	-	+	-	+	-	+	+	+	-	+	-	+	+
TL4	+	-	+	-	+	+	+	-	+	-	-	-	+	+
TL2	-	-	+	-	-	-	+	-	+	-	-	/	+	+
TL5	+	-	+	-	+	-	+	+	+	-	+	+	+	-
TR5	+	+	-	-	+	-	+	+	+	-	+	/	+	+

TS = Tlemcen stem, DS = Delys stem, TR = Tlemcen root, DR = Delys roots, TL = Tlemcen leaves, DL = Delys leaves, + = presence, - = absence.

nies was achieved. The distinction between the different bacteria was based on morphological criteria (form, area, elevation, size, chromogenesis, shape and opacity).

In order to have a first orientation on the identification of the bacterial species detected, we carried out microscopic examinations such as fresh observation and Gram staining. These tests were complemented by the study of some biochemical characteristics (catalase, oxidase, acetoin, indole, citrate, urease, nitrate, motility, mannitol, H<sub>2</sub>S, ONPG, TDA, glucose, lactose) that allowed to get closer and closer to the identity of each species.

**Identification by MALDI-TOF MS.** The matrix was prepared before each series of analysis by diluting a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) (Sigma H, Lyon, France) in 500  $\mu$ l of 50% (v/v) acetonitrile, 250  $\mu$ l of 10% (v/v) trifluoroacetic acid (TFA) and 250  $\mu$ l of HPLC water. The whole was stirred vigorously, sonicated for 10 min, centrifuged (13 000  $\times$ g, 5 min.) and then transferred to a clean polypropylene tube.

Each bacterial colony obtained from a young culture (18 to 24 h) was deposited in duplicate on the MALDI-TOF target plate (Bruker Daltonics TM, Wissembourg, France) and then covered with 1.5  $\mu$ l of the matrix solu-

tion. The whole (target plate and matrix) was dried at room temperature for a few min. and then analyzed (Pfleiderer *et al.*, 2013). A Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) was used for bacterial identification. The spectra of the bacteria obtained were compared with the Bruker computer database using the flexAnalysis v. 3.3 and MALDI-Biotyper v. 3.0 software for data analysis. The isolate was correctly and significantly identified at the species level when the logarithmic score (LSV) was greater than or equal to 1.9 (Seng *et al.*, 2009).

## Results

The study of the macroscopic and biochemical aspect gave us a first orientation towards the determination of the bacterial species (Tables I and II).

The efficiency of the disinfection was checked in the control box after 24 h of incubation at 37°C and showed no microbial growth, indicating that the epiphytes were completely removed according to the disinfection protocol.

Based on morphological and biochemical criteria, a total of 57 endophytic bacteria were isolated from

Table III  
Identification results by MALDI-TOF/MS.

Codes	Organism best match	Study Area	Numbers			%	Score value (LSV)	Color	Significance of results
			S	L	R				
TS1 TR2 DS2 DL2 DR3	<i>Bacillus anthracis</i>	T	2	0	5	20	2.317	G	Correct identification of the genus and species
		D	2	5	0	22.72	2.226	G	
TS2 TR1 DS3 DL3 DR3	<i>Bacillus megaterium</i>	T	0	0	5	14.28	2.07	G	
		D	1	2	2	22.72	2.302	G	
TL6 TS3 TR3 DS1 DL1 DR1	<i>Bacillus pumilus-ME</i>	T	3	1	1	14.28	2.233	G	
		D	5	2	2	40.90	2.404	G	
DR2	<i>Bacillus cereus</i>	D	0	0	1	4.54	2.26	G	
TL1 DL4	<i>Escherichia coli</i>	T	0	5	0	14.28	2.354	G	
		D	0	1	0	4.54	2.243	G	
TL5	<i>Pantoea agglomerans</i>	T	0	1	0	2.85	2.175	G	
TL3	<i>Enterobacter amnigenus</i>	T	0	1	0	2.85	2.293	G	
TL4	<i>Staphylococcus cohnii</i>	T	0	1	0	2.85	2.024	G	
TL2	<i>Enterococcus faecium</i>	T	0	1	0	2.85	2.405	G	
TR4	<i>Paenibacillus lautus</i>	T	0	0	1	2.85	1.875	Y	
TR5	<i>Paenibacillus glucanolyticus</i>	T	0	0	1	2.85	1.757	Y	
UN	?	T	2	1	4	20	<1.7	R	Unidentified
		D	0	0	1	4.54			

TS = Tlemcen stem, DS = Dellys stem, TR = Tlemcen root, DR = Dellys roots, TL = Tlemcen leaves, DL = Dellys leaves, D = Dellys, S = Stem, L = Leaves, R = Root, G = Green, Y = Yellow, R = Red, UN = Unidentified

*U. dioica* L., among them 35 bacteria from Tlemcen and 22 bacteria from Dellys. These bacteria belong to Bacillaceae, Enterobacteriaceae, Paenibacillaceae, Staphylococcaceae, Enterococcaceae.

The identification of the various isolated bacteria was proved by MALDI-TOF MS. The values of the scores obtained are noted in Table III.

Among the 57 isolates analyzed by MALDI-TOF MS, eight bacteria were not identified. The 11 species identified belong to different families. The results show a dominance of Bacillaceae, represented essentially by four species, namely *Bacillus pumilus-ME*, *Bacillus anthracis*, *Bacillus megaterium* and *Bacillus cereus*. There are followed by Enterobacteriaceae with 3 species (*Escherichia coli*, *Pantoea agglomerans* and *Enterobacter amnigenus*), Paenibacillaceae family with 2 species (*Paenibacillus lautus* and *Paenibacillus glucanolyticus*). The less frequent families are Staphylococcaceae and Enterococcaceae with *S. cohnii* and *E. faecium* respectively.

Analysis of the presence of endophytic bacteria in the two samples of the Great Nettle revealed a heterogeneous distribution of the identified germs.

It appears also that the leaves are richest in endophytic bacteria with 6 species isolated at Tlemcen and 4 species at Dellys. *B. pumilus-ME* is the common species in both regions of the different parts of the Great Nettle (leaves, stems and roots).

As for the effect of the biotope on diversity in endophytic bacteria, it seems that *U. dioica* L. collected in the region of Tlemcen is the richest in bacteria associated with seven genera and eleven species compared to that harvested from Dellys which is represented by 2 genera and 5 species. In addition, 4 bacterial species were isolated from the Great Nettle harvested in both regions. These include *B. anthracis*, *B. megaterium*, *B. pumilus-ME*, and *E. coli*. The 6 endophytic bacteria isolated only from Tlemcen are *P. lautus*, *P. glucanolyticus*, *P. agglomerans*, *E. amnigenus*, *E. faecium* and *S. cohnii*. Finally, *B. cereus* is detected only at Dellys.

## Discussion

Endophytic bacteria have already been isolated from medicinal plants by several authors. Indeed, El-deeb *et al.*, (2013) working on Shara «*Plectranthus tenuiflorus*», harvested from the Sahara of Saudi Arabia, revealed the presence of a multitude of endophytic bacteria including *Bacillus* sp., *B. megaterium*, *B. pumilus-ME* and *Paenibacillus* sp.

Similarly, Jasim *et al.*, (2014) showed the existence of *Bacillus* sp. and *Staphylococcus* sp. in the Ginger rhizome «*Zingiber officinale*». Coêlho *et al.*, (2011) isolated *B. cereus* and *B. anthracis* from the seeds and stems of Sumauma «*Ceiba pentandra*» and Mahogany «*Swietenia macrophylla*» from the Amazon.

Furthermore, all species of the genus *Pantoea* can be isolated from fecal matter, soil and plants (Andersson *et al.*, 1999), where they may be either pathogenic or commensal (Monier and Lindow, 2005). Among the bacteria of the genus *Pantoea*, *P. agglomerans* is used by plants as a biocontrol agent against phytopathogenic fungi and bacteria (Adriaenssens *et al.*, 2011). Although this bacterium is good for plant development, it may also become an opportunistic human pathogen. Cruz *et al.*, (2007) have shown that the same species can cause serious infections in children over 6 years of age. According to Kratz *et al.*, (2003), Ulloa-Gutierrez *et al.*, (2004) *P. agglomerans* is often isolated in humans from soft tissue or bone / joint infections. The transmission of the bacteria to humans is due to trauma caused by plants.

MALDI-TOF mass spectrometry is a technology of microbiology, which makes it possible to identify microorganisms by directly analyzing their proteins. Although MALDI-TOF MS was described by Tshikhudo *et al.*, (2013), as the ideal technique for the identification of bacterial cells by the easy determination of peptide fingerprints, De Bruyne *et al.*, (2011) report that various factors can influence the quality and reproducibility of bacterial fingerprints, particularly sample preparation, cell lysis method, matrix solutions and organic solvents, which justifies the use of alternative methods to ensure correct identification.

An investigation of the presence of endophytic bacteria from *U. dioica* L. was carried out. The results obtained demonstrate the presence of a diverse endophytic community in the internal tissues of the Great Nettle which are differently distributed within stems, leaves and roots in both regions.

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## The Very Low Frequency of Epstein-Barr JC and BK Viruses DNA in Colorectal Cancer Tissues in Shiraz, Southwest Iran

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### Abstract

Viruses including Epstein-Barr virus (EBV), JCV and BKV have been reported to be associated with some cancers. The association of these viruses with colorectal cancers remains controversial. Our objective was to investigate their infections association with adenocarcinoma and adenomatous polyps of the colon. Totally, 210 paraffin-embedded tissue specimens encompassing 70 colorectal adenocarcinoma, 70 colorectal adenomatous and 70 colorectal normal tissues were included. The total DNA was extracted, then qualified samples introduced to polymerase chain reaction (PCR). The EBV, JCV and BKV genome sequences were detected using specific primers by 3 different in-house PCR assays. Out of 210 subjects, 98 cases were female and the rest were male. The mean age of the participants was  $52 \pm 1.64$  years. EBV and JCV DNA was detected just in one (1.42%) out of seventy adenocarcinoma colorectal tissues. All adenomatous polyp and normal colorectal tissues were negative for EBV and JCV DNA sequences. Moreover, all the patients and healthy subjects were negative for BKV DNA sequences. The results suggested that EBV and JCV genomes were not detectable in the colorectal tissue of patients with colorectal cancer in our population. Hence, BKV might not be necessitated for the development of colorectal cancer. The findings merit more investigations.

**Key words:** Epstein-Barr virus, adenocarcinoma, colorectal cancer, JCV, BKV, adenomatous

### Introduction

Cancer remains among the most unresolved diseases for human being yet. Colorectal cancer (CRC) is among the most common malignancies and distinguished as a major cause of mortality among human beings. It ranked as the fourth cause of cancer-related death worldwide and responsible for near 1.4 million new cases annually (Arnold *et al.*, 2016).

In the growing list of cancer-related risk factors, viral infections have a special place with a lot of unsolved issues (Hagggar *et al.*, 2009; Jemal *et al.*, 2011; Mahmoudvand *et al.*, 2015). Viruses, especially DNA viruses such as polyomaviruses (JCV and BKV), herpesviruses (Epstein-Barr virus), human papilloma viruses and hepatitis B virus are the causative agents of 15–20% of human cancers including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, cervical cancer and hepatocellular carcinoma (Sarvari *et al.*,

2014; Polz-Gruszka *et al.*, 2015; Schafer *et al.*, 2015). Although each virus employs specific mechanisms of cancer induction, tumorigenic viruses contain oncogenes, which promote transformation of the infected cells, mostly by functional disruption of regulatory proteins, p53 and pRb (Chen *et al.*, 2014). Recently, a number of studies suggested that viruses such as human papillomavirus (HPV), BK, JC and EBV may be related to the outcome of colorectal cancer (Antonic *et al.*, 2013).

EBV, as a ubiquitous herpesvirus, has a widespread distribution among human populations (Moeini *et al.*, 2015). After primary infection through saliva, it establishes a persistent and lifelong infection in almost all individuals. While the direct role of EBV in the progression of a number of malignancies, including Burkitt lymphoma, nasopharyngeal carcinoma and gastric cancer has been revealed (Kadivar *et al.*, 2011), its impact on colorectal cancer development remains controversial (Fiorina *et al.*, 2014).

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Polyomaviruses including JCV, and BKV are prevalent small and non-enveloped viruses that contain a 5Kb circular double-stranded DNA genome (Pinto and Dobson 2014). The large or small T antigen (TAg or tAg) and agnoprotein of JCV have been implicated in blocking of p53 or pRb functions, interaction with  $\beta$ -catenin that consequently induces chromosomal instability, promoting gene damage and neoplastic conversion (Nosho *et al.*, 2009; Collins *et al.*, 2011). Moreover, JCV genome sequence as well as T-antigen expression have been detected in a broad range of brain tumors (Collins *et al.*, 2011; Mou *et al.*, 2012), indicating a possible association with its carcinogenesis. However, if JCV is attributed to the establishment or progression of colorectal carcinoma remained to be elucidated well (Rollison *et al.*, 2010; Sinagra *et al.*, 2014). BKV, another species of polyomaviruses, has also been proposed as a tumor virus, whose expression of oncoproteins, TAg and tAg transforms and immortalizes the rodent and human cells (Abend *et al.*, 2009). As the evidence supporting the strong association of BKV infection or expression of its proteins with tumor development in the case of colorectal cancer is limited, the subject needs to be delineated further (Giuliani *et al.*, 2008; Abend *et al.*, 2009).

Cancer is the third cause of death in Iran. Following lung and breast cancers, colorectal cancer is the third most common cause of cancer here (Mousavi *et al.*, 2009). The incidence rate of colorectal cancer in Iran is 5000 new cases annually, which lead a mortality rate of 2 per 100,000 cases (Esna-Ashari *et al.*, 2009). Although different reports regarding the possible correlation between viral infection and colorectal cancer have been published from Iran, studies which investigated the role of EBV, JCV and BKV in this kind of cancer are scanty. Therefore, this study was conducted to evaluate the association between EBV, JCV and BKV infections and adenocarcinoma/adenomatous colorectal cancer in a population from the southwest of Iran.

## Experimental

### Materials and Methods

**Patients and samples.** Two hundred ten paraffin-embedded biopsy specimens, including 70 adenocarcinoma colorectal tissues, 70 adenomatous colorectal tissues and 70 normal colorectal tissues were included in this study. The samples were collected from Faghihi Hospital, a teaching hospital affiliated to Shiraz University of Medical Sciences. The state of cases and relevant tissue samples had been examined by a specialist pathologist and the selected ones prepared according to the pathology reports (Fig. 1) from early 2012 to late 2013. The study was approved by the Ethics Committee of the University and the informed consent was obtained from all patients before sampling.

**DNA extraction.** Paraffin-embedded tissue samples were cut and deparaffinized by adding xylene as described before (Bakhtiyarizadeh *et al.*, 2017). Briefly, the samples were vortexed and incubated at room temperature and underwent centrifugation at 14,000 RPM for 5 min. Then, the supernatants were detached and absolute ethanol was added to each tube then incubated for 5 more minutes at room temperature. Finally, the tubes underwent high-speed centrifugation and the supernatants were discarded. Both steps were repeated once. In the final step, the tubes were incubated at 37°C until the ethanol evaporated. The total DNA was then extracted using a QIAamp DNA minikit (Qiagen Inc., Düsseldorf, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until use.

**Polymerase Chain Reaction (PCR).** To confirm the quality of the extracted DNA, all the DNA samples were primarily subjected to  $\beta$ -globin gene PCR with primers PCO3/PCO4 (Table I) and unsuitable negative samples were excluded from further experiments (Bakhtiyarizadeh *et al.*, 2017). The PCR reactions were performed in a total volume of 25  $\mu$ l, containing 1 mM MgCl<sub>2</sub>, 200  $\mu$ M

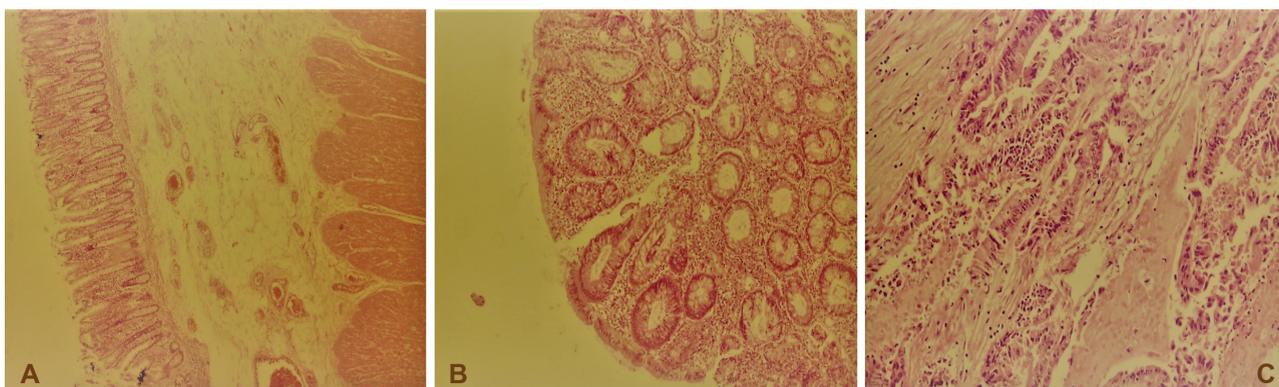


Fig. 1. Histopathology evaluation of different human colorectal tissues.

A: A photomicrograph of human normal colorectal mucosa. B: A photomicrograph of human colorectal adenomatous C: A photomicrograph of human colorectal adenocarcinoma. (H&E×250)

Table I  
The sequences and other characteristics of primers.

Target	Primers	5' to 3' Sequence	Product size, bp
β-globin	PCO3	5'-ACACAACCTGTGTTCACTAGC-3'	110
	PCO4	5'-CAACTTCATCCACGTTCCACC-3'	
JCV	PEP-1	5'-AGTCTTTAGGGTCTTCTACC-3'	173
	PEP-2	5'-GGTGCCAACCTATGGAACAG-3'	
BKV	BRP-1	5'-TTGAGAGAAAGGGTGGAGGC-3'	265
	BRP-2	5'-GCCAAGATTCCTAGGCTCGC-3'	
EBV	HIW1	5'-CCAGACAGCAGCCAATTGTC-3'	129
	HIW2	5'-GGTAGAAGACCCCTCTTAC-3'	

(each) deoxyribonucleotide triphosphates (dNTPs), 1 U Taq DNA polymerase (CinnaGene Inc., Iran) and 1 μM of each specific primers. Specific primers targeting LMP-1 for EBV, Tag for BK and JC viruses and regulatory regions of BKV were selected from previous studies (Nickleit, Klimkait *et al.* 2000, Hoshida, Tomita *et al.* 2004, Giraud, Ramqvist *et al.* 2008), as shown in Table I. To make sure of the validity of the tests, a set of control positive BKV, JCV and EBV DNA samples were also provided by other colleagues (Emami *et al.*, 2015) from transplant research center, affiliated to Shiraz University of Medical Sciences and included in all runs.

The screening PCR tests for β-globin were carried out as follows: primary 10 min initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 45 s, annealing at 44°C for 45 s, extension at 72°C for 1 min following a final extension at 72°C for 10 min. All the positive β-globin gene PCR samples were introduced into further confirmatory EBV, JCV and BKV PCRs, using specific primers. PCR program for amplification of LMP-1 region of EBV was set using HIW1/2 primer pair as follows: 10 min initial denaturation at 95°C, 50 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 90 sec following a final extension step at 72°C for 8 min. PCR tests for Large T-antigen coding region of JCV was performed by the help of PEP-1/2 as follows: 10 min initial denaturation at 95°C, 50 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, extension at 72°C 2 min, and a final extension at 72°C for 8 min. PCR targeting regulatory regions of BKV was performed with BRP-1/2 primers, as follows: 10 min initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 90 sec, and final extension at 72°C for 8 min. The PCR products were then loaded into 1.5% agarose gel, stained with 1% ethidium bromide, and visualized under UV exposure.

**Statistical analysis.** Data were analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA) software. Fisher's exact test was used for data analysis. A P-value below 0.05 was considered as statistically significant.

## Results

Totally, 217 samples were selected primarily for early assessment, and then 210 specimens were included based on β-globin gene positive signals in electrophoresis. Out of 210 subjects included in this study, 98 were female and the rest were male. The mean age was  $52 \pm 1.64$  SD years and the patients' age ranged between 22 and 87 years. Out of 140 patients of the study group, 63 and 77 were female and male, respectively. Out of 70 individuals in the control group, 35 were female and the rest were male. Anatomic locations of the samples were 89 (42.38%) colon tissue, 28 (13.33%) rectum tissue, 24 (11.42%) sigmoid tissue, and 69 (32.85%) other tissues.

On the bases of grading system, out of 70 colorectal adenocarcinoma, 48 well differentiated, 17 moderately differentiated, 3 poorly differentiated and 2 invasive sample were determined. Also, regarding the staging system of 70 colorectal adenocarcinoma 8 stage I, 1 stage II, 18 stage IIA, 1 stage IA, 1 stage IIIA, 1 stage IB, 1 stage IIB, 12 stage IIIB, 1 stage IIC, 3 stage IIC, 1 stage IV and 1 stage IVB were histologically recognized.

Those extracting DNAs that were positive for β-globin gene amplification reaction, showed a 110 bp band in electrophoresis indicative of the integrity of samples for further experiments. All the 210 suitable DNA samples were then subjected to EBV, JCV, and BKV DNA sequence detection. After electrophoresis of PCR results, detection of 129, 265 or 173 bp amplicons on electrophoresis were indicative of EBV, BKV and JCV, respectively, as depicted in Fig. 2. From all investigated specimens, the EBV and JCV genome was identified in only 1 of adenocarcinoma samples. All of adenomatous and normal biopsy specimens were negative for both viruses. Moreover, none of the 140 patients and 70 healthy biopsy specimens was positive for BKV using specific primer set.

Statistical analysis did not show any significant differences in the frequency of EBV, JCV and BKV DNA between adenocarcinoma/adenomatous, colorectal

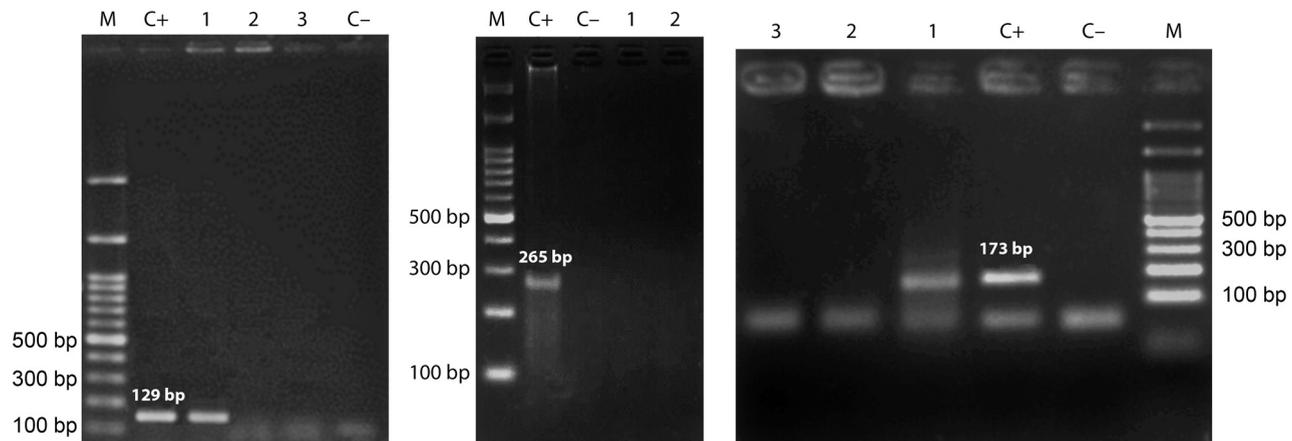


Fig. 2. Results of gel electrophoresis.

(A) PCR analysis of DNA samples using type-specific primers for EBV. M: 100 bp Marker, C+: Control positive, C-: Negative control, 1 (a positive sample), 2 and 3: negative samples. (B) PCR analysis of DNA samples using type-specific primers for BKV. M: 100 bp Marker, C+: Control positive, C-: Negative control, 1 and 2 and negative samples. (C) PCR analysis of DNA samples using type-specific primers for JCV. M: 100 bp DNA Marker; C+: positive control; C-: Negative control; 1(a positive sample), 2 and 3: Negative samples.

tissue and normal biopsy specimens. The analysis also revealed no differences in the frequency of EBV, JCV and BKV DNA between adenocarcinoma and normal colorectal tissues. Totally, EBV and JCV DNA were detected in only adenocarcinoma but not adenomatous or normal biopsy specimens. Regarding the sex of the infected subjects, both infected samples were female. Although the EBV and JCV DNA were detectable only in female patients, the difference was not statistically significant. Clearly, considering the very low frequency of positive samples, there was no significant correlation between grade and stages of malignancy and virus frequency. The age of the EBV and JCV positive patients were 56 and 58 years, respectively; which was a little older than the mean age of the participants. The anatomic location of EBV and JCV positive samples was colon tissue.

### Discussion

Among all the human tumors, CRC with more than 1 million new affected cases annually is categorized as the third most common cancer, worldwide (Parkin, Bray *et al.* 2005). In a similar pattern, in Iran, CRC is recognized as the third most common type of cancers following lung and breast cancers (Kolahdoozan *et al.*, 2010). While the role of viruses such as EBV, JCV, and BKV in tumor development has been suggested (Hollingworth *et al.*, 2015), their true impact on the development of CRC remained to be determined. Herein, we conducted a study to investigate EBV, BKV and JCV virus presence in colorectal tumor tissue, including 70 adenocarcinoma and 70 adenomatous samples in comparison to 70 normal colorectal tissues.

In our study, EBV DNA was detected in only 1 out of 70 (1.4%) adenocarcinoma colorectal tissues while the rest were negative. Regarding the very low frequency, statistical analysis did not show a significant difference among groups. In parallel with our result, Militello *et al.* reported a very low frequency of EBV DNA in 72 cancerous and cancer-adjacent mucous samples (4.2% versus 1.4%) (Militello *et al.*, 2009). Boguszakova *et al.* also failed to detect EBV DNA in the biopsy specimens from adenocarcinoma/adenomatous colorectal tissues (Boguszakova *et al.*, 1988). Similarly, Cho *et al.* reported no sign of EBER gene expression in colorectal tumor specimens, from 274 Korean patients. (Cho *et al.*, 2001). Differently, high frequency of EBV genome was recognized in a similar frequency in both cancerous and normal colorectal samples. In Italy, Fiorina *et al.* reported the presence of EBV DNA in 52% of CRC tissues without including a negative control group. They also showed that most of EBV infections are latent in infiltrating lymphocyte as just a small part of the cells was positive for BZLF-1, a marker of lytic replication (Fiorina *et al.*, 2014). In Iran, reports demonstrated the high but similar frequency of EBV at tumor samples, polyp specimens and non-malignant control group (Mehrabani Khasraghi *et al.*, 2015; Tafvizi *et al.*, 2015). These data are supportive for the absence of a significant correlation between EBV and CRC development.

In opposite, Liu *et al.* in China showed that EBV DNA is detectable in 21% (26/136) of adenocarcinoma/adenomatous specimens and introduced EBV as a carcinogenic agent in colorectal cancer, a finding that was achieved by performing 3 different detection methods (Liu *et al.*, 2003). In another study, EBV DNA was detected as a prevalent pathogen in 19% of 186 cases of sporadic colorectal cancer by using PCR assay albeit

no normal control specimens were included (Karpinski *et al.*, 2011). Ruschoff *et al.* found EBV genome in 15% of cases with colorectal malignancy by using PCR, and then suggested its association with colorectal tumor (Ruschoff *et al.*, 1997). Hence, from pioneer studies EBV has been suggested to be a colorectal cancer associated virus (Kim *et al.*, 1998; Grinstein *et al.*, 2002).

From all the findings, a clear controversy arose regarding the direct role of EBV in CRC development. Whereas reports suggesting the tumorigenic role of EBV are abundant, studies acclaiming no association with CRC are limited, as ours one. This type of controversy may lie in the differences of genetic background, geographical differences, possible co-factors effects as well as sample size. Otherwise, contamination during sample preparation as well as differences in the detection limit of the methods may inevitably explain these diversities (Mehrabani Khasraghi *et al.*, 2015). Separately of all the subjects, in some reports, the misleading achievements may be obtained due to tumor-infiltrating lymphocytes (TILs) and the site of sampling which may harbor latent viruses (Karpinski *et al.*, 2011).

In the case of JCV, only one cancerous sample was diagnosed to be infected with the virus. In agreement with our results, in two other large studies, the frequency of JCV infection was reported 1% and 0% of the investigated CRC tissues (Losa, Fernandez-Soria *et al.*, 2003; Newcomb *et al.*, 2004). Also, in studies from Italy, none of 72 and 71 cancerous and paired adjacent samples were positive for JCV (Giuliani *et al.*, 2008, Militello *et al.*, 2009). Moreover, Lundstig *et al.* showed no increased risk for colorectal cancer among subjects seropositive for JCV in a large prospective follow up in healthy Norwegian male subjects (Lundstig *et al.*, 2007). However, others reported the presence of JCV DNA with different frequencies in colon cancer specimens in different areas (Laghi *et al.*, 1999; Enam *et al.*, 2002; Mou *et al.*, 2012). The first report considering the implication of JCV in CRC establishment was presented by Laghi *et al.*, following an investigation on 24 specimens with 96% positive signal for virus DNA (Laghi *et al.*, 1999). In recent efforts, JCV infection in CRC tissue has been detected more prevalently than normal colon sample by using a molecular assay (Mou *et al.*, 2012; Ksaa *et al.*, 2015) Also in Portugal, Coelho *et al.* reported the rate of 40% and 90% JCV DNA in normal mucosa from the control and patient subjects, respectively (Coelho *et al.*, 2013). They suggested a selective advantage for expanding virus harboring cells toward colorectal tumor progression. Karpinski *et al.* reported 9% frequency of JCV infection among 186 tissues from colorectal cancer in Poland (Karpinski *et al.*, 2011). The possible reason behind the variation in findings is including differences in the detection limit of the assays between laboratories, patient genetic background, and

possible contamination of specimens (Rollison *et al.*, 2010). As a low copy-replicating virus, JCV viral load in the colorectal tissue is expected to be under the detection limit of some molecular methods, reasons behind the diversity of immunohistochemistry, PCR, and real-time PCR assays as described before (Laghi *et al.*, 1999). As mentioned for EBV, age, gender, area of study as well as the lifestyle could also contribute to different rates of JCV infection. Besides all, the site of sampling may also reflect different types of findings due to the diversity of resident cells.

In the case of BKV, none of the samples were positive for BKV genome. Although BKV and JCV are in the same family, clues regarding the role of BKV in tumor induction is not as much as for JCV. In agreement with this, in a study from Italy, no evidence for the BKV contribution in colorectal cancer development was reported (Militello *et al.*, 2009). Moreover, Lundstig *et al.* showed no increased risk for colorectal cancer among subjects seropositive for the BKV in a large prospective study of Norwegian men (Lundstig *et al.*, 2007). Others showed the presence of BKV DNA sequence in 9% (6/66) of Italian patients with colorectal cancer (Giuliani *et al.*, 2008). Regarding the above results, BKV infection might not be involved in the pathogenesis of colorectal cancer as none of the aforementioned studies reported an association with colorectal cancer development.

In our study, the age of only one EBV positive patient was 56 years that was a little older than the participants' mean age. In another report from Iran, Mehrabani *et al.* reported the highest prevalence of EBV in patients with colorectal cancer aged 35–55 years and over 55 years (Mehrabani Khasraghi *et al.*, 2015). Furthermore, the prevalence of EBV in men and women was determined 53.3% and 25%, respectively (Mehrabani Khasraghi *et al.*, 2015). In our study, the anatomic locations of EBV and JCV positive sample were from the colon tissue. Mehrabani Khasraghi *et al.* reported the highest prevalence rate of the virus in the proximal colon (Mehrabani Khasraghi *et al.*, 2015). In the present study, EBV and JCV positive samples were from females' subjects. Although the frequency of EBV positive cases was higher in the colorectal cancer tissues from females than males, the difference was not statistically significant ( $p = 0.41$ ). Collectively, statistical analysis showed no significant association between the frequency of EBV and age, gender and anatomic location in patients with colorectal cancer in our and others' studies.

While the results may be supported by some other aforesaid studies, limitation, including small malignant sample size, duration of sampling, employment of an endpoint PCR assay and a paraffin-embedded moiety of samples should be considered for future study as they all restrict the detection potency and final conclusion.

In conclusion, the results suggested that EBV and JCV infection is not common in patients with colorectal cancer in our population. Moreover, BKV might not be involved in the pathogenesis of colorectal cancer. The study collectively indicated a very low frequency of BKV, EBV and JCV in the colorectal cancer tissue among our population; however, the findings merit more investigations on a large number of cases.

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#### Conflict of interest

All the authors declared no conflict of interest.

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## Comparison of Methods Used for the Diagnosis of Epstein-Barr Virus Infections in Children

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### Abstract

The accurate diagnosis of Epstein-Barr virus (EBV) infections is important, as many other infectious agents or diseases can cause similar symptoms. In this study, sera of pediatric patients who were suspected to have an EBV infection, were sent to Eskisehir Osmangazi University Faculty of Medicine, Department of Clinical Microbiology, and investigated by IFA, ELISA, immunoblotting and Real-time PCR. The performances of these tests were compared with IFA. The rates of agreement between ELISA and IFA were found as 100% for seronegative, 100% for acute primary infection, 22.2% for late primary infection, 92.1% for past infection. The rates of agreement between immunoblotting and IFA were found as 80.8% for seronegative, 68.8% for acute primary infection, 55.6% for late primary infection, 86.6% for past infection. The sensitivity of immunoblotting for anti-VCA IgM was identical with ELISA, and higher for anti-VCA IgG, anti-EBNA IgG, anti-EA antibodies, while the specificity of immunoblotting for these antibodies were found to be lower. The sensitivity and specificity of Real-time PCR for detection of viremia in acute primary infection were found as 56.25% (9/16) and 97.89% (139/142), respectively. The diagnostic methods should be chosen by evaluating the demographic characteristics of patients and laboratory conditions together.

**Key words:** ELISA, Epstein-Barr virus, immunoblotting, Indirect Fluorescence Assay (IFA), Real-time PCR

### Introduction

EBV was discovered in 1964 by electron microscopy of suspension cultures of African Burkitt lymphoma cells (Epstein *et al.*, 1964). EBV is a member of Herpesviridae family and is a double stranded DNA virus (Martinez and de Gruijl, 2008). It is known as human herpesvirus 4 (Odumade *et al.*, 2011).

EBV is an important agent that affects nearly all adults throughout the world (Kreuzer *et al.*, 2013). In Turkey, seropositivity rates in adult age are found between 70–99.4% (Zeytinoglu *et al.*, 2005; Ozkan *et al.*, 2003). Primary EBV infection is usually seen as asymptomatic infection in childhood. Generally, younger children are moderately ill, and the severity of primary EBV infection in adult increases with age (Odumade *et al.*, 2011). During primary infection, EBV can cause a wide variety of symptoms, depending on the host age and immune status, ranging from asymptomatic infection

to severe infectious mononucleosis (IM) with complications (Ebell, 2004; Gärtner *et al.*, 2003). IM typically begins with malaise, and followed by fever, sore throat, swollen cervical lymph nodes and fatigue. Some patients experience an abrupt influenza-like onset, with fever, chills, body aches and sore throat (Odumade *et al.*, 2011). Cytomegalovirus (CMV), Rubella virus, *Toxoplasma gondii* infections and hematologic malignancies can cause similar symptoms. Therefore differential diagnosis of these agents is important.

EBV is intermittently shed from saliva. The main route of transmission is orally from person to person although transmission *via* blood products, transplantation, and sexual transmission were shown (Schooley, 1995; Woodman *et al.*, 2005). Being commonly transmitted through saliva; acute IM is called “the kissing disease” (Thompson, 2015). Virus can affect B and T lymphocytes, epithelial cells and smooth muscle cells, and can cause malign transformation. It is associated with

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Burkitt lymphoma, nasopharyngeal carcinoma, post-transplantation lymphoproliferative disease, Hodgkin and non-Hodgkin lymphoma, gastric carcinoma and leiomyosarcoma (Duca *et al.*, 2007).

In immunocompetent individuals, EBV infection is controlled by the humoral and cellular immune responses, in cooperation with the interferon system. EBV reactivation in immunocompetent individuals may not cause any specific symptoms. But in immunocompromised individuals such as solid organ or bone marrow recipients, and HIV infection, virus reactivation can cause serious complications (Jacobson and LaCasce, 2010).

Formerly, for the diagnosis of acute primary EBV infections, peripheral blood smear (atypical lymphocytes-Downey cells), monospot and Paul-Bunnell agglutination test could be used, but as these tests are not specific to the EBV infection and false negativity rates are high in children aged under 4 especially in Paul-Bunnell test (Horwitz *et al.*, 1981), evaluating specific antibodies by Immunofluorescence Assay (IFA), Enzyme Linked Immuno Sorbent Assay (ELISA), Immunoblotting and searching for DNA by molecular methods are used recently. In immunocompromised individuals, evaluating viral DNA by molecular methods should be used because of the inefficient antibody response.

In this study, serum samples of pediatric patients who were suspected to have an EBV infection were sent to Eskisehir Osmangazi University Medicine of Faculty, Department of Medical Microbiology, and investigated by IFA, ELISA, Immunoblot and Real-time PCR. The performances of these tests were compared with the gold standard test IFA and their sensitivities and specificities were evaluated.

## Experimental

### Material and Methods

**Design of study.** One hundred and seventy-eight consecutive samples that were received between February-June 2013 for EBV serology, from pediatric patients (median age 72 months; range: 24–216 months; 56.7% male, 43.3% female) applied to Eskisehir Osmangazi University Medical Faculty with lymphadenopathy, pharyngitis, fever, hepatomegaly and/or splenomegaly were included in this study. For the control group, 30 healthy pediatric individuals (median age 78 months; range: 24–204 months; 43.3% male, 56.7% female) were included. In a total of 208 serum samples, EBV specific antibodies were studied by IFA, ELISA, immunoblotting, and EBV DNA was evaluated by Real-time PCR.

**Methods.** EBV specific antibodies were evaluated by indirect IFA, ELISA and immunoblotting in serum

samples, and results were interpreted according to the manufacturers. Viremia was evaluated by real-time PCR in all patients except seronegatives.

**IFA.** EBV anti-viral capsid antigen (VCA) IgG, anti-VCA IgM, anti-early antigen (anti-EA) IgG, anti-Epstein-Barr nuclear antigen (EBNA) IgG and anti-VCA IgG avidity tests were evaluated simultaneously with commercial IFA kit (Euroimmun AG, Luebeck, Germany). The wells of the slides were prepared from human EBV transformed cell lines obtained from Burkitt lymphoma patients and the cell lines P3HR1 expressing VCA, Raji cell expressing EBNA complex, and EU-33 expressing EA antigen. The test was studied according to the manufacturer's instructions and the slides were evaluated under  $\times 20$  and  $\times 40$  objectives of fluorescent microscope in accordance with the manufacturer's instructions.

**ELISA.** Anti-VCA IgG, anti-VCA IgM, anti-EBNA IgG, anti-EA IgG antibodies and anti-VCA IgG avidity were evaluated by Euroimmun AG, Luebeck, Germany ELISA kits according to the recommendations of manufacturer. For anti-VCA IgG avidity, serum samples were studied duplicated one with urea (8 M concentration) and one without. At the end of the study, relative avidity index was calculated, and values up to 40 were considered as low avidity (indicating recently acquired infection) and those exceeding 60 were considered as indicative of high avidity. Values between 40 and 60 were evaluated as grey zone.

**Immunoblotting.** Euroline anti-EBV profil 2 Immunoblot kit (Euroimmun, Germany) was used to detect EBV specific anti-VCA IgM, anti-VCA IgG, anti-EBNA-1 IgM, anti-EBNA-1 IgG, anti-p22 IgM, anti-p22 IgG, anti-EA IgM and anti-EA IgG antibodies. This kit contains test strips coated with parallel lines of different antigens. In the case of positivity, the specific IgG or IgM antibodies bind to the corresponding antigenic site, and then to detect the antibodies, another incubation is performed using an enzyme-labelled anti-human Ig to create a color change. Test was performed and at the end of the study the strips were scanned and evaluated by software according to the instructions of manufacturer.

**Real-time PCR.** EBV Real-time PCR Kit, Exicycler™ 96 thermal block, ExiCycler 96 Real-time PCR (Bioneer, Korea) system were used to detect EBV DNA quantitatively in serum samples. DNA isolation was performed by ExiPrep viral DNA/RNA extraction kit and device (Bioneer, Korea). In PCR stage, lyophilized PCR mix was diluted and DNA was added. Then patient samples and five standards were loaded to the system. In PCR stage the cycle was as following: predenaturation at 95°C for 5 min., 45 cycles of denaturation at 95°C for 5 s and annealing and detection at 55°C for 5 s. Data were evaluated by ExiDiagnosis (Diagnostic Soft. ver. 1.27.3).

## Results

EBV infectious serology was evaluated in patient (n=178) and control (n=30) groups by ELISA and immunoblotting tests, and results were compared with IFA as the reference method. EBV DNA was searched in sera by real-time PCR to evaluate viremia.

EBV infection stages were determined by IFA reference method, according to the EBV diagnosis standards (Linde and Falk, 2007). In patient group, 26 (14.6%) seronegativity, 16 (9.0%) acute primary infection, 9 (5.1%) late primary infection, 127 (71.3%) past infection were determined by IFA. In control group, 25 (83.4%) past infection, 4 (13.3%) seronegativity and 1 (3.3%) late primary infection stages were determined.

EBV infection stages were also determined by ELISA and immunoblotting methods, and these results were compared with IFA results (Table I and Table II).

In patient group, ELISA and IFA concordance was as 100% for seronegativity, 100% for acute primary infection, 22.2% for late primary infection, 92.1% for past infection. Immunoblotting and IFA concordance was as 80.8% for seronegativity, 68.8% for acute primary infection, 55.6% for late primary infection, 86.6% for past infection. In control group, the concordance of ELISA and immunoblotting with IFA were as 100%.

When considering specific antibodies, the results obtained by ELISA and immunoblotting were compared with IFA results, and the sensitivities, specificities, positive predictive values and negative predictive values of methods were evaluated (Table III and IV).

The sensitivity of immunoblotting method was found similar with ELISA method for anti-VCA IgM, and was higher than ELISA for the other three antibodies, but the specificity was lower than ELISA for all four antibodies. Table V and Table VI show the PCR results and viral loads according to the infection stages, respectively. DNA was not detected in the control group. The sensitivity of real-time PCR in detecting viremia dur-

Table I  
Comparison of stages determined by ELISA and IFA in patient group.

ELISA serological stage	IFA serological stage				
	SN	API	LPI	Past	Total
SN	26	–	–	2	28
API	–	16	5	1	22
LPI	–	–	2	7	9
Past	–	–	2	117	119
Total	26	16	9	127	178

SN = seronegative, API = acute primary infection, LPI = late primary infection

Table II  
Comparison of stages determined by immunoblotting and IFA in patient group.

ELISA serological stage	IFA serological stage				
	SN	API	LPI	Past	Total
SN	21	–	–	1	22
API	–	11	2	2	15
LPI	2	5	5	14	26
Past	3	–	2	110	115
Total	26	16	9	127	178

IB = immunoblotting, SN = seronegative, API = acute primary infection, LPI = late primary infection

ing acute primary infection is 56.25% (9/16), and the specificity is 97.89% (139/142).

## Discussion

In immunocompetent individuals, the main aim to evaluate serological antibodies is to diagnose acute primary infection (Gärtner *et al.*, 2003). Three antibodies are essential for the detection of infection stage in

Table III  
Comparison of antibody results obtained by ELISA with IFA results in patient group.

ELISA		IFA		ELISA				Kappa value
		Pos.	Neg.	Sens.	Spec.	PPV	NPV	
Anti-VCA IgM	Pos.	20	7	83.33	95.45	74.07	97.35	0.748 (Substantial agreement)
	Neg.	4	147					
Anti-VCA IgG	Pos.	145	1	96.03	96.30	99.32	81.25	0.858 (Almost perfect agreement)
	Neg.	6	26					
Anti-EBNA IgG	Pos.	114	5	91.20	90.57	95.80	81.36	0.792 (Substantial agreement)
	Neg.	11	48					
Anti-EA IgG	Pos.	6	2	26.09	98.71	75	90	0.343 (Fair agreement)
	Neg.	17	153					

Pos. = positive, Neg. = negative, sens. = sensitivity, spec. = specificity, PPV = positive predictive value, NPV = negative predictive value

Table IV  
Comparison of antibody results obtained by immunoblotting with IFA results in patient group.

IB		IFA		IMMUNOBLOTTING				
		Pos.	Neg.	Sens.	Spec.	PPV	NPV	Kappa value
Anti-VCA IgM	Pos.	20	15	83.33	90.26	57.14	97.20	0,617 (Substantial agreement)
	Neg.	4	139					
Anti-VCA IgG	Pos.	150	6	99.34	77.78	96.15	95.45	0.835 (Almost perfect agreement)
	Neg.	1	21					
Anti-EBNA IgG	Pos.	120	15	96	71.70	88.89	88.37	0.716 (Substantial agreement)
	Neg.	5	38					
Anti-EA IgG	Pos.	17	25	73.91	83.87	40.48	95.59	0.427 (Moderate agreement)
	Neg.	6	130					

IB = immunoblotting, Pos. = positive, Neg. = negative, sens. = sensitivity, spec. = specificity, PPV = positive predictive value, NPV = negative predictive value

Table V  
Evaluation of real-time PCR results based on infectious stages by IFA, ELISA and immunoblotting methods in patient group.

		Real-Time PCR			Total
		Detected	Not detected	N/A	
IFA	Seronegative	–	6	20	26
	Acute primary infection	9	7	–	16
	Late primary infection	2	7	–	9
	Past infection	1	126	–	127
ELISA	Seronegative	–	8	20	28
	Acute primary infection	11	11	–	22
	Late primary infection	–	9	–	9
	Past infection	1	118	–	119
IB	Seronegative	–	2	20	22
	Acute primary infection	7	8	–	15
	Late primary infection	5	21	–	26
	Past infection	–	115	–	115
Total		12	146	20	178

IB = immunoblotting, N/A = not applied

immunocompetent patients: anti-VCA IgM, anti VCA IgG and anti-EBNA-1 IgG. Presence of anti-VCA IgM and anti-VCA IgG, absence of anti-EBNA IgG means acute primary infection, and presence of anti-VCA IgG and anti-EBNA with absence of anti-VCA IgM means past infection (De Paschale and Clerici, 2012). Isolated anti-VCA IgG positivity, isolated anti-EBNA positivity and the presence of all three parameters are difficult to interpret. In these situations, to evaluate IgM and IgG antibodies with immunoblotting, anti-VCA IgG avidity test, anti-EA/D antibodies and to investigate viral genome with molecular methods are recommended (De Paschale and Clerici, 2012). In many instances different methods can be simultaneously used to detect the correct infection stage.

In this study, we compared the results of different methods with gold standard IFA test. In our patient

group, the ratio of acute primary infection stage was as 9.0%, and late primary infection consisted of 5.1% of patients. All of the acute primary infection patients (n:16) were also interpreted as acute primary infection by ELISA method. But six other patients were interpreted as acute primary infection by ELISA although five of them were late primary, and one was past infection by IFA. The compliance value of ELISA and IFA was as 100% for seronegativity and acute primary infection, and 92.1% for past infection, but this value was low for late primary infection. Only two of 9 late primary infections were interpreted correctly by ELISA. Five patients were interpreted as acute primary infection, and 2 were as past infection.

By ELISA, four specific antibodies are evaluated (anti VCA IgM, anti VCA IgG, anti EBNA IgG and anti EA IgG). When solely anti VCA IgG is positive, it is recom-

Table VI  
Distribution of real-time PCR positive results on the basis of stages according to different methods.

Real-time PCR Viral load (copies/ml)	IFA Stage	ELISA Stage	Immuno- blotting Stage
92	Past	Past	Late primary
96.9	Acute	Acute	Late primary
283	Acute	Acute	Acute
1120	Acute	Acute	Acute
2470	Acute	Acute	Acute
5440	Acute	Acute	Late primary
6450	Acute	Acute	Late primary
6450	Late primary	Acute	Late primary
8550	Acute	Acute	Acute
8730	Late primary	Acute	Acute
10300	Acute	Acute	Acute
16700	Acute	Acute	Acute

mended to test anti VCA IgG avidity to distinguish late primary infection from past infection without anti EBNA. The avidity testing of VCA IgG may also help to resolve cases in which VCA IgM persists for a long term (Bauer, 2001). In our study, all of the patients with solely anti VCA IgG positivity ( $n=9$ ) had low avidity index values, therefore they were interpreted as late primary infection by ELISA. Two of them were interpreted as late primary infection, and seven were past infection by IFA. Four of these 9 patients were also solely positive for anti VCA IgG by IFA. They were interpreted according to their avidity results. In the literature, the presence of isolated anti VCA IgG profile occurs in 2–8% of immunocompetent patients tested for EBV (Klutts *et al.*, 2009; De Paschale *et al.*, 2009), and most of these cases are thought to correspond to past infections. In our patient group, isolated anti VCA IgG positivity was seen in 10 (5.6%) patients by standard method IFA. Two patients were diagnosed as late primary infection due to their avidity index values. Eight patients were interpreted as past infections. Seven of these eight patients were positive for anti-EBNA IgG, and negative for anti-EBNA IgM by immunoblotting, so they were interpreted as past infection. One of them had anti-EBNA IgM but not anti-EBNA IgG and was interpreted as late primary infection. Isolated anti-VCA IgG can be found in patients with past infection as non-appearance or loss of EBNA-1 IgG, and in patients with acute infection with the delayed appearance or early loss of anti-VCA IgM (De Paschale *et al.*, 2009). It is reported that anti-VCA IgM may appear 1–2 weeks after anti-VCA IgG or lasts for a short time, or anti-VCA IgM can be at low concentrations therefore it cannot be detected. Furthermore, some patients are negative for anti-EBNA

IgG after recovery (De Paschale *et al.*, 2009; Bauer, 1995). In a study that evaluated EBV serological markers of 2422 patients with suspected EBV infection by ELISA, isolated anti-VCA IgG positivity rate is found as 7% (De Paschale *et al.*, 2009). In some instances, second sampling of blood is preferred to correctly diagnose the infection stage, but especially in children it may be difficult. It is important to correctly diagnose the stage in one sample. It may be possible by evaluating the anti-VCA IgG avidity simultaneously.

As mentioned above, all of the acute primary infection cases were diagnosed correctly by ELISA. Kreuzer *et al.*, (2013) compared two different ELISA assays with IFA method in 537 samples and reported the agreement values of assays as 100% for acute infection. The agreement values in seronegativity and past infection were as 89.7–100% and 98.1–99.1%, respectively. Schaade *et al.* (2001) compared the performances of EBV specific ELISA with indirect immunofluorescent reference method in 66 children patients and concluded that the ELISA system used were suitable for the diagnosis of seronegativity and acute EBV infection by that system could not determine recent primary and past infections correctly and had a high rate of indeterminate results. Also, they suggested that the ELISA system used was not applicable to the diagnosis of reactivated EBV infections. Devanthery and Meylan (2010) evaluated EBV infection stages of 387 serum samples by three different methods and determined a consensus stage. They reported ELISA/consensus concordance as 85% (329/387). In 85 samples the consensus stage was acute infection, but ELISA could detect 67 of them correctly. In 18 samples the ELISA could give indeterminate or grey zone results. On the other hand, all of the acute infection results determined by ELISA were evaluated as acute infection by consensus. The sensitivity and specificity of ELISA kits are critical in stage evaluation. Especially the sensitivity of anti-VCA IgM is very important to detect acute infections. The EBV-specific tests differ in the substrates or antigens used, and the interpretation criteria are different among various manufacturers (Hess, 2004). The reference method IFA usually uses EBV-transformed B cell lines from Burkitt's lymphoma patients; on the contrary, most ELISA kits use purified native or recombinant antigens (Gärtner, *et al.*, 2003; De Paschale and Clerici, 2012). The most important thing in determining the correct stage is to choose high quality kits. ELISA is preferred in many laboratories as it is practical. As these assays can be automated, they can improve speed and efficiency of EBV testing, especially in high-volume laboratories.

When we compared immunoblotting stage results with IFA, we realized that the concordance rates were low. Five of 16 (31.2%) acute primary patients were evaluated as late primary infection. The ages of these

patients were 3, 3, 5, 10, and 12. Three of these patients were positive by PCR (96.9, 5440, 6450 copies/ml respectively) and all of these five patients were diagnosed as acute primary infection by ELISA. Immunoblotting method in our study uses recombinant antigens (VCA p19, EBNA-1, p22, EA-D) and one native purified antigen by chromatography (VCA gp125). Recombinant EBV specific antigens are accepted to be superior to lysate antigens, because they are not influenced by potential anti-cellular antibodies (Bauer, 2001; Gärtner *et al.*, 2001). Basically, infection stages can be evaluated according to anti-VCA IgM, anti-VCA IgG, anti-EBNA-1 IgG, anti-EA-D IgG and anti-p22 IgG. This method is highly specific and used mostly as a confirmatory method (Hess, 2004).

In our study, with the use of anti-VCA IgG avidity, we defined late primary infection stage by ELISA and IFA. To make accurate comparisons, we defined stages compatible with late primary infection by evaluating the antibodies detected by immunoblotting. As it is reported in the literature, during convalescence, anti-VCA IgM antibodies decrease, while anti VCA IgG antibodies rise and persist for life. Between the third and sixth months, anti-VCA IgM antibodies disappear but anti-EBNA-1 IgG antibodies become detectable. Therefore, anti-VCA IgM, anti-VCA IgG and anti-EBNA IgG may be present simultaneously in late primary infection and reactivation (Nystad and Myrnel, 2007). We also evaluated the anti-EBNA-1 IgM to be able to make correct comments on stages of infections. It is reported as an indicator of recent primary infection, and it is positive for 2–4 months after primary infection. Although there are a few commercially available anti-EBNA-IgM ELISA kits, anti-EBNA IgM is not used in routine diagnosis of EBV infections. In our study, we could be able to evaluate anti-EBNA IgM by immunoblotting IgM strips. 26 patients and 1 individual of the control group were evaluated as late primary infection. 20 of them were positive for anti-VCA IgM, anti-VCA IgG and anti-EBNA IgG, and in 10 anti-EBNA IgM was positive. In 7 samples, anti-VCA IgM and anti-EBNA IgG were negative but anti-VCA IgG was positive. According to their ages and/or anti-EBNA IgM results they were evaluated as late primary infection. By immunoblotting, some of the results were difficult to interpret. In routine diagnosis additional tests should be performed to diagnose the correct stage. But in our study, we aimed to evaluate the sufficiency of the tests alone. Although immunoblotting is suggested as a confirmatory test (Hess, 2004), we had some serious problems especially when anti VCA IgM, IgG and anti-EBNA IgG were simultaneously or when only anti VCA IgG was positive. Maybe we should report these results as “not determined” but we evaluated them as “late primary infection” and this may be our limitation.

We also aimed to measure the agreement of different methods in detecting the specific antibodies correctly as well as the correct staging, and evaluated sensitivity and specificity values. To interpret the degree of agreement, the guidelines reported by Cohen and Viera were used: Kappa value between 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, 0.81–0.99 almost perfect agreement (Cohen, 1960; Viera and Garrett, 2005). According to these criteria, ELISA method had substantial or almost perfect agreement values for anti VCA IgM, IgG and anti-EBNA IgG. But this value was very low for anti-EA IgG. Therefore, we suggest that in immunocompetent patients, it is appropriate and sufficient to evaluate the infection stage by these three antibodies but not anti-EA IgG. Likewise, the agreement value of anti-EA IgG of immunoblotting method was low as moderate agreement. As immunoblotting is recommended as a confirmatory method, this low value is disappointing.

In the literature, anti-EA IgG is reported positive in approximately 85% of patients with acute infection, but in some cases, it can still be positive for years after acute primary infection. During reactivation or nasopharyngeal carcinoma, high titers of anti-EA IgG are seen (de Paschale and Clerici, 2012). It is also reported that, IgG reactivity against EA can be seen in all phases of infection, although it is predominantly seen during lytic infection (Nystad and Myrnel, 2007). Since an increase in the titer of anti-EA IgG can be considered as a marker of reactivation, it is suggested to evaluate anti-EA IgG only if serial sampling is possible (De Paschale and Clerici, 2012). In a study by Altuglu *et al.*, (2007), they reported the kappa value of anti-EA IgG of the same immunoblotting system as 0.67 (substantial agreement). But this value was as 0.20 for anti-EA IgG with automated bead assay. Klutts *et al.*, (2009) evaluated specific EBV antibodies and determined all possible phases that may occur. They reported that except for the relatively small number of primary acute infection patients, anti EA IgG was of little utility. Also Hess (2004) reported the positivity rate of anti-EA IgG in primary infection as 60–80% and suggested that in 20% of healthy individuals it is present. In this literature it is reported that EA specific serological parameters are not helpful to confirm any stage specific diagnosis.

In addition to the comparison of the methods in evaluating individual parameters, sensitivity and specificity values were also compared. The sensitivity of these two methods was the same for anti VCA IgM, and immunoblotting had slightly high values for anti VCA IgG and anti EBNA IgG. But the sensitivity performance of immunoblotting was significantly better for anti EA IgG. When we considered the specificity values, immunoblotting method had lower values. Immuno-

blotting assays are considered to have high specificity for EBV serology but unfortunately we had lower values than ELISA, especially for anti EBNA IgG (false positive results; 15/53). This means that, anti EBNA negative acute primary infection serologic profiles could be diagnosed as past infection, according to the anti EBNA IgG positivity. The IFA method is labor-intensive and requires experience. To prevent or minimize the false positive or negative results, the slides were evaluated by two different researchers, and confirmed by a specialist in this topic. Besides, to make accurate comparison and to eliminate the differences between manufacturers, we used ELISA, IFA and immunoblotting kits from the same manufacturer. The antigens used by IFA for anti-VCA antibodies were antigen expressing P3HR1 cells and, native gp125 and recombinant p19 antigen. The antigens used by ELISA and immunoblotting were similar. For anti-EBNA IgG and anti-EA, ELISA and immunoblotting used recombinant antigens, but IFA used cells expressing EA antigen and Raji cells expressing EBNA.

In the active phase of the infection, EBV DNA is present in plasma or serum samples. In addition to active infection, EBV DNA viremia is present in reactivation, EBV associated malignancies, and posttransplant lymphoproliferative disease. Our study group consisted of pediatric patients; therefore the most common causes of the EBV serologic tests in routine practice were to diagnose the acute primary infection, and to exclude EBV infection in patients with lymphadenopathy. In our study, we detected EBV DNA in 9 of 16 acute primary infection patients. Therefore, we determined the sensitivity in active infection as 56.25%. EBV DNA was positive in 2 (viral loads were 6450 and 8730 copies/ml) of 9 late primary and 1 (viral load 92 copies/ml) of 127 past infection patients. The latter patient was a 3-year old child with classic triad of EBV infection as sore throat, cervical lymphadenopathy and fever. Interestingly, the stage determined by immunoblotting was as late primary infection with anti VCA IgM positivity. This finding made us think that whether ELISA and IFA methods could not detect the anti VCA IgM. But as IFA was considered as the gold standard test for the EBV infections, we suggested the anti-VCA IgM of immunoblotting as false positive. In routine practice, real time PCR would not be performed for this patient as it was considered as past infection. Detecting viremia by molecular methods is necessary for immunosuppressed individuals, as serological assays are insufficient due to their deficiencies in generating antibodies. Therefore, in immunosuppressed patients, detection of viral load by PCR is recommended (Hess, 2004). We suggest that the duration of viremia and the viral load may vary between patients, as we could not detect in some of acute primary patients but detected in late pri-

mary stage. Our positivity rate of EBV DNA in acute primary infection was lower than studies reported previously. Chan *et al.* (2001) and Gartzonika *et al.* (2012) reported the sensitivity of PCR in acute infections as 80% and 93.5%, respectively. The PCR system we used is a closed system that prevents the contamination, and the range of detection of the kit is  $10-10^{10}$  copies/reaction. In the literature, it is reported that, in immunocompetent infectious mononucleosis patients, EBV DNA is detectable in serum for only approximately seven days after onset of symptoms (Fafi-Kremer *et al.*, 2005). In addition, Berth *et al.* (2011) reports that EBV DNA can be undetectable while the serological profile can be acute EBV infection. Therefore, we commented that the low sensitivity rate could be due to the short duration of viremia in our patients.

In our study, we aimed to evaluate the performances of different diagnostic methods on determining the infection stages and detecting the specific antibodies in serum samples of patients with suspicious of EBV infections. But as a limitation of our study, we did not evaluate the anti-CMV IgM and IgG antibodies to exclude cross reactivation on anti VCA IgM positive samples.

In conclusion, IFA is the gold standard test for the diagnosis of EBV infections, but the most important disadvantages are the high cost and the requirement of experienced staff. The compliance of ELISA with IFA is high, and this method provides advantages in terms of ease of use as it is practical and can be automated. Although immunoblotting is recommended as a confirmatory test in EBV diagnosis, high false positivity rates should be kept in mind. Especially in immunocompromised patients that serological methods are insufficient, real-time PCR is recommended to detect viremia. The most suitable diagnostic method should be decided according to the demographic characteristics of patient groups, experience of the staff, financial facilities, working conditions of the laboratory, and when necessary the correct conclusion should be achieved using more than one diagnostic method as a test battery.

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## Isolation of Sabin-like Polioviruses from Sewage in Poland

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### Abstract

As a complement to the active search for cases of acute flaccid paralysis, environmental sampling was conducted from January to December 2011, to test for any putative polio revertants and recombinants in sewage. A total of 165 environmental samples were obtained and analyzed for the presence of polioviruses by use of cell culture (L20B, RD and Caco-2) followed by neutralization and reverse-transcription polymerase chain reaction. Out of the 31 CPE positive samples, 26 contained one and 5 two different serotypes, yielding a total of 36 PVs. The microneutralization test revealed the presence of 7, 10 and 19 strains belonging to poliovirus serotype 1, 2 and 3, respectively. The genomic variability of 36 poliovirus strains was examined by the restriction fragment length polymorphism assay (RFLP). By combined analyses of two distant, polymorphic segments of the viral genome, one situated in the capsid protein VP1 coding region and the other in the 3D-polymerase coding region, we screened for the putative poliovirus revertants and recombinants. All detected PVs were classified as vaccine strains on the basis of RFLP-VP1 test. None of wild-type PVs or vaccine derived polioviruses were detected. RFLP assay also revealed the presence of 11 recombinants in 3D-polymerase coding region. Nine isolates appeared to be S3/S2, one S3/S1 and S1/S2 recombinant in analyzed 3D<sub>pol</sub> region. This study revealed, through environmental monitoring, the introduction of SL PVs into the population associated with the routine use of OPV in Poland before the April 2016. Our findings demonstrate the usefulness of environmental surveillance in the overall polio eradication program.

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**Key words:** environmental surveillance, oral polio vaccine (OPV), poliovirus recombinants and revertants in sewage

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### Introduction

The three serotypes of poliovirus (PV) are members of the family *Picornaviridae*. The viruses possess a single-stranded RNA genome of approximately 7,400 nucleotides (nt). Poliovirus is a causative agent of poliomyelitis, commonly known as polio (Landsteiner and Popper 1909). Polioviruses are transmitted by the fecal-oral route, they multiply in the gastrointestinal tract and are excreted in large numbers in the feces of infected persons, whether or not they are symptomatic. Virus infects sensitive cells of lymphoid tissue in the mouth, nose and throat. The incubation period lasts from 2 to 35 days. It leads to a transient viremia and the virus spreads to the reticuloendothelial system without causing clinical symptoms (Sabin, 1956). In very rare cases, 1–2% of infected individuals, the virus enters the central nervous system (CNS) and replicates in motor neurons within the spinal cord, brain stem, or motor cortex.

The Global Polio Eradication Initiative (GPEI) was launched in 1988. The basic strategy involves high levels of immunization, routine vaccination and poliovirus

surveillance based on investigation of acute flaccid paralysis (AFP) cases. Although AFP surveillance is still the gold standard for GPEI, under certain circumstances supplementary information can be obtained by environmental poliovirus surveillance (ES). The rationale for ENV is based on the fact that infected individuals, whether they presenting the symptoms or not, excrete poliovirus in faeces up to several weeks. ES is used for monitoring of PV transmission in human populations by examining environmental samples contaminated by human faeces.

About 30 years ago, the effective control of poliomyelitis was achieved by the introduction of two polio vaccines: inactivated poliovirus vaccine (IPV) and the oral polio vaccine (OPV), both of which contain all three PV serotypes. The OPV consists of live attenuated Sabin strains obtained from wild PVs (Sabin and Boulger 1973). Since 2004, three doses of IPV and one dose of OPV have been used in Poland. According to WHO Global Action Plan III recommendations, Poland was obligated to switch to the IPV-only schedule before April 2016.

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Both vaccines (OPV and IPV) provide protection from poliomyelitis. However, OPV strains actively multiply in the gut and induce local secretory IgA response that is not seen with IPV. A major problem with the OPV is that it is genetically unstable. Sabin strains are subject to genetic variation during their replication in human intestine. Mutation in VP1 region encoding capsid protein may result in recovery of the capacity for higher neurovirulence of OPV strains. The neurovirulent revertants – vaccine derived polioviruses (VDPVs) may cause paralysis in under-immunized population and develop sustained circulation. VDPVs display 1–15% nucleotide difference within VP1 from parental vaccine strain (CDC 2007; Kew *et al.* 2005). Such changes were observed also by alterations of indirect markers such as thermosensitivity (ts-phenotype). Higher capacity to replication at supraoptimal temperature (40°C) of VDPVs and wild type (WT) polioviruses has been attributed to the loss of the ts-phenotype (Zurbriggen *et al.* 2008). Genetic recombination is another mechanism frequently observed in Sabin strains. The administration of trivalent OPV provides optimal conditions for recombination between heterotypic viral genomes. Thus, poliovirus strains are subject to genetic variation during their multiplication in humans (Kew and Nottay 1984). This prompted the initiation of the a study to investigate the prevalence of poliovirus in sewage samples collected in Poland. The study led us to create a comprehensive monitoring system for the environmental surveillance of PVs. Our results provide valuable information about the prevalence of PVs in sewage samples collected from 14 locations in Poland during 12-month period. Our study highlights the importance of environmental surveillance for detection of PVs during the OPV-to-IPV transition period.

## Experimental

### Materials and Methods

**Sewage samples.** A total of 165 raw sewage samples were collected between January and December 2011, one sample per month, from 14 sewage disposal systems in Poland. The sample-processing protocol has been described earlier (Zurbriggen *et al.*, 2008). To describe the process briefly,  $\text{AlCl}_3$  (final concentration, 0.5 mM) was added to 500 ml of sewage sample, and the pH was adjusted to 3.5. Following the addition of 250  $\mu\text{l}$  of an  $\text{SiO}_2$  slurry, the samples were stirred for 30 min followed by centrifugation at room temperature and 1500  $\times$ g for 10 min to pellet the  $\text{SiO}_2$ . The virus was recovered by rocking the pellet for 20 min with 3 ml of 50 mM glycine (pH 9.5) containing 3% (wt/vol) beef extract. After centrifugation for 5 min

at 4°C and 1500  $\times$ g, the supernatants were treated with 3 ml chloroform with rocking for 20 min. After a final centrifugation, the concentrates were used to inoculate cell cultures.

**Isolation and identification of viruses.** RD, L20B and Caco-2 cells were used throughout the experiment. RD and L20B cells were cultivated in minimal essential medium supplemented adequately with 3.5 and 10% fetal bovine serum (FBS). Caco-2 cells were cultivated in EMEM supplemented with 10% FBS. A volume of 200  $\mu\text{l}$  sewage concentrate was inoculated into tubes with RD and L20B cells according to WHO procedure (WHO 2004) and additionally into tubes with Caco-2 cells. The tubes were incubated for 7 days at 36°C and examined daily for the appearance of cytopathic effect (CPE). After 7 days, the tubes were frozen at –20°C, thawed and 200  $\mu\text{l}$  volumes passaged in cultures of the same cell type. Each specimen underwent three passages in RD, L20B and Caco-2 cells. Isolates obtained from specimens that are negative in L20B cells but positive in RD or Caco-2 cell line were re-passaged in L20B to include the possibility of poliovirus. Samples demonstrating characteristic viral CPE were identified by neutralization assay using a selected set of polyclonal antisera against poliovirus type 1, 2 and 3 developed by National Institute of Public Health and the Environment, the Netherlands. The typing of polioviruses was performed according to WHO polio laboratory manual (WHO 2004). Briefly, using the micro-neutralization (microtitre plate) technique, the serum/isolate mixtures are incubated for one hour at 36°C to allow the antibodies to bind to the virus. Subsequently, suspensions of L20B cells are added to the microtitre plates which are examined daily for the presence of CPE. The anti-serum that prevents the development of CPE indicates the identity of the virus.

**Molecular characterization of viruses.** Viral RNA was purified from supernatants of tubes that showed cytopathic effect, using Qiamp Viral RNA Mini Kit following the manufacturer's instructions. RT-PCR for intratypic differentiation (ITD) was carried out with Sabin-type specific primers according to WHO manual (WHO 2004). Three primer pairs (Sabin1.re/Sabin1.fw, Sabin2.re/Sabin2.fw, Sabin3.re/Sabin3.fw) targeting the sequence of VP1 region characteristic for each Sabin strain. These primers allow the amplification of genomic fragments of 97 bp, 71 bp, 53 bp from PV Sabin 1, Sabin 2 and Sabin 3 strains, respectively. RT-PCR was performed: 1 cycle of reverse transcription at 45°C for 20 min; 1 cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 70°C for 30 s followed by 1 cycle of elongation at 70°C for 7 min. Amplification products were analyzed in 2% agarose gels, stained with GelRed and visualized under UV light.

**Restriction Fragments Length Polymorphism (RFLP) assays.** Recombinant genomes were detected with a double RFLP assay that was performed as described elsewhere (Balanant *et al.* 1991, Furione *et al.* 1993) with slight modifications. Briefly, two distant regions of the viral genome were subjected to RT-PCR amplification followed by restriction enzyme digestion. The first pair of primers UC1 and UG1 was used for amplification of a 480 bp segment in VP1 capsid-encoding region in the RFLP-VP1 test. Another set of primers UC8 and UG7, was used to amplify a 291 bp segment in the 3D polymerase-coding region (RFLP-3D assay). The one step RT-PCR amplification was performed: 1 cycle of reverse transcription at 42°C for 30 min, 1 cycle of denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 10s, annealing at 45°C for 60 s, elongation at 70°C for 60s followed by 1 cycle of elongation at 70°C for 5 min. Amplification products of 490 bp and 291 bp were analyzed in 2% agarose gels, stained with GelRed and visualized under UV light.

Restriction enzymes were selected to differentiate between the genomes of the recombinants and the parental Sabin strains. RFLP-VP1 patterns of poliovirus strains were obtained by digesting a fraction of 480 bp amplified fragment with *Hae*III (10U), *Dde*I (5U) and *Hpa*II (14U). The 291 bp amplicon was digested by *Hae*III (10U), *Dde*I (10U) and *Rsa*I (10U) to obtain the RFLP-3D profiles. Reaction mixtures were incubated overnight at 37°C and digestion products were analyzed by electrophoresis in 3% agarose gel.

**Determination of temperature sensitive (ts) phenotype.** Tubes with confluent monolayer of L20B cells were inoculated with 200 µl of undiluted virus stocks: poliovirus isolates (no 1–36) and Sabin 1, 2 and 3 strains. After adsorption for 1 h at 36°C or 40°C, the unabsorbed virus inoculum was removed. The maintenance medium was added and the tubes were incubated in parallel at 36°C or 40°C. After 24 h, the tubes were frozen and thawed three times. The infectivity of isolates and Sabin strains was measured by titration on L20B cells according to WHO procedure (WHO 2004). In a previous study Zubriggen *et al.* (2008) showed a titer reduction of 2 log<sub>10</sub> units with reference wild type PV1/Mahoney strain. Therefore, the titer reduction of >2 log<sub>10</sub> units was considered to reflect temperature sensitivity.

## Results

**Isolation and identification of polioviruses.** The wastewater samples were collected monthly from 14 sampling sites in Poland between January and December, 2011. In total, 31 out of 165 (18.8%) sewage samples were positive for poliovirus isolation in L20B, RD, Caco-2 cells and resulted in recovery of 36 PV strains. Polioviruses were isolated from samples collected in all months except April, May and June (Fig. 1). The highest monthly percentage of positive samples was observed in December (57.1%) and September (42.9%). The percentage of samples positive in cell culture

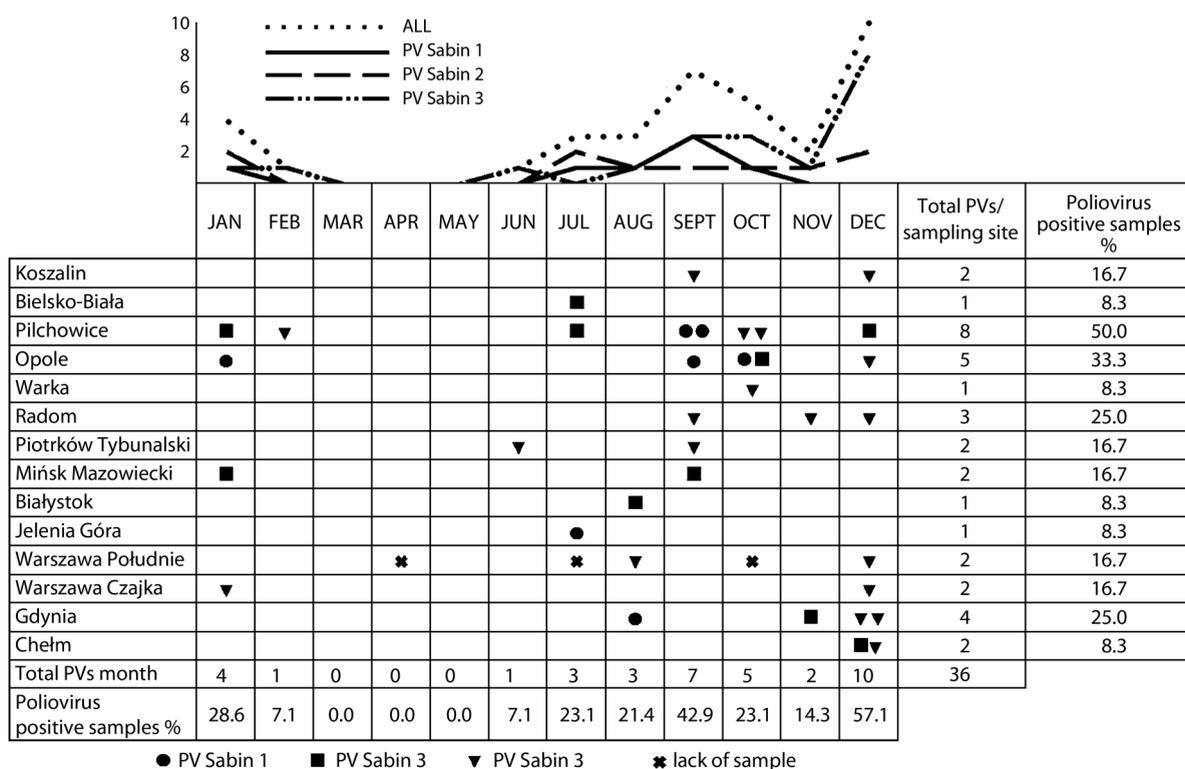


Fig. 1. Seasonal distribution of polioviruses isolated in cell culture from sewage samples in Poland.

isolation at the different sampling sites ranged between 8.3% and 50.0% (Fig. 1).

Out of the 31 CPE positive samples, 26 contained one and 5 two different serotypes, yielding a total of 36 PVs. All poliovirus strains were isolated in three cell lines: L20B (17 strains), RD (2 strains) and Caco-2 (17 strains). The identification of poliovirus isolates, was performed using a selected set of polyclonal antisera against poliovirus types 1, 2 and 3. The microneutralization test revealed the presence of 7, 10 and 19 strains belonging to poliovirus serotype 1, 2 and 3, respectively.

**Intratypic differentiation of polioviruses.** Further characterization of isolates was carried out by intratypic differentiation using Sabin type-specific primers. The results obtained by RT-PCR assay for ITD confirmed the serotypes identified in microneutralization test and showed that all isolates are poliovirus Sabin-like (SL) strains (Table I).

Most of identified PVs come from samples collected during the June-December period (Fig. 1). The most frequently identified PV type 3 was detected in the following months: January, February, June, August (7.1%), September (21.4%), October (23.1%) and November (7.1%) with a peak on December (57.1%). Detection of PVs type 2 was mainly observed from July to Decem-

ber. PVs type 1 were isolated almost on the same level as PVs type 2, without a clear peak. All samples collected in March, April and May were negative for polioviruses.

The geographical distribution of polioviruses identified in sewage samples is shown in Figure 2. The number of detected PVs differed from 8 to 1, depending on the sampling site.

**Determination of ts phenotype.** In contrast to WT polioviruses, SL strains display ts phenotype and do not grow well at the elevated temperature of 40°C. For this reason, all isolates were tested for the replication capacity in supraoptimal temperature. Almost all poliovirus strains were temperature sensitive as expected, with titer reduction of  $\sim 2 \log_{10}$  in 36°C/40°C. Only two strains showed  $< 2 \log_{10}$  reduction in titer.

**Identification of vaccine (Sabin) origin by RFLP-VP1.** The vaccine origin of isolates was determined first by ITD assay. To confirm the vaccine origin of the strains, viral genomes were analyzed by RFLP-VP1 test. A 480-nucleotide fragment in the VP1 capsid protein-coding region was amplified by PCR from the viral genome. RFLP patterns specific for the isolates were generated by digestion of the PCR product with *HaeIII*, *DdeI*, and *HpaII* endonucleases and compared with the patterns obtained with the reference strains: Sabin type 1, 2, 3 (Table I). The RFLP profiles of all iso-

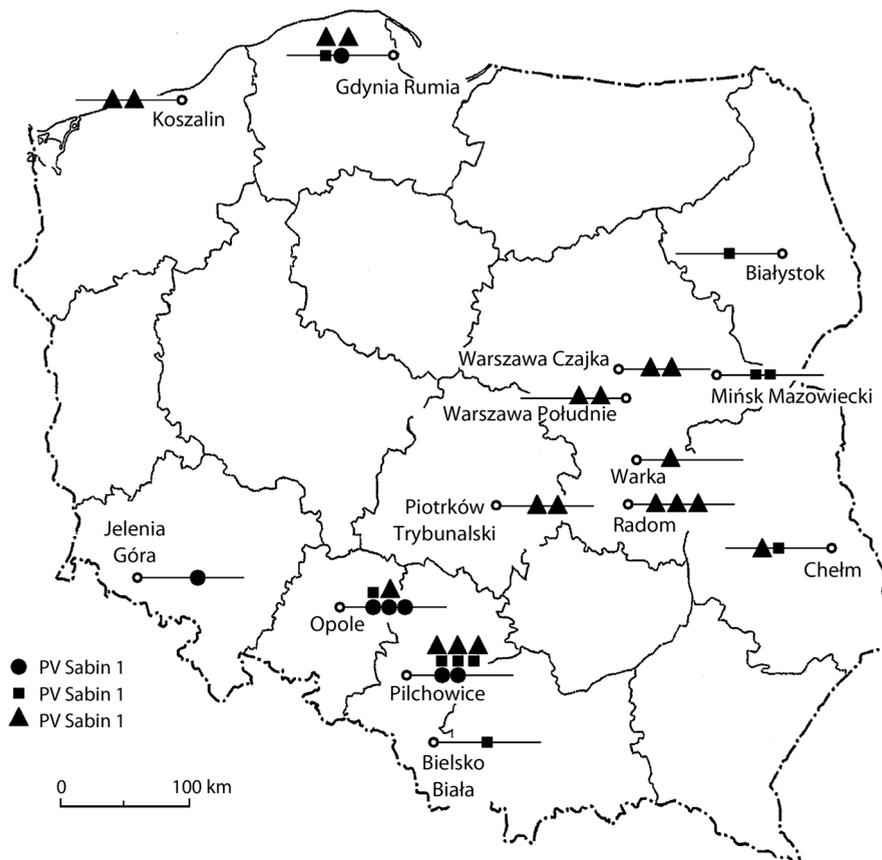


Fig. 2. Geographical distribution of polioviruses identified in sewage samples in Poland.

Table I  
Polioviruses identified in sewage samples in Poland.

Isolate no./Month	Cell line	City	ITD	RFLP assay					
				RFLP-VP1			RFLP-3D		
				HaeIII	DdeI	HpaII	HaeIII	DdeI	RsaI
1/VIII	CaCo-2	Gdynia	S1	S1	S1	S1	S1	S1	S1
2/VII	L20B	Jelenia Góra	S1	S1	S1	S1	S1	S1	<u>S2</u>
3/I	L20B	Opole	S1	S1	S1	S1	S1	S1	S1
4/IX	RD	Opole	S1	S1	S1	S1	S1	S1	S1
5/X	L20B	Opole	S1	S1	S1	S1	S1	S1	S1
6/IX	CaCo-2	Pilchowice	S1	S1	S1	S1	S1	S1	S1
7/IX	L20B	Pilchowice	S1	S1	S1	S1	S1	S1	S1
<b>Control strain: PV Sabin 1</b>			S1	S1	S1	S1	S1	S1	S1
8/VIII	CaCo-2	Białystok	S2	S2	S2	S2	S2	S2	S2
9/VII	RD	Bielsko-Biała	S2	S2	S2	S2	S2	S2	S2
10/XII	CaCo-2	Chełm	S2	S2	S2	S2	S2	S2	S2
11/XI	CaCo-2	Gdynia	S2	S2	S2	S2	S2	S2	S2
12/I	L20B	Mińsk Maz.	S2	S2	S2	S2	S2	S2	S2
13/IX	L20B	Mińsk Maz.	S2	S2	S2	S2	S2	S2	S2
14/X	CaCo-2	Opole	S2	S2	S2	S2	S2	S2	S2
15/I	L20B	Pilchowice	S2	S2	S2	S2	S2	S2	S2
16/VII	CaCo-2	Pilchowice	S2	S2	S2	S2	S2	S2	S2
17/XII	CaCo-2	Pilchowice	S2	S2	S2	S2	S2	S2	S2
<b>Control strain: PV Sabin 2</b>			S2	S2	S2	S2	S2	S2	S2
18/XII	CaCo-2	Chełm	S3	S3	S3	S3	S3	S3	S3
19/XII	CaCo-2	Gdynia	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
20/XII	L20B	Gdynia	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
21/IX	L20B	Koszalin	S3	S3	S3	S3	S3	S3	S3
22/XII	L20B	Koszalin	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
23/XII	CaCo-2	Opole	S3	S3	S3	S3	S3	S3	S3
24/II	CaCo-2	Pilchowice	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
25/X	CaCo-2	Pilchowice	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
26/X	L20B	Pilchowice	S3	S3	S3	S3	S3	S3	S3
27/VI	L20B	Piotrków Tryb.	S3	S3	S3	S3	S3	S3	S3
28/IX	L20B	Piotrków Tryb.	S3	S3	S3	S3	<u>S1</u>	<u>S1</u>	<u>S1</u>
29/IX	L20B	Radom	S3	S3	S3	S3	S3	S3	S3
30/XII	L20B	Radom	S3	S3	S3	S3	S3	S3	S3
31/XI	CaCo-2	Radom	S3	S3	S3	S3	S3	S3	S3
32/X	CaCo-2	Warka	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
33/I	L20B	W-wa Czajka	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
34/XII	L20B	W-wa Czajka	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
35/VIII	CaCo-2	W-wa Płd.	S3	S3	S3	S3	S3	S3	S3
36/XII	CaCo-2	W-wa Płd.	S3	S3	S3	S3	<u>S2</u>	<u>S2</u>	<u>S2</u>
<b>Control strain: PV Sabin 3</b>			S3	S3	S3	S3	S3	S3	S3

S1, S2, S3: Poliovirus Sabin type 1, 2 and 3, respectively.

lates, after cleavage with 3 endonucleases were identical to those of the Sabin type 1, 2 and 3 reference strains. However, one exception was recorded. The restriction profiles after cleavage with *DdeI* and *HpaII* of the strain 25 were similar to those obtained with Sabin 3. Cleav-

age with *HaeIII* yielded one additional DNA fragment (<300 bp), different from those obtained with Sabin 3. This RFLP pattern may suggest genetic variation and introduction additional restriction site recognized by endonuclease *HaeIII*.

**Detection of recombinant strains by RFLP-3D.** We screened for recombinant strains by RFLP-3D assay, which analyzes the 5' part of the 3D polymerase coding region. RFLP-3D profiles were generated by digestion of the 291 bp PCR product with *Hae*III, *Dde*I, and *Rsa*I endonucleases and compared with the patterns obtained with the reference strains: Sabin type 1, 2, 3 (Table I). Among the 7 strains belonging to PV1, 6 had RFLP-3D profiles similar to those obtained with the Sabin 1. One strain (2) had restriction profile similar to those of the Sabin 2 with one of the three endonucleases used (*Rsa*I), which strongly suggested the relatedness to the Sabin 2. None of the serotype 2 strains was found to have a recombinant genome. Out of the 19 Sabin 3-derived strains, only 9 had restriction profiles identical to those obtained with Sabin 3. Nine strains (19, 20, 22, 24, 25, 32, 33, 34, 36) had RFLP-3D profiles indistinguishable from those obtained with Sabin 2, suggesting that their genomes consisted of segments derived from both Sabin 3 and Sabin 2. One strain (28) had restriction profile obtained after digestion with three endonucleases, identical to those of the Sabin 1, suggesting close relatedness.

### Discussion

Environmental surveillance has been used successfully in monitoring poliovirus circulation and assessing the extent or duration of epidemic poliovirus circulation in specific populations. In several countries, wild polioviruses and VDPVs have been detected in the sewage, sometimes in the absence of reported AFP cases. The list of countries routinely employing environmental surveillance includes Czech Republic, Egypt, Estonia, Finland, India, Israel, Japan, Latvia, The Netherlands, New Zealand, Pakistan, Russia, Slovakia and Switzerland. In Finland and Israel it is considered to be the main approach of PV surveillance (Hovi *et al.*, 2010). Polioviruses are frequently isolated from sewage samples in countries using OPV in routine immunization schedule. In Finland, where IPV is used exclusively for polio immunization, Sabin strains are isolated from environmental samples annually. This phenomenon involves traveling tourists from neighbouring Estonia and Russia, where OPV has been used recently. Moreover, importations of wild polioviruses was documented by ENV in the Israel and Gaza district in the absence of AFP cases (Manor *et al.*, 1999; Manor *et al.*, 2007). As a complement to the active search for cases of AFP, ENV has a role in the new WHO strategy (The Global Polio Eradication Strategic Plan, 2010–2012) for intensified efforts to complete poliomyelitis eradication.

After eradication of WPV transmission, the risk of re-emerge of polio remains is high (especially for under-

immunized populations) as long OPV continues to be used routinely. Attenuated strains of the Sabin OPV tend to vary during their natural multiplication in the human gut and in rare cases cause vaccine-associated paralytic poliomyelitis (VAPP). Thus oral polio vaccine provides a potential source of circulating neurovirulent VDPV strains. Environmental contamination with feces and increasing use of wastewater in agriculture are likely risk factor for transmission of PVs (Dowdle *et al.*, 2006). Highly divergent and neurovirulent VDPVs have been isolated from sewage samples in several countries: Greece, Finland, Slovakia, Switzerland (Dedepšidīs *et al.*, 2007; Roivainen *et al.*, 2010; Cernáková *et al.*, 2005). ENV can be important tool for monitoring the emergence of VDPVs, re-emergence of WT PVs or disappearance of Sabin strains after OPV cessation.

The present study used SiO<sub>2</sub>-based protocol for concentration of sewage samples, followed by isolation of viruses in cell lines: L20B, RD and Caco-2. Compared to other methods of concentration, this protocol is simple, fast and easy to implement in environmental microbiology, especially for large volumes of the analyzed samples. Moreover, Zurbriggen *et al.* demonstrated a higher recovery rate (60%) in silicon dioxide assay than the two-phase separation protocol (30%) recommended by WHO (WHO 2003).

Culture-based systems for virus isolation have been the “gold standard” in clinical virology for decades. Although this technique is often slow and requires considerable technical expertise, WHO recommends L20B and RD cell lines for isolation of polioviruses (WHO 2004). In this study, Caco-2 cell line was used to obtain the maximum sensitivity for isolation of poliovirus from the sewage samples. Our earlier studies and other authors showed that Caco-2 cell line support growth of astroviruses, adenoviruses, rotaviruses and enteroviruses including poliovirus (Hamza *et al.*, 2011; Pinto *et al.*, 1995; Wiczorek *et al.*, 2013). In total, we isolated 36 poliovirus strains out of 165 (isolation rate 18.78%) sewage samples in L20B, RD, Caco-2 cells. Interestingly, 47% of poliovirus strains were isolated in Caco-2 cell line. The use of Caco-2 in the present study significantly increased the poliovirus detection.

The seasonal distribution of polioviruses is well documented, its presence being common in the late summer and fall. In our study the distribution of PVs followed the seasonal pattern with a clear peak in September and December. Polioviruses were isolated from samples collected in all sampling sites during 12-month period except April, May and June. In fact, scheduled OPV immunizations determine the frequency of Sabin-like PVs isolation. Other studies have reported a rapid decline in PV isolation around OPV-to-IPV transition period, with a disappearance of PV vaccine strains from wastewater within 2 to 3 months after the cessa-

tion of OPV administration (Mueller *et al.*, 2009; Wahajuhono *et al.*, 2014).

The serotypes of the isolates were determined by the microneutralization assays. The serotyping test revealed the presence of 7 and 10 strains belonging to poliovirus serotype 1, 2 respectively. The most frequently detected polioviruses were PV3 (19 strains) and these accounted 53% of total serotyped strains. Other authors also indicate that PV3 is frequently isolated from sewage samples (Sarijlou *et al.*, 2007; Yoshida *et al.*, 2000). In previous studies Stanway *et al.* (1984) showed that the difference between attenuated Sabin 3 and wild-type PV3 Leon strains is restricted to only 10 nucleotides and three amino acids. In addition, it has been shown that P1 domain of PV3 Sabin strain harbors only two nucleotide changes correlating with the apparent mitigation of neurovirulence. Therefore, most cases of VAPP result from vaccination with PV3 (Minor 2004).

Mutations known to be involved in restoring neurovirulence or eliminating the temperature-sensitive phenotype of vaccine derivatives are associated with capsid coding region of poliovirus genome (Macadam *et al.*, 1989; Westrop *et al.*, 1989; Ren *et al.*, 1991; Macadam *et al.*, 1993). The ts phenotype correlates with the attenuated Sabin strains. A titer reduction of  $> 2 \log_{10}$  units at 40°C is considered to reflect temperature sensitivity (Zurbriggen *et al.*, 2008). In the present study we showed a  $\geq 2 \log_{10}$  reduction in titer for most of poliovirus isolates propagated at 40°C. All analyzed isolates grew poorly in supraoptimal temperature, what is characteristic for Sabin-like strains. Similar findings presented by other authors, also demonstrate that ts is an important viral phenotypic marker, as it may be involved in virulence of attenuated vaccine strains (Blomqvist *et al.*, 2003; Zurbriggen *et al.*, 2008). On the other hand, Bouchard *et al.* (1995) suggest that temperature sensitivity may not always be an acceptable method for evaluating the presence of attenuating mutations. Although intratypic differentiation revealed the presence of 36 SL PVs, two strains showed  $< 2 \log_{10}$  reduction in titer. Our results also show that that determinants of attenuation and temperature sensitivity can be genetically separated.

Recombination is a very frequent event in poliovirus evolution (Agol 2006). Moreover, the trivalent OPV provides ideal conditions for intertypic recombination between the attenuated strains of the three PV serotypes. In order to understand the evolution of vaccine strains during their replication in humans, our study focused on the characterization of PV genomes by double RFLP assay and revealed the presence of 11 recombinants in 3D-polymerase coding region. Nine isolates appeared to be S3/S2, one S3/S1 and S1/S2 recombinant in analyzed 3D<sub>pol</sub> region. The low degree of changes found in the genome of the 36 SL PVs may suggest that

these strains have undergone only limited circulation in the population.

In summary, the isolation of 36 SL PVs demonstrated that environmental surveillance is an effective supplemental support to the AFP surveillance to verify the absence of wild or VDPV strains in the country. No WT and VDPV strains were isolated from the environmental samples examined during the study period. In Slovakia, high percentage of PV strains (SL and VDPV) were also observed in sewage water during 2001–2006. Thereafter, PV isolates were gradually replaced by non-polio enteroviruses (NPEVs). A transition was due to the change in the childhood vaccination programme in which OPV was substituted by IPV (Klement *et al.*, 2013). In contrast, other authors have showed the disappearance of SL PVs from the environment even before the OPV immunization has ceased (Nakamura *et al.*, 2015).

In conclusion, our study revealed, through environmental monitoring, the introduction of SL PVs into the population associated with the routine use of OPV in Poland before the April 2016. Detection of polioviruses in sewage samples of the local community reflects the presence of virus-shedding individuals. ENV should be continued in the critical period between interruption of WPV transmission and certification of polio eradication. Environmental surveillance should be also available in the post-eradication and OPV cessation periods. Even if the OPV-to-IPV transition is successful, the risk of PV infection for a susceptible population should be monitored with an appropriate surveillance system.

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## Evaluation of the Carba NP Test for the Detection of Carbapenemase Activity in *Bacteroides* Species

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### Abstract

We evaluated the usefulness of the Carba NP test for rapid detection of carbapenemase activity in *Bacteroides* spp. The minimum inhibitory concentration (MIC) for imipenem was determined with gradient test strips, and *cfiA* gene was investigated by polymerase chain reaction for 27 clinical *Bacteroides* spp. isolates. Carba NP test was performed according to recommendations of the Clinical and Laboratory Standards Institute. Among three *cfiA* gene harboring clinical isolates, two imipenem resistant isolates were Carba NP test positive, while the imipenem intermediate isolate was negative. Our preliminary results suggest that the Carba NP test can be useful as a rapid test to detect carbapenemases in *Bacteroides* species.

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Key words: *Bacteroides fragilis*, carbapenemase, *cfiA*, Carba NP, imipenem

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*Bacteroides fragilis* is one of the most important causes of intraabdominal infections and bacteremia which might be fatal if not properly treated (Wexler *et al.*, 2007). Carbapenems are generally used for the treatment of anaerobic infections, but production of carbapenemases by *Bacteroides* spp. renders these antimicrobials ineffective (Sóki *et al.*, 2000). An important mechanism of carbapenem resistance in *Bacteroides* spp. is the acquisition of the *cfiA* gene encoding a metallo-beta-lactamase enzyme that is able to hydrolyse carbapenems. If present, the *cfiA* gene may or may not cause clinical resistance depending on expression level of enzyme, or else the gene may stay completely silent (Ang *et al.*, 2007). The molecular detection of the *cfiA* gene by itself is not indicative of clinical carbapenem resistance; supplementary phenotypic tests are needed to determine the carbapenem susceptibility of clinical *Bacteroides* isolates, such as performance of the minimal inhibitory concentration (MIC) testing for carbapenems.

Laboratory detection of the carbapenemase genes is challenging, moreover carbapenemase-producing bacteria are spreading throughout the world (Cantón *et al.*, 2012). Many strains have carbapenem MICs in the susceptible range, and different phenotypic methods designed to detect such strains lack specificity and

sensitivity such as the modified Hodge test, and combined disk tests with different inhibitors (Nordmann *et al.*, 2012). Detection of carbapenemase genes and demonstration of the ability of a strain to hydrolyse carbapenems still remain the gold standard methods for identification of carbapenemase-producing isolates (Poirel *et al.*, 2013). The recently published Carba NP test is described as a biochemical method based on imipenem hydrolysis (Nordmann *et al.*, 2012). Without any specialized equipment, the CARBA NP test detects the pH change by colorimetric pH indicator caused by the breakdown of imipenem in a solution containing lysed test bacteria. The test's specificity and sensitivity were initially reported as 100% when results were compared with those of molecular-based methods, the reference standard for identifying carbapenemase genes (Nordmann *et al.*, 2012).

The usefulness of Carba NP test has been demonstrated for many Gram-negative bacteria, including *Enterobacteriaceae*, *Acinetobacter* spp. and *Pseudomonas aeruginosa* (Nordmann *et al.*, 2012; Dortet *et al.*, 2012; Dortet *et al.*, 2014). In this study, our aim was to investigate the carbapenemase activity in clinical *Bacteroides* spp. isolates by Carba NP test and compare the results obtained from the Carba NP test with phenotypic (carbapenem MICs) and genotypic (presence or absence

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Table I  
Distribution of the clinical *Bacteroides* spp. isolates and sources.

Source	Number of isolates				Total
	<i>B. fragilis</i>	<i>B. vulgatus</i>	<i>B. thetaiotaomicron</i>	<i>B. caccae</i>	
Blood	8	1	1	2	12
Abdominal infection	4	1	2	–	7
Rectal, anal, perirectal abscess	4	–	–	–	4
Wound	1	1	–	–	2
Abscess	1	–	–	–	1
Urine	–	1	–	–	1
Total (%)	18 (66.7)	4 (14.8)	3 (11.1)	2 (7.4)	27

of the *cfiA* gene), and thus assess the usefulness of the Carba NP test for the detection of carbapenemase activity in *Bacteroides* spp.

A total of 27 clinical *Bacteroides* spp. isolates collected between 2011 and 2016 were included in this study. The distribution of strains to isolation sites is presented in Table I. All isolates were stored at  $-80^{\circ}\text{C}$  in cryopreservation vials (Salubris, Turkey). Frozen stock cultures were used to inoculate subcultures which were routinely grown on Schaedler agar (bioMérieux, France) and incubated at  $35\text{--}37^{\circ}\text{C}$  in anaerobe pouch system (GasPak™ EZ Anaerobe Pouch System, Becton Dickinson, USA). For species identification matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany) was used.

The susceptibility of the *Bacteroides* spp. to imipenem (IMP) was determined by gradient strips according to the manufacturer's recommendations. In the current study, bacterial suspension matched to 1 McFarland standard was prepared from fresh colonies and inoculated onto Brucella agar with 5% sheep blood supplemented with hemin and vitamin K1 (Salubris, Turkey), then an IMP gradient strip was placed onto agar surface for each isolate (Oxoid™ M.I.C.Evaluator™ Strips, Thermo Scientific, UK). The plates were incubated in anaerobe pouch system at  $35\text{--}37^{\circ}\text{C}$  for 24–48 hours. *B. fragilis* ATCC 25285 was used as a reference strain. The clinical breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used to interpret the results (EUCAST, 2016).

Bacterial DNA was extracted by a commercial kit (High Pure PCR Template Preparation Kit, Roche, Germany). The carbapenemase coding gene *cfiA* was amplified by polymerase chain reaction (PCR) with the following primers: *cfiA*-RT1 (5'-AATCGAAGGA TGGG GTATGG-3') and *cfiA*-RT2 (5'-CGGTCCGGTG AATCG-GTGAAT-3') which amplify 300 bp of the 750 bp *cfiA* gene (Sóki *et al.*, 2013). A *B. fragilis* strain with previ-

ously documented *cfiA* positivity was used as a positive control in PCR studies (Toprak *et al.*, 2012). The PCR cycles were as follows: 2 minutes at  $94^{\circ}\text{C}$ , 35 cycles (45 seconds at  $94^{\circ}\text{C}$ , 45 seconds at  $51^{\circ}\text{C}$ , 45 seconds at  $72^{\circ}\text{C}$ ) and 2 minutes at  $72^{\circ}\text{C}$ . For the visualisation of PCR products, 2% agarose gel was used which were stained with SYBR gold and monitored using ORTE device (Salubris Technica, Istanbul, Turkey).

Carba NP test was performed according to CLSI recommendations (CLSI, 2016). For each strain two microcentrifuge tubes (1.5 ml) labeled as "a" and "b" were used. The bacterial colonies grown on Schaedler agar were collected after 24 hours and a loopful of bacteria (approximately 10  $\mu\text{l}$ ) were added into 100  $\mu\text{l}$  of bacterial protein extraction reagent (B-PERII, Thermo Scientific, Pierce) and stirred for 5 seconds. From the solutions A (containing zinc sulphate and phenol red, pH:  $7.8 \pm 0.1$ ) and B (containing solution A and 6 mg/ml imipenem), 100  $\mu\text{l}$  solution was added to a, and b tubes, respectively, and then incubated at  $35 \pm 2^{\circ}\text{C}$  for up to two hours. The results were interpreted according to the color changes of the tubes. The carbapenemase-producing *B. fragilis* strain TAL 2480 was used as positive control and *B. fragilis* ATCC 25285 was used as negative control. The microcentrifuge tubes which contain only bacterial protein extraction reagents were prepared as reagent control. When imipenem was hydrolysed, the color of the tube's content turned from red to orange or yellow which was interpreted as a positive Carba NP test, whereas tubes containing bacterial extracts of isolates with no carbapenemase activity remained red. The color changed from red to yellow as early as 30 minutes after incubation with *B. fragilis* TAL 2480 (Fig. 1).

Imipenem MIC values determined by gradient strips ranged from 0.03 to  $\geq 32$  mg/l among study isolates. Majority (24 out of 27; 88.9%) of the strains were found susceptible to imipenem ( $\text{MIC} \leq 2$  mg/l) with EUCAST breakpoints, one strain showed intermediate susceptibility ( $\text{MIC} = 4$  mg/l) and two of the strains were found resistant to imipenem ( $\text{MIC} \geq 16$  mg/l). The *cfiA*

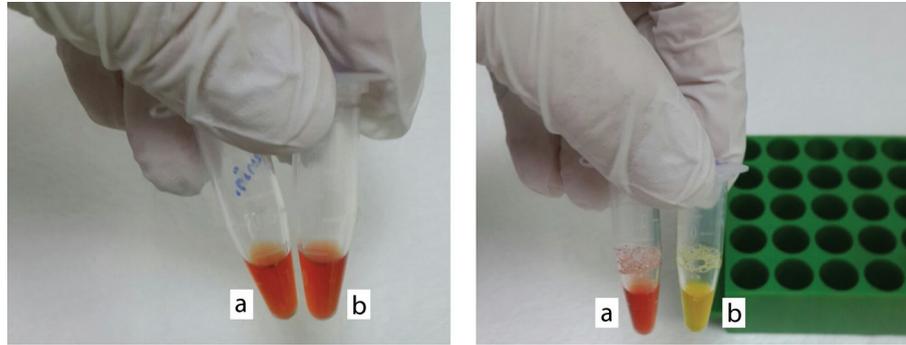


Fig. 1. Interpretation of the Carba NP test results: *B. fragilis* ATCC 25285 with no carbapenemase activity (left) and carbapenemase-producing *B. fragilis* strain TAL 2480 (right). The tubes denoted as “a” contain solution A + bacterial lysate, tubes “b” contain solution B (solution A + imipenem) + bacterial lysate. If acidification occurs as a result of hydrolysis of imipenem, the pH indicator (phenol red) causes a color change from red to yellow in tube b.

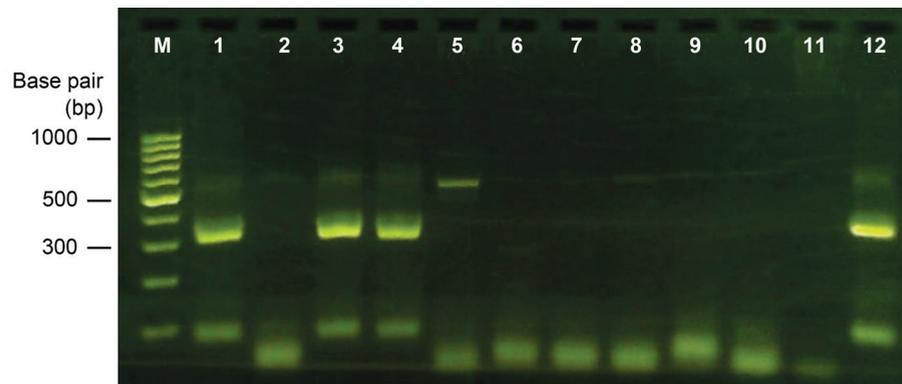


Fig. 2. The PCR amplification results for the *cfiA* gene in clinical *Bacteroides* spp. isolates. Lanes 1, 3 and 4 are clinical *B. fragilis* isolates demonstrating the presence of the *cfiA* gene, lanes 2, 5–10 are clinical *Bacteroides* spp. lacking the *cfiA* gene, lane 11 is negative control (water), lane 12 is a *cfiA* positive *B. fragilis* strain used as positive control. Molecular sizes in base pair (bp) are indicated at the left. (M: Molecular mass standard) This PCR assay was used to amplify a 302 bp fragment of the *cfiA* gene.

gene was detected in three isolates (11.1%), all *B. fragilis* blood isolates with imipenem MIC  $\geq 4$   $\mu\text{g/ml}$ . A correlation between imipenem MIC being  $\geq 4$   $\mu\text{g/ml}$  and *cfiA* gene positivity was observed for the tested *Bacteroides* sp. isolates. Carba NP test results were obtained in 2 hours in clinical isolates and in 30 minutes for the reference strain. The two clinical strains with imipenem MIC  $\geq 32$  mg/l and the reference *cfiA* positive strain were tested positive in the Carba NP test, however the

imipenem intermediate (MIC = 4 mg/l), *cfiA* positive strain was found negative (Table II).

In the present study, we aimed to evaluate the applicability of the Carba NP test, which was originally developed for *Enterobacteriaceae*, to investigate the *cfiA*-mediated carbapenemase production in *Bacteroides* spp. The *cfiA* gene was investigated in a collection of clinical *Bacteroides* sp. isolates ( $n=27$ ) and we found three (11.1%) *cfiA* positive *B. fragilis* isolates.

Table II  
Correlation of *cfiA* gene presence and Carba NP test positivity with imipenem MICs in clinical *Bacteroides* spp. isolates ( $n=27$ ).

	Imipenem Susceptibility (Imipenem MIC)		
	S* (MIC $\leq 2$ mg/l)	I* (MIC = 4 – 8 mg/l)	R* (MIC $\geq 16$ mg/l)
Carba NP test positive	0	0	2
<i>cfiA</i> positive	0	1	2
Total	24	1	2

\*According to EUCAST breakpoints, S = susceptible, I = intermediate, R = resistant

The Carba NP test yielded positive results for two of the *cfiA* positive isolates both having imipenem MICs of  $\geq 16$  mg/l but the test gave negative results for the *cfiA* positive isolate with imipenem MIC of 4 mg/l. No positive Carba NP test results were obtained in imipenem susceptible (MIC  $\leq 2$  mg/l) and *cfiA* negative isolates. Our results with the limited isolate collection suggest that Carba NP test can also be used for *Bacteroides* spp. to have preliminary information on carbapenem susceptibility. The positive results with the Carba NP test are very likely to be related with high carbapenem MIC values which might be taken as a warning for treatment failure if carbapenems are used.

*Bacteroides* spp. are the predominant members of the human intestinal microbiota and constitute approximately 30% of the total colonic bacteria. They are also among the most commonly isolated anaerobic bacteria from clinical specimens. The *B. fragilis* group is of special medical importance since they are often involved in polymicrobial infections, bear the potential to express  $\beta$ -lactamases and are associated with a high mortality when causing bloodstream infections (Wexler *et al.*, 2007). A French multicenter study determined the role of anaerobe bacteria in the aetiology of all bacteremias as 0.5% to 9%, of which 60% being caused by *Bacteroides* spp. (Zahar *et al.*, 2005). These factors emphasize the importance of identification and rapid antimicrobial susceptibility testing for clinical *Bacteroides* spp. isolates for the administration of appropriate antibiotic therapy to achieve optimal outcomes. Access to rapid susceptibility results is important in the case of infections due to *Bacteroides* spp. since the susceptibility of these bacteria cannot be predicted due to the ability of the organism to acquire resistance mechanisms. The *B. fragilis* group are uniformly resistant to penicillin and first generation cephalosporins which leaves limited number of alternatives such as metronidazole, imipenem, piperacillin-tazobactam, moxifloxacin and clindamycin with reported resistance rates of <1%, 1.2%, 3.1%, 13.6%, and 32.4%, respectively (Nagy *et al.*, 2011). A Europe-wide study showed that imipenem retains its activity against *B. fragilis* isolates, but the percentage of isolates with reduced susceptibility (MIC:  $\geq 4$  mg/L) are steadily increasing (Nagy *et al.*, 2011). Carbapenem resistance in *B. fragilis* is most commonly related to the acquisition of *cfiA* gene which – if expressed at high levels due to acquisition of an insertion sequence upstream of the gene – can lead to high-level carbapenem resistance (Thompson *et al.*, 1990; Edwards *et al.*, 2000). The frequency of *cfiA* positivity in *Bacteroides* spp. has been reported to be as high as 27% in a Turkish study (Toprak *et al.*, 2012), raising concern as an emerging resistance trait that may limit the empirical use of carbapenems in infections due to *Bacteroides* spp.

Our study findings revealed the rate of *cfiA* positivity as 11.1% in a small collection of clinical *Bacteroides* sp. isolates. This is in concordance with previously published literature in which rates ranging between 0 and 13% was observed for a collection of 161 *B. fragilis* group strains isolated from nine different European countries (Eitel *et al.*, 2013). The high rate of 27% *cfiA* positivity reported from Istanbul, Turkey in this study, might be related with the institutional spread of a resistant clone.

We also had the opportunity to test other *Bacteroides* species by including a few *B. thetaiotaomicron*, *B. caccae* and *B. vulgatus* isolates, however the study collection mostly consisted of clinical *B. fragilis* isolates. The total number of isolates tested is relatively low for a validation study, but the collection included *cfiA* gene positive and negative isolates with varying imipenem MIC values that enabled us to investigate the performance of the test. Additionally, the high concordance observed between the phenotypic and genotypic test results further supports the applicability of CARBA NP test in *Bacteroides* spp.

In our study, we demonstrated the usefulness of the recently described Carba NP test to detect carbapenemase activity in clinical *Bacteroides* spp. isolates. Although further studies are needed, with our preliminary results, we can suggest that the Carba NP test holds the potential to be used as a rapid phenotypic carbapenemase detection test for *Bacteroides* species. The rapid detection of carbapenemase activity in *Bacteroides* spp. might provide early insight into susceptibility of the organism before the final results of the conventional antimicrobial susceptibility are obtained, and thus serve as an important tool to manage antimicrobial therapy.

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#### Conflict of interest

The authors have no conflict of interest (commercial or otherwise) to declare regarding this study.

#### Literature

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## Rapid Detection of Bloodstream Pathogens in Oncologic Patients with a FilmArray Multiplex PCR Assay: a Comparison with Culture Methods

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### Abstract

The results of the FilmArray® Blood Culture Identification Panel (BCID) (BioFire Diagnostics) and the culture with susceptibility testing of 70 positive blood cultures from oncologic patients were compared. The multiplex PCR assay (BCID) identified 81 of the 83 isolates (97.6%), covered by the panel. The panel produced results in significantly shorter time than standard identification methods, when counted from receiving positive blood cultures bottles to the final results. It is an accurate method for the rapid identification of pathogens and resistance genes from blood culture in oncologic patients

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Key words: bloodstream infection, FilmArray, multiplex PCR, oncologic patients

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Sepsis in oncologic patients is a serious complication in the course of primary treatment. Surgery, often an extensive one, involves opening of the digestive tract, urinary tract, anastomosis of the colon, use of vascular lines, catheters in the bladder, parenteral nutrition, stay in the ICU, immunocompromised immune systems, and favours systemic infections (Encina *et al.*, 2016; Alkhamis *et al.*, 2014; Smit *et al.*, 2016; Mahdi *et al.*, 2014). Generalized infections in cancer patients are burdened with high mortality; therefore, time is one of the important factors in their diagnosis and treatment (Namendys-Silva *et al.*, 2010; Rosolem *et al.*, 2012). Classic diagnosis of these infections, including identification, determination of antibiotic susceptibility and detection of resistance mechanisms of the cultured microorganisms, takes 2–5 days from the delivery of samples for microbiological examination. Due to the relatively long period of waiting for the test results, empiric therapy is implemented. The etiologic agent is often not grown, due to the sensitivity of the culture method. Empiric therapy carries the risk of not including the etiologic agent of the infection within its coverage, it can lead to overuse of antibiotics with a wide spectrum, prolongs hospitalization, increases the cost of treatment, and selects for multidrug-resistant strains in units of health care, which ultimately leads to increased mortality (Kumar, 2011).

In 2013, the Food and Drug Administration (FDA) issued a positive opinion on a FilmArray® Blood Culture Identification Panel (BCID) (BioFire Diagnostics, Salt Lake City, UT) for rapid identification of aerobic microorganisms in positive blood culture. The application of the multiplex PCR method in the diagnosis of bloodstream infections is designed to reduce the time for identification of the microorganisms grown from the positive samples to 24–48 hours, as pathogens are identified directly from a positive blood sample.

There have been several papers published on the usefulness of the test in diagnosis of sepsis in adults, children and patients undergoing organ transplantation in relation to conventional methods, but there are no papers defining the efficacy of the test in diagnosis of bloodstream infections in cancer patients, including patients after surgery (Blaschke *et al.*, 2012; Zheng *et al.*, 2014; Otlu *et al.*, 2015).

The aim of the study was to compare two methods of identification of microorganisms from positive blood cultures: the classical method – culture and the genetic method – multiplex PCR as well as the time from the receipt of positive samples to communicating the result of PCR and uploading the microbiological report into the hospital information system.

The examination involved 70 positive blood samples obtained in BacT/Alert 3D instrument, between

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Table I  
Microorganism identified with BCID.

Organism Gram-negative	Organism Gram-positive	Yeast	Antibiotic resistance
<i>Acinetobacter baumannii</i>	<i>Enterococcus</i>	<i>Candida albicans</i>	<i>mecA</i>
<i>Haemophilus influenzae</i>	<i>Listeria monocytogenes</i>	<i>Candida glabrata</i>	<i>vanA/B</i>
<i>Neisseria meningitidis</i>	<b>Staphylococcus</b>	<i>Candida krusei</i>	<i>bla<sub>KPC</sub></i>
<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i>	
Enterobacteriaceae	<b>Streptococcus</b>	<i>Candida tropicalis</i>	
<i>Enterobacter cloacae</i> complex	<i>Streptococcus agalactiae</i>		
<i>Escherichia coli</i>	<i>Streptococcus pyogenes</i>		
<i>Klebsiella pneumoniae</i>	<i>Streptococcus pneumoniae</i>		
<i>Klebsiella oxytoca</i>			
<i>Serratia marcescens</i>			
<i>Proteus</i> sp.			

August 2015 and November 2016. Diagnostic materials were collected from 55 patients suffering from cancers, who were treated in clinical departments of Prof. F. Łukaszczyk Oncology Centre in Bydgoszcz. Among the patients from which blood was drawn, 40 patients (72.7%) were treated surgically, with different extensity of the surgical procedure, including pelvic exenteration in the case of advanced ovarian cancer, and 15 patients (27.3%) underwent conservative treatment. Positive aerobic blood cultures, confirmed by the microscopic preparation stained with the Gram method, underwent multiplex PCR analysis with the use of BCID. Microorganisms and resistance genes covered by BCID are presented in Table I. Positive blood cultures were also passaged on solid media and the isolated microorganisms were identified and analysed for antibiotic susceptibility with the use of VITEK 2 Compact and E-test® system (bioMérieux, USA). Due to the fact that there is a 12-hours shift system in the Microbiology Department, 13 test samples (18.6%) underwent genetic analysis in more than 8 hours after the signal from BacT/Alert 3D instrument was observed. Validation of the method did not confirm that the extension of bottles incubation time to 15 hours had a negative impact on the reliability of the results. As a gold standard, the culture method was used in the research. Quantitative data were developed with the use of U Mann-Whitney test, value  $p < 0.05$  was acknowledged as statistically significant.

Eighty-nine isolates were cultured from 70 samples. In 55 (78.6%) cultures microbial growth in monoculture was obtained. The multiplex PCR assay revealed all bacterial species and types present in positive blood cultures bottles that have been included in the panel. However, for two isolates identified using BCID, despite the increase in cultivation time, the presence of the microorganism in the samples was not confirmed using multiplex PCR method; this concerned *Staphylococcus hominis* ssp. *hominis* present in monoculture

and *Escherichia coli* present in mixed culture with three other microorganisms. In 6 (6.7%) cases, the microorganisms that are not covered by BCID were cultured: *Lactobacillus* spp., *Lactobacillus plantarum*, *Candida lusitanae*, *Stenotrophomonas maltophilia*, *Acinetobacter lwoffii* and *Haemophilus parainfluenzae*. In one case, in a mixed culture (1.3%), *Haemophilus influenzae* was identified in PCR while *H. parainfluenzae* was cultured. Antibiotics resistance determined by detection of the resistance genes and by phenotypic methods showed good concordance. The presence of the *mecA* gene was confirmed in 30 strains of coagulase-negative staphylococci, and in two strains of *Staphylococcus aureus*. In one case (3.3%) a *mecA* gene was found, without confirmation of growth of methicillin-resistant strain in the culture. Among six enterococci identified, no strains with *vanA/B* gene were detected. Moreover, no Gram-negative bacteria with resistance to carbapenems resulting from the presence of the *bla<sub>KPC</sub>* gene were cultured from the blood in the analysis period. Comparison of the results obtained using the multiplex PCR method (BCID) and the classical method is shown in Table II.

The average time of the positive samples detection in the BactAlert 3D system was 23.1 h (SD ± 14.2 h), with a period of time from detection of positive sample to start of BCID – 3.6 hours (SD ± 4.18 h). Information about the positive PCR results was submitted to a doctor within 4.9 hours (SD ± 4.2 h) while the report on the culture results was presented on average in 67.7 h (SD ± 22.9 h). Blood culture is the most commonly used microbiological method in the diagnosis of sepsis. In recent years, however, research is being conducted on the use of rapid, more sensitive tests for the detection of microorganisms directly from the blood. Rapid identification of microorganisms in sepsis is crucial for the selection of appropriate treatment. An adequate and early treatment significantly reduces mortality (Dellinger *et al.*, 2012). BCID con-

Table II  
Comparison of blood culture and multiplex PCR (BCID) results.

Microorganism group	Culture result (no)	BCID result (no)
Gram-positive bacteria	<i>Staphylococcus hominis</i> ssp. <i>hominis</i> MR (9)	<i>Staphylococcus</i> (8) <i>mecA</i> (8)
	<i>Staphylococcus hominis</i> ssp. <i>hominis</i> MS (3)	<i>Staphylococcus</i> (3) <i>mecA</i> (1) <sup>1</sup>
	<i>Staphylococcus epidermidis</i> MR(16)	<i>Staphylococcus mecA</i> (15) <sup>1</sup>
	<i>Staphylococcus epidermidis</i> MS (4)	<i>Staphylococcus</i> (4)
	<i>Staphylococcus haemolyticus</i> MR (6)	<i>Staphylococcus mecA</i> (6)
	<i>Staphylococcus capitis</i> MS (2)	<i>Staphylococcus</i> (2)
	MSCNS (1)	<i>Staphylococcus</i> (1)
	<i>Staphylococcus aureus</i> MS (4)	<i>Staphylococcus</i> ; <i>Staphylococcus aureus</i> (4)
	<i>Staphylococcus aureus</i> MR (2)	<i>Staphylococcus</i> ; <i>Staphylococcus aureus mecA</i> (2)
	<i>Enterococcus faecalis</i> (4)	<i>Enterococcus</i> sp. (4)
	<i>Enterococcus faecium</i> (2)	<i>Enterococcus</i> sp. (2)
	<i>Streptococcus salivarius</i> (1)	<i>Streptococcus</i> sp. (1)
	<i>Lactobacillus plantarum</i> (1)	ND <sup>2</sup>
	<i>Lactobacillus</i> spp. (1)	ND <sup>2</sup>
Gram-negative bacteria	<i>Pseudomonas aeruginosa</i> (9)	<i>Pseudomonas aeruginosa</i> (9)
	<i>Enterobacter cloacae</i> (1)	Enterobacteriaceae; <i>Enterobacter cloacae</i> (1)
	<i>Escherichia coli</i> (9)	Enterobacteriaceae; <i>Escherichia coli</i> (8)
	<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i> (3)	Enterobacteriaceae; <i>Klebsiella pneumoniae</i> (3)
	<i>Stenotrophomonas maltophilia</i> (1)	ND <sup>2</sup>
	<i>Proteus mirabilis</i> (2)	Enterobacteriaceae; <i>Proteus</i> sp.(2)
	<i>Acinetobacter lwoffii</i> (1)	ND <sup>2</sup>
	<i>Haemophilus parainfluenzae</i> (1)	ND <sup>2</sup>
	ND	<i>Haemophilus influenzae</i>
Yeast	<i>Candida glabrata</i> (2)	<i>Candida glabrata</i> (2)
	<i>Candida albicans</i> (1)	<i>Candida albicans</i> (1)
	<i>Candida parapsilosis</i> (2)	<i>Candida parapsilosis</i> (2)
	<i>Candida lusitaniae</i> (1)	ND <sup>2</sup>

<sup>1</sup> in one sample two morphological different strains of *S. epidermidis*; <sup>2</sup> not detectable in BCID panel spectrum; ND – not detected; MR – methicillin resistant; MS – methicillin susceptible; MRCNS – methicillin resistant coagulase negative *Staphylococcus*

tains a wide panel of microorganisms: bacteria and fungi responsible for approx. 90.0% of bloodstream infections, detects the most common mechanisms of resistance in short time, and is an important tool in the surveillance of bloodstream infections (Blaschke *et al.*, 2012; Otlu *et al.*, 2015; Altun *et al.*, 2013.). In our study on cancer patients, BCID covered 93,3% of the microorganisms isolated from the blood. Similar results were obtained by Zheng *et al.* (2014) in a study of 166 positive blood cultures from 138 children, where BCID covered approximately 93.0% of cultured microorganisms. In most of the samples, approx. 97.0%, we obtained consistent identifications with both methods used: the genetic method and the culture method. In one case, we observed a growth of *S. hominis* ssp. *hominis* methicillin-resistant in a culture, without confirmation of presence of the microorganism with the multiplex PCR method. The similar results were obtained by Zheng *et al.* (2014); this microorganism was also not

detected in one sample analysed. BCID did not detect the following microorganisms: *C. lusitaniae*, *S. maltophilia*, *A. lwoffii*, *L. spp.*, *L. plantarum*, *H. parainfluenzae*, which do not fall within the spectrum of BCID. According to the manufacturer's instructions, the limitation of this method is that in mixed cultures BCID may not correctly identify all microorganisms. In our study, 15 blood cultures were positive for more than one species of bacteria. Blaschke *et al.* (2012) suggested that the uneven growth of two species in liquid culture could cause that despite the positive signal from the BacT/ALERT detection system one of the species may not be detected with the multiplex PCR. However, according to Altun *et al.* (2013), the lower limit of detection (LOD) in BCID, is generally sufficient to detect pathogens. According to the characteristics of BCID specified by the manufacturer, the density of bacteria in positive blood culture during the test was  $\sim 10^7$ – $10^8$  CFU/ml. In our study, usually Gram-positive cocci grown in mixed

cultures as follows: enterococci and coagulase-negative staphylococci, two species of coagulase-negative staphylococci as well as enterococci and yeast-like fungi. In these cases, we obtained full compliance of results in both methods. In mixed cultures of Gram-negative bacilli and Gram-positive cocci in one case, the system did not detect *E. coli*, despite longer culture time and identified *H. parainfluenzae*, which was not included in BCID. The oxacillin resistance determined by the presence of the *mecA* gene was correctly identified in both coagulase-negative strains of *Staphylococcus* and methicillin-resistant strains of *S. aureus*. Furthermore, in one case, the PCR system also detects the presence of the *mecA* gene, which could not be confirmed phenotypically. BCID correctly indicated negative results for *vanA* and *vanB* among enterococci. However, it should be noted that the system does not distinguish between *Enterococcus faecalis* and *Enterococcus faecium*, the most common species of enterococci. This is associated with the choice of antibiotic for treatment, as the majority of *E. faecium* is resistant to ampicillin. This is a certain limitation of the panel, especially in the case of sepsis after surgery in the abdominal cavity. MacVane *et al.* (2016) confirmed that BCID is useful in the diagnosis of sepsis caused by vancomycin-resistant strains of *Enterococcus* spp. The study included 68 patients with bacteremia caused by VRE. The authors showed statistically significant differences in the time to identify the microorganism by culture and genetic methods (47.7 h versus 18.2 h,  $p < 0.001$ ), and statistically significant difference was also shown in the time required to evaluate the susceptibility to vancomycin and the time for the implementation of effective therapy ( $p < 0.001$ ). The authors also pointed to a significant reduction in the cost of a patient's stay in hospital, when using a genetic method. Otlu *et al.* (2015) evaluated the usefulness of BCID in the diagnosis of sepsis in patients undergoing liver transplantation in order to shorten the time needed to obtain a result in relation to the classical methods, automatic Vitek II and mass spectrometry – Vitek MS system. These differences were significant, and the time to obtain the results was as follows: the classical method – 36.2 h (SD  $\pm$  19.2 h), automatic method Vitek II – 23.6 (SD  $\pm$  2.23 h) and Vitek MS system 19.5 h (SD  $\pm$  15.1 h). BCID identified pathogen within 65 to 100 minutes.

In our study, the difference in amount of time needed to inform the physician about the detection of the microorganism and its mechanisms of resistance using BCID compared to the time needed to obtain the same results by VITEK 2 Compact and E-test® was also statistically significant ( $p < 0.05$ ). However, due to the 12-hours shift system, the time to transfer the result was longer than in the above-cited studies.

Inglis *et al.* (2016) studied 149 blood cultures derived from 143 patients and evaluated the usefulness of BCID depending on the hospital referral level. The authors believe that BCID is particularly suitable for small laboratories in regional hospitals, dominated by the most common microorganisms. Our research confirms that the panel can also be used among cancer patients after surgery and conservative treatment of cancer.

In conclusion, BCID identified most of the microorganisms present in positive blood cultures in cancer patients, including patients undergoing abdominal surgery and pelvic exenteration. It can be a very useful tool in the surveillance of bloodstream infections providing information on the etiological agent and the basic mechanisms of microbial resistance. It should be noted, however, that the essential component of genetic diagnostics is culture, which remains the gold standard. Application of BCID speeds up the decision on the selection of appropriate treatment, because it significantly shortens the time to provide essential information to the doctor.

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## Changes of Microbial Diversity During Swine Manure Treatment Process

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### Abstract

We investigated microbial diversity in a manure storage tank (MST) storing untreated manure and an aeration tank (AT) during swine manure treatment process using the next-generation sequencing in order to find the aeration effect on microbial diversity. *Proteobacteria* were more abundant in the AT group than in the MST group and may include denitrifying bacteria contributing to nitrous oxide (N<sub>2</sub>O) emission or aerobic bacteria stimulated by oxygen. The opposite held true for the phyla *Bacteroidetes* and *Firmicutes* that may include anaerobic bacteria inhibited under aerobic conditions in the AT group.

**Key words:** biological swine manure treatment, manure storage tanks, microbial communities in swine manure, next-generation sequencing

Swine manure is often considered as contaminant to soil, air, and water even though well treated swine manure has been a good source of nutrients for agricultural products during cultivation history. Recently malodor complaint and greenhouse gas (GHG, especially methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) from livestock agriculture) emissions are the main targets for air pollution. A well-known efficient means to decrease odor, CH<sub>4</sub>, and N<sub>2</sub>O from swine manure is to treat it aerobically (Williams *et al.*, 1989; Park *et al.*, 2011) as anaerobic and anoxic environments are preferable to microbes generating odor and CH<sub>4</sub>, and N<sub>2</sub>O (Zhu, 2000; Yu *et al.*, 2001). Rassamee *et al.* (2011) indicated that both incomplete nitrification and incomplete denitrification could result in N<sub>2</sub>O emission under anoxic-aerobic and intermittent aeration conditions. Harper *et al.* (2000) reported that N<sub>2</sub>O production with NO<sub>3</sub><sup>-</sup> increase indicated denitrification process in swine lagoons. Biological swine manure treatment system in Korea often consists of liquid-solid separation process for stored manure, aerobic process (composting system, activated sludge system, *etc.*), anoxic process and advanced process for discharge. In Korea, 80.27% of swine barns had a manure storage tank (MST) storing untreated manure and an aeration tank (AT) (Korea Pork Producers Association, 2014) in order to solve the

manure and odor problems. However, little research on microbial communities during swine manure treatment processes in Korea has been conducted even though microbe populations and activity are the most important variables to evaluate the efficiency of that system. In this study, we examined the effects of the mechanical aeration on microbial communities in swine manure storage using the next-generation sequencing of 16S rRNA gene amplicons.

Total community DNA was extracted from the MST and the AT groups using NucleoSpin<sup>®</sup>Soil Kit (Macherey-Nagel, Düren, Germany) as described previously (Han *et al.*, 2016). 16S rRNA gene amplicon sequencing was conducted using the Illumina MiSeq sequencer (Roche, Mannheim, Germany) for the V4 region libraries that were constructed using the 515f-806r bacterial/archaeal primer set (Caporaso *et al.*, 2011; Walters *et al.*, 2016). The QIIME software package v.1.9.1 (Caporaso *et al.*, 2010) was used to conduct sequence processing and bioinformatics analysis.

A total of 10,509 16S rRNA gene sequences comprising 10,042 bacterial and 467 archaeal sequences were identified from samples that were obtained from the MST group (5,486 sequences) and the AT group (5,023 sequences) in swine wastewater purifying facilities. The 10,509 bacterial sequences were classified into

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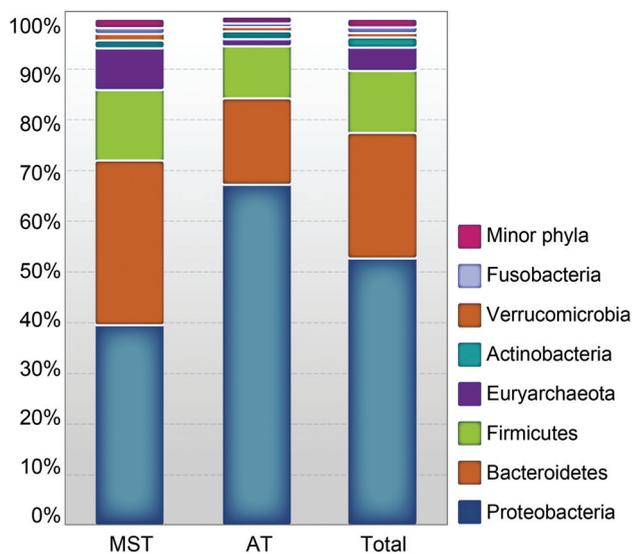


Fig. 1. Proportions of phyla for each group.

Phyla represented by <1% of total sequences across all 2 groups were combined and regarded as minor phyla. "Total" is the proportion of total sequences across all 2 groups. MST, manure storage tank; AT, aeration tank

24 phyla where *Proteobacteria* was the first predominant phylum and accounted for 53% of all the 10,509 sequences. *Bacteroidetes* and *Firmicutes* were the second and the third predominant phyla and accounted for 25% and 12% of all the sequences, respectively. *Actinobacteria* was the fourth predominant phylum and accounted for 2% of all the 10,509 sequences, while *Verrucomicrobia* and *Fusobacteria* each accounted for 1% of all the 10,509 sequences. The rest of 17 phyla were *Tenericutes*, *Spirochaetes*, *Thermi*, *WWE1*, *Gemmatimonadetes*, *Chloroflexi*, *Planctomycetes*, *Lentisphaerae*, *Synergistetes*, *TM7*, *Acidobacteria*, *Chlorobi*, *Cyanobacteria*, *FBP*, *BRC1*, *Chlamydiae* and *SR1*. Each of these 17 phyla represented <1% of all the 10,509 sequences,

and the 17 phyla were regarded as "minor" phyla. On the other hand, all the 655 archaeal sequences were assigned to *Euryarchaeota* that are mostly composed of methanogens, accounting for 4% of all the 10,509 sequences. Proportions of phyla for each group and collective data indicating the proportion of total sequences across all 2 groups were shown in Fig. 1.

The 10,042 sequences were assigned to 226 genera where 19 genera accounted for at least  $\geq 0.5\%$  of the total sequences in at least 1 of the 2 sample groups were regarded as "major genera". The 19 dominant genera were *Corynebacterium*, *Bacteroides*, *Paludibacter*, *Porphyromonas*, *Prevotella*, *Aequorivita*, *Gelidibacter*, *Turicibacter*, *Clostridium*, *Megasphaera*, *Cupriavidus*, *Thauera*, *Desulfuromonas*, *Campylobacter*, *Sulfurimonas*, *Rhodanobacter*, *Treponema* and 2 putative genera (B-42 and vadinCA11). A total of 4,960 OTUs were identified across the MST and the AT groups where 18 OTUs each accounted for at least 0.5% of the total sequences in at least 1 of the 2 groups and were regarded as "major OTUs" (Fig. 2). These 18 OTUs were assigned to *Proteobacteria* (11 OTUs), *Bacteroidetes* (6 OTUs) and *Euryarchaeota* (1 OTU).

The proportion of phylum *Proteobacteria* was slightly increased in the AT group compared to the MST group, indicating aerobic conditions in the aeration tank might stimulate the growth of aerobic bacteria placed within *Proteobacteria*. Of the 19 dominant genera, the proportion of genus *Rhodanobacter* was more abundant (3.5-fold) in the AT group than in the MST group. Prakash *et al.* (2012) indicated that *Rhodanobacter denitrificans* is facultative anaerobic and involved in denitrification. The growth of *Rhodanobacter* in the current study may be stimulated under anoxic conditions in aeration and contribute to  $N_2O$  emission in swine manure. The proportions of genera *Thauera* and

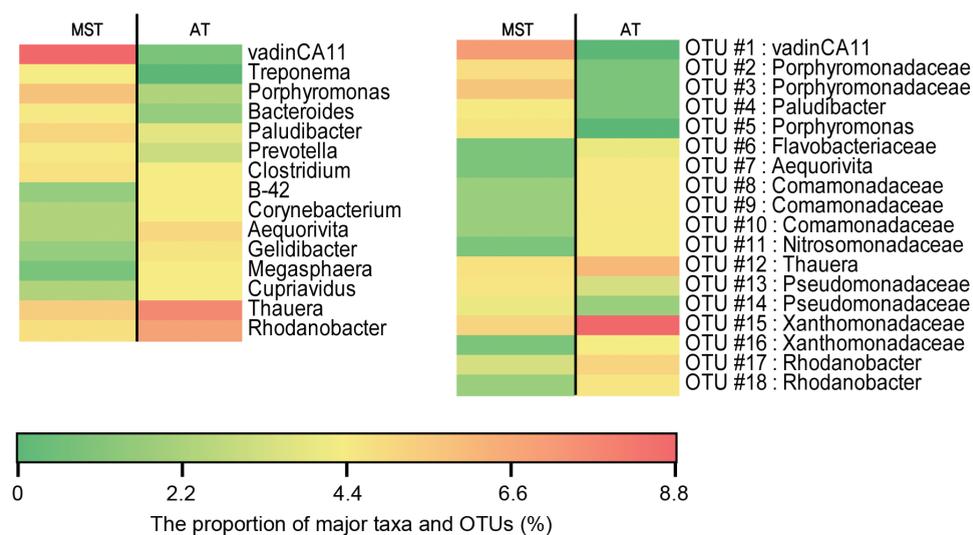


Fig. 2. Heatmap showing the proportion of major taxa (a) and OTUs (b). Taxa and OTUs accounting for  $\geq 0.5\%$  of total sequence reads in at least 1 of the MST and the AT groups were regarded as "major" taxa and OTUs. MST, manure storage tank; AT, aeration tank

*Cupriavidus* also were more than 2-fold abundant in the AT group than in the MST group (Fig. 2). *Thauera* spp. are aerobic denitrifying bacteria that produce  $N_2O$  under aerobic conditions (Scholten *et al.*, 1999; Yamashita *et al.*, 2011). In the current study, the growth of *Thauera* may be stimulated by oxygen available under anoxic conditions in aeration and contribute to  $N_2O$  emission. It was reported that *Cupriavidus necator* is a denitrifying bacterium (Lykidis *et al.*, 2010), indicating that it also may contribute to  $N_2O$  emission. Eight of the 18 dominant OTUs were assigned to families *Comamonadaceae*, *Nitrosomonadaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* but could not be assigned to any known genus (Fig. 2). The proportions of the 8 OTUs were more abundant in the AT group than in the MST group, indicating that putative species corresponding to these 8 OTUs may be denitrifying bacteria contributing to  $N_2O$  emission or aerobic bacteria stimulated by oxygen.

The proportion of *Bacteroidetes* was slightly decreased in the AT group compared to the MST group (Fig. 1). At the genus level, the proportions of *Porphyromonas*, *Bacteroides*, *Paludibacter* and *Prevotella* were decreased in the AT group compared to the MST group (Fig. 2). It seems that *Porphyromonas*, *Bacteroides* and *Prevotella* are anaerobic pathogens and phylogenetically close to each other (Falagas and Siakavellas, 2000). An anaerobic *Bacteroides* strain was isolated from a swine manure storage pit (Land *et al.*, 2011), while anaerobic *Prevotella* strains were isolated from swine fecal samples (Nograšek *et al.*, 2015). *Paludibacter* strains also were anaerobic bacteria (Ueki *et al.*, 2006; Qiu *et al.*, 2014). Therefore, the growth of these 4 anaerobic genera seems to be inhibited by oxygen available under anoxic conditions in the aeration group. On the other hand, genera *Aequorivita* and *Gelidibacter* placed were more than 5-fold abundant in the AT group than in the MST group (Fig. 2). Because *Aequorivita* and *Gelidibacter* are aerobic (Liu *et al.*, 2013; Bowman, 2016), aeration seems to stimulate the growth of these two genera with breakdown of organic compounds in swine manure. Of the 18 dominant OTUs, 3 OTUs were assigned to *Porphyromonadaceae* (2 OTUs) and *Flavobacteriaceae* (1 OTU) that were decreased in the AT group compared to the MST group (Fig. 2). Putative species corresponding to these 3 OTUs will need to be identified in future studies.

The proportion of *Firmicutes* was slightly decreased in the AT group compared to the MST group (Fig. 1). At the genus level, the proportion of *Megasphaera* was increased in the AT group compared to the MST group while the opposite held true for *Clostridium*. Although *Megasphaera* sequences were identified from swine manure compost (Guo *et al.*, 2007), the role of *Megasphaera* during aeration remains to be elucidated.

Table I  
Alpha diversity indices.

Group	# of observed OTUs	Chao1	PD_whole_tree	Chao1
MST <sup>1</sup>	3046	17978	189.79	10.14
AT <sup>2</sup>	2694	15102	157.85	9.90

<sup>1</sup> MST = manure storage tank

<sup>2</sup> AT = aeration tank

Anaerobic *Clostridium* in the MST group may originate from the swine gut (Holman *et al.*, 2017) and be decreased by oxygen produced by aeration.

The proportion of putative genus vadinCA11 in family *Methanomassiliicoccaceae* was greatly decreased in the AT group compared to the MST group (Fig. 2). This result indicates that anaerobic methanogens are inhibited by oxygen produced by aeration.

Alpha diversity indices, which are OTU richness, Chao1 estimate, PD\_whole\_tree distance and the Shannon diversity index, were greater in the MST group than in the AT group (Table I). These results indicate that microbial communities are more diverse in the MST group than in the AT group. Microbial diversity in the AT group might be reduced because the growth of anaerobic microbes was inhibited by oxygen produced by aeration.

In conclusion, we demonstrated that microbial diversity in the manure storage tank is changed by the mechanical aeration of swine manure including changes of denitrifying bacteria contributing to  $N_2O$  emission. It may partly answer the microbiological question why  $N_2O$  emission is increased by the mechanical aeration. Our study may further indicates the possibility of finding the way to reduce greenhouse gases through manipulation of microbial diversity during swine manure treatment process.

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## Comparative Seroprevalence of Hepatitis A And E Viruses in Blood Donors from Wielkopolska Region, West-Central Poland

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### Abstract

The objective of the present study was to investigate the seroprevalence of HAV and HEV in Polish blood donors (BDs). One hundred and ten randomly selected healthy BDs, living in Wielkopolska Region were tested for anti-HAV IgG and anti-HEV IgG with commercial assays. The seroprevalence of anti-HAV was 11.8%; anti-HEV were detected in 60.9% of BDs ( $p < 0.0001$ ). Consumption of risky food was more common in anti-HEV-positive BDs (59.1% vs. 33.3%;  $p = 0.01$ ). Twelve out of 20 BDs (60%) with no history of travel abroad were exposed to HEV. Wielkopolska Region, Poland should be regarded as a new HEV infection-hyperendemic area in Europe.

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**Key words:** blood donors, HAV, HEV, Poland, seroprevalence

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The prevalence of hepatitis E virus (HEV) infections in Central Europe has been described only partially and few studies report such data for this part of the European continent (Lapa *et al.*, 2015). The knowledge of this subject in Poland is limited to two reports discussing the seroprevalence of IgG antibodies in 182 patients from Poznan center (anti-HEV-positivity in 15.9% patients, EIA-gen HEV IgG kits; Adaltis, Milano, Italy) (Bura *et al.*, 2015) and in 1016 hunters from all 16 Polish provinces (anti-HEV detected in 20.3% participants with ELISA recomWell HEV IgG kits; Microgen, Neuried, Germany) (Sadkowska-Todys *et al.*, 2015).

For another primary hepatotropic virus transmitted by the fecal-oral route, that is, hepatitis A virus (HAV), in the 21<sup>st</sup> century, Poland has experienced a shift toward very low endemicity (Magdzik and Czarkowski, 2004) and is currently one of the countries with the lowest hepatitis A incidence among the European Union member states (European Centre for Disease Prevention and Control, 2014).

A comparative study of HAV and HEV exposure markers (the presence of IgG antibodies against respective viruses) in 110 randomly selected healthy (HBV, HCV, HIV and syphilis-negative; no jaundice in the past) unpaid voluntary blood donors (BDs; 75 men and 35 women) living in Wielkopolska Region (Greater

Poland; one of the largest regions in Poland, located in the west-central part of our country) was performed. The BDs were 19–58 years of age ( $38.4 \pm 9.6$ ; median age – 40 years).

Anti-HAV IgG (anti-HAV) seroprevalence was determined by a chemiluminescent microparticle immunoassay (CMIA; ARCHITECT HAV Ab-IgG kits; Abbott Laboratories, Wiesbaden, Germany) whereas for anti-HEV IgG (anti-HEV) WANTAI HEV-IgG ELISA kits (Wantai Biological Pharmacy Enterprise, Beijing, China) were used, both according to the manufacturers' instructions. These tests are accepted diagnostic tools for the assessment of exposure to HAV and HEV, respectively.

The serum samples were collected at the Regional Blood Center in Poznan, Poland, during the first 6 months of 2015 and were stored at  $-20^{\circ}\text{C}$  prior to testing.

All the BDs gave their written informed consent. The study was approved by the local Bioethics Committee (reference number: 263/15) and was funded by Poznan University of Medical Sciences (number of funds: 502-01-02205314-04519).

Numerical data were presented as mean and standard deviation. The comparison of age was performed by t-Student test. The assumption if data follow normal

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distribution was checked by Shapiro-Wilk's test. The homogeneity of variances was checked by Levene's test. Nominal data were compared by Chi-square test of independence. For the factors that might influence the HEV seroprevalence odds ratios (OR) and 95% confidence intervals (CI) were calculated. Statistical analysis was performed with Statistica 12 (Statsoft, Inc.) software.

All tests were considered significant at  $p < 0.05$ .

The seroprevalence of anti-HAV was 11.8% (13/110), whereas anti-HEV were detected in 60.9% of BDs (67/110) ( $p < 0.0001$ ); in 2 cases a borderline HEV antibodies testing result was found and included as negative in further analysis.

The distribution of combined HAV and HEV IgG assessment results was as follows: double (anti-HAV/anti-HEV) positive – 5 persons (4.5%), only anti-HAV-positive – 8 BDs (7.3%), double negatives – 35 participants (31.8%) and only anti-HEV-positive – 62 persons (56.4%).

Anti-HAV-positive BDs were older ( $46.7 \pm 6.8$  years, range 38–58) than seronegatives ( $37.2 \pm 9.4$  years, range 19–58) ( $p = 0.0006$ ).

For anti-HAV positive samples the mean  $\pm$ SD relative light units (RLU) signal/cut off (s/co) ratio value was  $11.03 \pm 3.16$  (range 4.12–14.47; the positive result was defined as RLU s/co  $> 1.00$ ) and for anti-HEV positive samples the mean optical density/cut off (OD/co) ratio value was  $5.932 \pm 3.454$  (range 1.221–14.458; the positive result was defined as OD/co  $> 1.100$ ; values between 0.9 and 1.100 were considered borderline); it is worth to know that in only 9 HEV seropositive persons (13.4% out of all HEV-positive BDs) the OD/co ratio was lower than 2.000. The prevalence of some variables in relation to anti-HEV IgG status is shown in the Table I.

The main conclusion from the above analysis is that the culinary habits indicating the consumption of some

risky food (raw / undercooked meat or seafood) were more common in the BDs exposed to HEV. This factor has been recognized as the source of infections with the zoonotic genotypes of the virus (Said *et al.*, 2009; Mansuy *et al.*, 2011; Guillois *et al.*, 2013; Meng, 2013). In addition, a trend toward a more frequent prevalence of anti-HAV in HEV-seronegative (*vs* HEV-seropositive) BDs in conjunction with a significantly less common presence of HAV exposure marker among the study participants suggest that the main modes of transmission for these viruses in our region are different.

Although there was no mean age difference between the study participants exposed to HEV and anti-HEV-negatives (in our opinion, it was only because the group of BDs was small), the number of HEV-seropositive BDs aged  $> 30$  tended to be higher than the prevalence of these antibodies in younger persons (see Table I). An evident relationship between the seroprevalence of HEV and age is reported in most available studies among BDs (Dreier and Juhl, 2014).

It should be stressed that in case of 20 persons with no history of travel abroad anti-HEV positivity was found in 60% of them ( $n = 12$ ). It supports some previous suggestions (Bura *et al.*, 2015) that autochthonous (locally acquired) HEV infections do occur also in Poland.

Despite an obvious limitation of this study, that is, a small number of participants, its results are surprising because they indicate that Wielkopolska Region is a hyperendemic area for HEV infections. In Europe, similar seroprevalence of anti-HEV (52.5%) was found only in the Toulouse region, southwestern France (Mansuy *et al.*, 2011). Such a high frequency of HEV-seropositivity, significantly more important than found in previous surveys from our country in specific populations (patients of Infectious Diseases Department

Table I  
Status of anti-HEV and selected variables in 110 BDs from western Poland.

Variable	Anti-HEV+ (n = 67)	Anti-HEV – (n = 43)	OR [95% CI]	p
Mean age $\pm$ SD [years]	$39.5 \pm 8.9$	$36.6 \pm 10.3$	NA <sup>b</sup>	0.1198
Median age (IQR <sup>a</sup> )	41 (34–46)	37 (27–45)		
BDs aged $> 30$	56 (83.6%)	29 (67.4%)	2.46 [0.99–6.09]	0.0523
Men	45 (67.2%)	30 (69.8%)	0.89 [0.39–2.02]	0.7749
Current place of residence – city	55 (82.1%)	35 (81.4%)	1.05 [0.35–3.29]	0.9233
Childhood place of residence – city	46 (69.7%)	29 (67.4%)	0.90 [0.37–2.25]	0.8038
Travel abroad during lifetime	55 (82.1%)	35 (81.4%)	1.05 [0.39–2.82]	0.9266
Contact with swine	12 (17.9%)	8 (19.1%)	0.93 [0.34–2.50]	0.8814
Anti-HAV positivity	5 (7.5%)	8 (18.6%)	2.83 [0.86–9.33]	0.0866
Consumption of raw / undercooked meat or seafood	39 (59.1%)	14 (33.3%)	2.89 [1.29–6.48]	0.0100

<sup>a</sup>IQR – interquartile range; <sup>b</sup>NA – not assessed.

and hunters) (Bura *et al.*, 2015; Sadkowska-Todys *et al.*, 2015), can be explained by a very good sensitivity of the Wantai assay. Some seroprevalence assessments across Europe have shown that the use of these tests can result in several times higher ratios of anti-HEV in comparison to other manufacturers' assays (Bendall *et al.*, 2010; Mansuy *et al.*, 2011; Wenzel *et al.*, 2013).

Faced with such unexpected data, at least one question of fundamental importance must be asked: what is the practical significance of such a common occurrence of HEV infections in Wielkopolska Region?

The answer is difficult because to the best of our knowledge none of the Polish hepatology centers has been performing tests for hepatitis E to date and there are no data on the prevalence of acute hepatitis of unknown etiology neither in our region nor Poland-wide. Due to a very common subclinical exposure to HEV revealed by this study, a potential danger of HEV-infected blood transfusions, particularly to immunocompromised recipients, should also be taken into account (Matsubayashi *et al.*, 2004; Hewitt *et al.*, 2014; Huzly *et al.*, 2014). This issue should be rapidly clarified.

In conclusion, the results of the current report suggest that Wielkopolska Region, west-central Poland, should be regarded as a new HEV infection-hyperendemic area in Europe.

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## The Prevalence of *Campylobacter* spp. in Polish Poultry Meat

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### Abstract

The prevalence, count and molecular identification of *Campylobacter* spp. in Polish poultry meat were analysed. 181 samples of meat from chicken (70), turkey (47), duck (54) and goose (10) were studied. *Campylobacter* spp. was found in 64% of meat samples. The highest prevalence of this pathogen was detected for duck meat. On average 80% of duck samples were contaminated with *Campylobacter* spp. The counts of *Campylobacter* spp. in positive samples remained under ten colony forming units per gram of product in 59% of poultry meat. *C. jejuni* was more frequently detected in poultry meat than *C. coli*.

**Key words:** *Campylobacter* spp., microbiological quality, poultry meat

During the last few decades, the global production of poultry meat has increased rapidly from 58.5 million tonnes in 2000 to 95.5 million tonnes in 2014. Production is not equally distributed; the Americas accounted for 43% of the total production, Asia (mainly China) for 34%, Europe for 17% and Africa and Oceania for 5% and 1% of the whole production in 2012 (93 million tonnes), respectively. In 2023, poultry meat is expected to be the largest meat sector by around 130.7 million tonnes (Skarp *et al.*, 2016). Chicken meat is currently the first most widely produced poultry meat followed by turkey meat, duck meat and goose meat. Although much attention has focused on microbiological safety of poultry meat, this type of product remains a significant cause of foodborne disease in the world. The most reported poultry-borne gastroenteric disease is campylobacteriosis. In 2015 there were 229,213 cases of campylobacteriosis diagnosed (EFSA, 2016). Infection in humans is mainly caused by the zoonotic pathogen *Campylobacter* spp. Poultry is a natural host for *Campylobacter* spp. in general, and that colonized birds are the primary vector for transmitting this pathogen to humans (Bless *et al.*, 2014; Rozynek *et al.*, 2009).

Although poultry meat is becoming increasingly popular, relatively little research has been done inves-

tigating the presence and count of *Campylobacter* spp. in other than chicken types of poultry meat. In order to add more insight to these issue the objective of this study was to determine the prevalence, count and genetic diversity of *Campylobacter* spp. in different kind of poultry meat available in local trade network.

One hundred and eighty one samples of four types of commercially available fresh poultry meat were microbiologically analysed from 2013 to 2015. The samples of meat were transported to the Laboratory of Microbiology in isothermal containers, maintaining the temperature at 0–2°C, and tested immediately on reaching the laboratory. A total of 70 chicken, 47 turkey, 54 duck and 10 goose meat portions were examined in terms of the prevalence and count of *Campylobacter* spp. isolation and count were performed according to PN-ISO 10272-1:2007+Ap1:2008 and PKN ISO/TS 10272-2:2008. To confirm isolates and identify the species, polymerase chain reaction (PCR) methods was applied (Maćkiw *et al.*, 2012). For quality control, *C. jejuni* ATCC 33291 and *C. coli* ATCC 33559 strains were used. Prevalence data for *Campylobacter* spp. sorted by meat type, and species were analyzed using the analysis of variance test ANOVA (Statistica 6.0 PL). The significance level was  $P < 0.05$ . In case of finding

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Table I  
*Campylobacter* spp. presence and counts in different types of poultry meat.

Meat type	No of samples	No / % of positive samples	No / % of identified strains		No of positive samples Counts [CFU/g]		
			<i>C. jejuni</i>	<i>C. coli</i>	<10	≤100	>100
chicken	70	49/70	36/31	13/11	23	10	16
turkey	47	18/38	12/10	6/5	12	6	0
duck	54	43/80	27/23	16/14	28	5	10
goose	10	6/60	4/3	2/2	5	1	0
total	181	116/64	79/68	37/32	68	22	26

significant differences the post-hoc analysis was done using the Tukey test.

The frequency of *Campylobacter* spp. detection and counts in the tested poultry meat is shown in Table I. Examination of the meats revealed that the vast majority of samples (64%) were contaminated with *Campylobacter* spp. The prevalence of this genus ranged from 38% to 80%, respectively for turkey and duck. The direct plating method yielded enumeration results from < 10 CFU/g to  $1.0 \times 10^3$  CFU/g. Enumeration data showed the greater number of samples were positive only after enrichment (68%) indicating low microbiological load of *Campylobacter* on analysed poultry meat (Table I).

Of the 116 positive samples, isolates originating from a variety of poultry meat were lost in the course of freeze storage, leaving isolates from 97 samples for inclusion in the PCR analysis. Of the 97 *Campylobacter* spp. isolates, 61 and 36 were confirmed based on PCR as *C. jejuni* and *C. coli*, respectively (Table II). Variability in *C. jejuni* and *C. coli* prevalence observed in samples obtained from different types of poultry meat was not statistically significant.

Due to the lack of regulation in the EU legislation routine tests of poultry meat for the presence of *Campylobacter* spp. are not carried out in Poland (Commission Regulation (EC) No 2073/2005 as amended). Therefore, the above quantitative and qualitative assessment results of *Campylobacter* spp. prevalence in different types of poultry meat, available in Polish trade are a valuable source of information on this pathogen contamination.

In this study *Campylobacter* spp. was isolated from 64% of poultry meat. Within the tested meat types, highest *Campylobacter* spp. prevalence was found in duck (80%) followed by chicken (70%), goose (60%), and turkey (38%). Similar results were obtained by Korsak *et al.* (2015). Polish studies at the retail level revealed that 49.3% of poultry samples were contaminated with *Campylobacter* spp. Our results on the prevalence of *Campylobacter* spp. in raw poultry meat are in agreement with data from other countries (Adzitey *et al.*, 2012; Guyard-Nicodeme *et al.*, 2015; Hansson *et al.*, 2015). During the seven years of the study in the United States the average prevalence of *Campylobacter* spp. in retail broiler meat was 41%, with no statistical differences in the prevalence by year ( $P > 0.05$ ) (Williams and Oyarzabal, 2012). In this study the prevalence of *Campylobacter* spp. in chicken meat was 70% and was lower than the frequency of contamination detected in research performed on chicken in Germany or Ireland, respectively, 87% and 91%. (Luber and Bartelt, 2007; Madden *et al.*, 2011; Moran *et al.*, 2009). The percentage obtained in our experiment for duck samples positive for this pathogen is similar to findings reported from Great Britain (Colles *et al.*, 2011), Tanzania (Nonga and Muhairwa, 2010) and South Korea (Wei *et al.*, 2014). According to Colles *et al.* (2011) and Wei *et al.* (2014) the percentage of contaminated duck samples was 93.3–100.0% and 96.6% respectively. Lower values were found by Jamali *et al.* (2015) and Rahimi *et al.* (2011). These authors detected *Campylobacter* spp. in 39.2% and 35.5% duck samples, respec-

Table II  
 Genotypic identification of *Campylobacter* spp.

Meat type	No of contaminated samples	No / % of strains identified to species	
		<i>C. jejuni</i>	<i>C. coli</i>
chicken	37	25/68	12/32
turkey	14	8/57	6/43
duck	40	24/60	16/40
goose	6	4/67	2/33
total	97	61/63	36/37

tively. The differences among results might be due to diverse isolation methods, geographic, and seasonal factors (Adzitey *et al.*, 2012; Jamali *et al.*, 2015). With regard to the range of *Campylobacter* sp. – positive samples in turkey meat, the results of Atanassova *et al.* (2007) and Rahimi and Tajbakhsh (2008) are similar to the results obtained in this investigation. Of the turkey meat examined, 34.0% and 24.7% samples were *Campylobacter* sp. positive (Atanassova *et al.*, 2007; Rahimi and Tajbakhsh, 2008). Other authors have described higher levels. Cakmak and Erol (2012) detected *Campylobacter* spp. from 45.6% of the turkey meat samples. On the other hand Noormohamed and Fakhr (2014) found in their study that 17% of the turkey samples were positive for *Campylobacter* spp. There are very few data about prevalence of microbial contamination on goose meat. The first study has shown the occurrence of *Campylobacter* spp. in 26.5% goose samples (Rahimi *et al.*, 2011). In later research reported by Jamali *et al.* (2015) prevalence was 26.1%.

Our findings showed that *C. jejuni* was more prevalent than *C. coli* in poultry meat that is in agreement with data from other countries (Ghafir *et al.*, 2007; Jamali *et al.*, 2015; Noormohamed and Fakhr, 2014; Rahimi *et al.*, 2011; Wei *et al.*, 2014; Williams and Oyarzabal, 2012). The higher prevalence of *C. jejuni* in poultry meat is contrary to the findings conducted by researchers from India, Reunion Island and Poland. Malik *et al.* (2014) observed a shift in the prevalence of important species of *Campylobacter* spp. *C. coli* were prevalent in 93.75% (30/32) and *C. jejuni* in 6.25% (2/32) among broilers slaughtered at chicken shop. Henry *et al.* (2011) also detected *C. coli* as a predominant species in chicken flocks. Maćkiw *et al.* (2012) reported that *C. coli* was the most ubiquitous. Its presence was determined in 75.5% samples of chicken meat and giblets, whereas *C. jejuni* was found in 24.5% of samples.

The quantitative results from present study showed low *Campylobacter* spp. contamination level of examined poultry meat. *Campylobacter* spp. counts were <10 CFU/g in 68% of positive cases. 22% and 26% samples showed a pathogen concentration with a range of  $\geq 10$  to <100 CFU/g and with  $\geq 100$  CFU/g, respectively. Our findings are similar to data from the Belgian monitoring program where 58% of the samples were contaminated with <10 CFU/g, 29% of the samples were contaminated with a range of  $\geq 10$  to <100 CFU/g and 11% of the samples were contaminated with  $\geq 100$  CFU/g. The average *Campylobacter* spp. concentration was  $4.8 \times 10^1$  CFU/g (Habib *et al.*, 2008). The higher *Campylobacter* spp. load were found on Estonian broiler chicken products. Enumeration data, conducted by Mäesaar *et al.* (2014) showed that the overall arithmetic *Campylobacter* spp. CFU mean was  $1.6 \times 10^3$  CFU/g of product. Relatively low counts

obtained in our study and in research conducted by Habib *et al.* (2008) might also be considered hazardous. In a restaurant-associated outbreak, the number of *C. jejuni* bacteria in the causative chicken meal was estimated to range from 53 to 750 CFU/g. Additionally, *in vitro* models indicate that the efficiency with which some *Campylobacter* strains invade intestinal cell lines is optimal at the lowest range of multiplicities of infection, which suggests that species is a highly efficient solitary invader (Habib *et al.*, 2008). Our study revealed that fresh poultry meat is often contaminated with *Campylobacter* spp. that decreases the quality of this kind of meat and constitutes a public health hazards.

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**III Ogólnopolskie Sympozjum Mikrobiologiczne**

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Celem Sympozjum jest podsumowanie dotychczasowych badań i przedstawienie najnowszych osiągnięć z zakresu metagenomiki, mikrobiologii, oddziaływań pomiędzy mikro- i makroorganizmami różnych środowisk oraz ich znaczenia dla rozwoju rolnictwa i ochrony środowiska. Podczas konferencji zostaną zaprezentowane oraz przedyskutowane wyniki badań prowadzonych w ośrodkach naukowych, w tym także postęp w wykorzystaniu nowoczesnych metod badawczych w obszarze mikrobiologii środowiskowej. Sympozjum będzie miejscem wymiany doświadczeń, nawiązywania kontaktów do realizacji wspólnych projektów badawczych oraz wydarzeniem naukowym na temat kompleksowego rozpoznania bioróżnorodności mikroorganizmów w różnych środowiskach oraz najnowocześniejszych metod badawczych stosowanych w mikrobiologii, biologii molekularnej i biotechnologii. Przewiduje się organizację następujących sesji naukowych: nowoczesne metody identyfikacji mikroorganizmów, metagenomika środowisk ekstremalnych, metagenomika aplikacyjna, metagenomika a jakość środowiska.

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**KONFERENCJA POD PATRONATEM PTM****V edycja konferencji****VIRUSES OF MICROBES V: BIODIVERSITY AND FUTURE APPLICATIONS**

Wrocław, 9–13 lipca 2018 r.

Jest to piąte już spotkanie z serii międzynarodowych konferencji towarzystwa International Society for Virus of Microbes (ISVM) poświęconej wirusom drobnoustrojów. Konferencje Virus of Microbes odbywają się co dwa lata, a rozpoczęły się w 2010 roku w Instytucie Ludwika Pasteura w Paryżu, a następnie kontynuowane były w Brukseli (2012), Zurichu (2014), i Liverpoolu (2016), gromadząc na każdym spotkaniu ok. 400–500 uczestników z całego świata.

Spotkanie zatytułowane jest „Biodiversity and future application”. Tematyka pięciodniowej konferencji poświęcona będzie podstawowym i aplikacyjnym badaniom naukowym nad wirusami mikroorganizmów (algi, archaea, bakterie, grzyby, pierwotniaki i wirusy). Wirusy są kluczowym elementem warunkującym bioróżnorodność i ewolucję mikrobiologiczną, jak również służą jako narzędzie w biologii molekularnej. W ostatnich czasie coraz większym zainteresowaniem cieszą się badania nad bakteriofagami, które stanowią obiecującą alternatywę leczenia infekcji wywołanych zwłaszcza przez wielooporne szczepy patogenów człowieka, a są stosowane już w konserwacji żywności, hodowli zwierząt i produkcji roślin uprawnych.

Uniwersytet Wrocławski (prof. dr hab. Zuzanna Drulis-Kawa) wraz z Laboratorium Bakteriofagowym Instytutu Immunologii i Terapii Doświadczalnej we Wrocławiu (prof. dr hab. Krystyna Dąbrowska), mają zaszczyt organizować kolejną międzynarodową edycję tej konferencji w 2018 roku.

Konferencja porusza tematykę niezwykle aktualną i będzie dedykowana do wszystkich osób zajmujących się zagadnieniami z zakresu zwalczania zakażeń bakteryjnych, jak również ekologii i różnorodności biologicznej drobnoustrojów.

Konferencja została objęta patronatem przez European Molecular Biology Organization jako EMBO Workshop „Viruses of microbes 2018” <http://www.embo.org/events>

**Lokalizacja:**

Uniwersytet Wrocławski, Wydział Prawa, Administracji i Ekonomii  
ul. Uniwersytecka 22/26, 50-145 Wrocław

**Organizatorzy:**

Uniwersytet Wrocławski, Instytut Immunologii i Terapii Doświadczalnej PAN

**Kontakt:**

prof. Zuzanna Drulis-Kawa, [zuzanna.drulis-kawa@uwr.edu.pl](mailto:zuzanna.drulis-kawa@uwr.edu.pl), tel. 71 375 62 90  
prof. Krystyna Dąbrowska, [dabrok@iitd.pan.wroc.pl](mailto:dabrok@iitd.pan.wroc.pl), tel. 71 337 11 72 wewn. 316

## INTERNATIONAL CONFERENCE

**VIRUSES OF MICROBES V: BIODIVERSITY AND FUTURE APPLICATIONS**

9–13 July 2018 Wrocław, Poland

Institute of Genetics and Microbiology University of Wrocław & Hirschfeld Institute of Immunology and Experimental Therapy Polish Academy of Sciences On behalf of the organising committee of the ISVM conference on the Viruses of Microbes, we are pleased to invite to the fifth meeting in an international series that began in 2010 at the Pasteur Institute.

This event is one that focuses on basic and applied scientific research on viruses infecting microbes (algae, archaea, bacteria, fungi, protozoa and viruses). Viruses have always been a key element of microbial diversity and evolution, as well as a tool for the molecular biologist to learn more about how the host cell functions, but this information has also been put to productive use in latter days to control infections and fouling in many areas of our modern life.

The conference is included to the EMBO Workshop list <http://www.embo.org/events>

**Location:**

Uniwersytet Wrocławski, Wydział Prawa, Administracji i Ekonomii  
ul. Uniwersytecka 22/26, 50-145 Wrocław

**Organizers:**

Uniwersytet Wrocławski; Instytut Immunologii i Terapii Doświadczalnej PAN

**Contact:**

prof. Zuzanna Drulis-Kawa, [zuzanna.drulis-kawa@uwr.edu.pl](mailto:zuzanna.drulis-kawa@uwr.edu.pl), tel. 71 375 62 90  
prof. Krystyna Dąbrowska, [dabrok@iitd.pan.wroc.pl](mailto:dabrok@iitd.pan.wroc.pl), tel. 71 337 11 72/ 316

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**Organizatorzy wszystkich konferencji współorganizowanych  
przez Polskie Towarzystwo Mikrobiologów i obejmowanych patronatem PTM powinni zagwarantować  
zniżkowe opłaty rejestracyjne dla członków PTM**

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**INFORMACJA O PRACY PREZYDIUM ZARZĄDU GŁÓWNEGO PTM**

Warszawa, 02.01.2018 r.

Szanowni Członkowie Polskiego Towarzystwa Mikrobiologów,

Zaczął się Nowy Rok 2018. Życzymy Państwu sukcesów osobistych i zawodowych, rozwoju Oddziałów Terenowych PTM, a także poprawy kondycji całego naszego Stowarzyszenia.

Poniżej szereg informacji z ostatnich prac Prezydium ZG PTM

1. Materiały z Nadzwyczajnego Walnego Zgromadzenia Delegatów PTM, które odbywało się w ramach Konferencji Naukowej „PTM wczoraj – dziś – jutro”, Kraków 22–23 września 2017 r., wraz z nowym statutem PTM zostały skompletowane, podpisane, przekazane do kancelarii prawnej i złożone do Krajowego Rejestru Sądowego. Oczekujemy na decyzję Sądu.

2. Polskie Towarzystwo Mikrobiologów składa ogromne podziękowanie **Zgromadzeniu Fundatorów Założycieli Fundacji na Rzecz Polskiej Mikrobiologii**, w osobach:

prof. dr hab. Danuta Daniela Dzierżanowska-Madalińska

prof. dr hab. Jerzy Franciszek Hrebenda

prof. dr hab. Marek Aleksander Jagielski

prof. dr hab. Adam Jan Jaworski

prof. dr hab. Stanisław Mirosław Kałużewski

prof. dr hab. Józef Feliks Kubica

dr hab. Jan Andrzej Patzer

dr Bohdan Jerzy Starościak

dr Jolanta Elżbieta Szych

za podjętą po likwidacji Fundacji decyzję i przekazanie dnia 14 grudnia 2017 r. kwoty 27 527 zł na konto PTM na cele statutowe PTM związane z rozwojem polskiej mikrobiologii.

3. Tuż przed Świątami Bożego Narodzenia PTM otrzymało dofinansowanie Konferencji „90 lat PTM” z Ministerstwa Nauki i Szkolnictwa Wyższego, z puli przeznaczonej na działalność upowszechniającą naukę. Dotacja ta umożliwi pokrycie kosztów konferencji. W trybie bardzo pilnym musieliśmy sporządzić sprawozdania merytoryczne i finansowe, aby rozliczyć otrzymane dofinansowanie. Dnia 12.01.br. przekazano materiał do MNiSW i obecnie czekamy na decyzję.

4. Złożono wniosek do MNiSW na działalność upowszechniającą naukę – o dofinansowanie modernizacji w latach 2018–2019 czasopism PTM: *Polish Journal of Microbiology* i *Postępów Mikrobiologii*.

5. Objęto patronatem PTM Międzynarodowe Sympozjum Naukowe „Non-conventional Yeasts: from basic aspects to applications” organizowane przez Uniwersytet Rzeszowski, zaplanowane w dniach 15–18 maja 2018 r. w Rzeszowie (Uchwała nr 42-2017 z dnia 15.11.2017 r.), a także III Ogólnopolskie Sympozjum Mikrobiologiczne „Metagenomy różnych środowisk” zaplanowane w dniach 28–29 czerwca 2018 r. w Lublinie (Uchwała nr 5-2018 z dnia 15.01.2018 r.). Konferencje: „**Viruses of Microbes V: Biodiversity and future applications**” i „**Non-conventional Yeasts: from basic aspects to applications**”, dzięki poparciu PTM, w tym finansowemu, uzyskały dofinansowanie z FEMS w kwocie 5,000 Euro, każda.

6. Przypominamy, że członkowie PTM począwszy od stycznia 2018 r. nie będą otrzymywać bezpłatnych zeszytów kwartalników *Postępy Mikrobiologii* i *Polish Journal of Microbiology*. Czasopisma te są dostępne w wersji elektronicznej. Zgodnie z uchwałami 29-2017 i 30-2017 ZG PTM członkowie PTM mają dostęp do wszystkich, w tym bieżących numerów, kwartalników *Postępy Mikrobiologii* oraz *Polish Journal of Microbiology* w 2018 r., po zarejestrowaniu się w systemie na stronie internetowej PTM. Jednocześnie informujemy, że osoby, które będą chciały posiadać wersje papierowe tych czasopism w roku 2018 muszą zamówić i wykupić prenumeratę *Postępów Mikrobiologii* oraz *Polish Journal of Microbiology* bezpośrednio w Zakładzie Wydawniczym Letter Quality, e-mail: roma.walendzewicz@gmail.com.

Cena jednego zeszytu kwartalników w 2018 r. będzie wynosiła 40 zł + 5% VAT, tym samym roczna prenumerata (w 2018 r.) każdego z czasopism będzie wynosiła 160 zł + 5% VAT (Uchwała nr 10-2018 z dnia 15.01.2018 r.). Wysokość ceny jest taka sama dla wszystkich chętnych prenumeratorów. Nowa cena jeszcze w pełni nie rekompensuje kosztów wydania zeszytu, lecz zmniejsza straty wydawnicze PTM.

7. Uruchomiono system logowania się na stronie PTM przez członków PTM z opłaconymi składkami członkowskimi za 2017 r. i lata wcześniejsze. Uaktualniane są przy okazji dane osobowe członków i ich adresy. Rozważamy różne warianty informacji, które mogłyby być dostępne dla członków PTM po zalogowaniu się na stronę. Na dzień 31.01.2018 r. 200 członków już się zalogowało.

8. Przypominamy, że dla manuskryptów otrzymywanych przez redakcję *Polish Journal of Microbiology* od 01.07.2018 r. zostaną podniesione opłaty redakcyjne

za publikację w PJM, odpowiednio:

dla autorów korespondencyjnych, członków PTM z: 125 USD + 23% VAT do 250 USD + 23% VAT

oraz dla pozostałych osób z 250 USD + 23% VAT do 500 USD + 23% VAT

(Uchwała nr 40-2017 z dnia 31.10.2017 r.).

9. W związku ze zgłoszoną przez Panią prof. Jolantą Solecką rezygnacją z funkcji Redaktora Naczelnego czasopisma *Polish Journal of Microbiology* oraz rezygnacją z funkcji sekretarzy Pani mgr Joanny Ziemskiej i Pana mgr Adama Guśpiela z NIZP-PZH, po rozeznaniu istniejącej sytuacji, postanowiono przychylić się do prośby Pani prof. Jolanty Soleckiej z zespołem i po skompletowaniu numeru 1/2018 PJM, z dniem 28.02.2018 r. powierzyć funkcję Redaktora Naczelnego PJM Pani prof. Elżbiecie Annie Trafny, członkowi Prezydium ZG PTM (Uchwała nr 2-2018 z dnia 15.01.2018 r.). Ponadto postanowiono zatrudnić nową osobę do prowadzenia spraw sekretarskich związanych z wydawaniem *Polish Journal of Microbiology*. W 2018 r. ulega także zmianie firma świadcząca usługi wydawania przez PTM czasopisma PJM drogą elektroniczną, została nią amerykańska firma Exeley (Uchwała nr 3-2018 r. z dnia 15.01.2018 r.).

10. W związku z pismem Pana prof. Waldemara Rastawickiego Redaktora Naczelnego kwartalnika *Medycyna Doświadczalna i Mikrobiologia* w sprawie współfinansowania przez PTM wydawania tego czasopisma, podjęto uchwałę (nr 11-2018 z dnia 15.01.2018 r.) w sprawie niefinansowania kwartalnika oraz rezygnacji z zapisu dotyczącego PTM w treści informacji „organ Narodowego Instytutu Zdrowia Publicznego – Państwowego Zakładu Higieny w Warszawie oraz Polskiego Towarzystwa Mikrobiologów” (pozostaje zapis dotyczący tylko NIZP-PZH). Wnioskowaliśmy, aby informacja „W latach 1949–2017 kwartalnik *Medycyna Doświadczalna i Mikrobiologia* ukazywał się jako organ Państwowego Zakładu Higieny (obecnie Narodowy Instytut Zdrowia Publicznego – Państwowy Zakład Higieny) w Warszawie oraz Polskiego Towarzystwa Mikrobiologów” wraz z logo PTM, zamieszczone zostały na wewnętrznej stronie okładki wszystkich zeszytów kwartalnika.

Do końca 2017 r. koszty związane z wydawaniem kwartalnika *Medycyna Doświadczalna i Mikrobiologia* w całości pokrywał Państwowy Zakład Higieny, obecnie NIZP-PZH.

11. Toczą się rozmowy pomiędzy PTM, FEMS i Firmą Global Congress w sprawie organizacji FEMS Council w Warszawie w dniach 7–8. września 2018 r. w Warszawie.

12. W odpowiedzi na prośbę Ministerstwa Zdrowia, PTM zgłosiło kandydaturę Pani prof. Katarzyny Dzierżanowskiej-Fangrat, zastępcy dyrektora ds. klinicznych Instytutu-Pomnika Centrum Zdrowia Dziecka na Konsultanta Krajowego w dziedzinie mikrobiologii lekarskiej.

13. Uchwałami nr 43-2017 z dnia 15.11.2017 r. oraz nr 6-2018 z dnia 15.01.2018 r. przyjęto 16 nowych członków zwyczajnych do Polskiego Towarzystwa Mikrobiologów.

14. Jeden członek naszego Towarzystwa otrzymał FEMS Travel Grant umożliwiający uczestnictwo w konferencji “Challenges and new concepts in antibiotics research” w dniach 19–21.03.2018 r. w Instytucie Pasteur’a w Paryżu.

15. Jesteśmy na kolejnym etapie porządkowania spraw opłat składek członkowskich PTM.

Tabele z informacjami o zaległych składkach członków Oddziałów Terenowych PTM przekazano do Przewodniczących Oddziałów.

Zgodnie z nowym statutem PTM, przyjętym na Nadzwyczajnym Walnym Zgromadzeniu Delegatów PTM w Krakowie w dniu 23.09.2017 (statut jest obecnie w trakcie rejestracji w sądzie) członkiem zwyczajnym PTM w 2018 r. może być osoba, która ma uregulowane wszystkie składki przed 2018 r. Osoby, które nie zapłaciły składki za 2015, 2016 lub 2017 rok są informowane o konieczności niezwłocznego uregulowania zaległych składek do końca stycznia 2018 r.

Na dzień 31.01.2018 r. jeszcze 243 osób (z ogólnej liczby 1005 członków PTM) nie uregulowało zaległych składek.

16. W Uchwale nr 9-2018 z dnia 15.01.2018 r. ustanowiono, zgodnie z par. 15 nowego statutu PTM z dnia 23.09.2017 r., dodatkowy miesięczny (do końca lutego) termin do uregulowania opłat za zaległe składki członkowskie PTM za lata 2015–2017. W ciągu lutego zostanie wysłane kolejne przypomnienie o konieczności uregulowania wszystkich zaległości do dnia 01.03.2018 r. Jeżeli nie zostaną one uregulowane, na planowanym zebraniu Zarządu Głównego PTM w marcu br., zostanie podjęta uchwała o usunięciu osób z nieopłaconymi składkami za poprzednie lata (2015–2017) z grona członków PTM. Powinniśmy dążyć do sytuacji, gdy składki członkowskie są regularnie wpłacane na konto PTM. Jednocześnie przypominamy o obowiązku opłacenia składki członkowskiej za 2018 rok, zgodnie ze statutem, w pierwszym kwartale 2018 r.

Wysokość składki członkowskiej w 2018 r. nie uległa zmianie i nadal wynosi 100 zł.

17. Pani prof. Ewa Augustynowicz-Kopeć, wiceprezes PTM, opracowuje projekt regulaminu pracy Zarządu Głównego PTM, wymagany przez statut PTM. Projekt regulaminu będzie przedstawiony na marcowym zebraniu ZG PTM.

18. Ustalono, na tym samym poziomie, co w 2017 r., wynagrodzenia dla zespołów redakcyjnych *Postępy Mikrobiologii* (Uchwała nr 1-2018), *Polish Journal of Microbiology* (Uchwała nr 4-2018) oraz Pań księgowej PTM (Uchwała nr 7-2018) i sekretarki PTM (Uchwała nr 8-2018).

SEKRETARZ  
Polskiego Towarzystwa Mikrobiologów  
*A. Laudy*  
dr n. farm. Agnieszka F. Laudy

PREZES  
Polskiego Towarzystwa Mikrobiologów  
*Stefan Tyski*  
prof. dr hab. Stefan Tyski

**CZŁONKOWIE WSPIERAJĄCY PTM****CZŁONEK WSPIERAJĄCY PTM – ZŁOTY**

od 27.03.2017 r.



HCS Europe – Hygiene & Cleaning Solutions  
ul. Warszawska 9a, 32-086 Węgrzce k. Krakowa  
tel. (12) 414 00 60, 506 184 673, fax (12) 414 00 66  
[www.hcseurope.pl](http://www.hcseurope.pl)

Firma projektuje profesjonalne systemy utrzymania czystości i higieny dla klientów o szczególnych wymaganiach higienicznych, m.in. kompleksowe systemy mycia, dezynfekcji, osuszania rąk dla pracowników służby zdrowia, preparaty do dezynfekcji powierzchni dla służby zdrowia, systemy sterylizacji narzędzi.

**CZŁONEK WSPIERAJĄCY PTM – SREBRNY**

od 07.06.2017 r.



Aesculap Chifa Sp. z o.o.  
ul. Tysiąclecia 14  
64-300 Nowy Tomyśl  
tel. (61) 44 20 100, fax (61) 44 23 936  
[www.chifa.com.pl](http://www.chifa.com.pl)

Aesculap Chifa Sp. z o.o. jest członkiem grupy B. Braun, jednej z wiodących na świecie firm medycznych, produkującej i dystrybuującej między innymi preparaty do antyseptyki rąk, skóry, błon śluzowych, do mycia i dezynfekcji wyrobów medycznych oraz powierzchni.

**CZŁONEK WSPIERAJĄCY PTM – SREBRNY**

od 12.09.2017 r.



Firma Ecolab Sp. z o.o. zapewnia:  
najlepszą ochronę środowiska pracy przed patogenami powodującymi zakażenia  
podczas leczenia pacjentów, bezpieczeństwo i wygodę personelu,  
funkcjonalność posiadanego sprzętu i urządzeń.  
Firma jest partnerem dla przemysłów farmaceutycznego, biotechnologicznego i kosmetycznego.

**CZŁONEK WSPIERAJĄCY PTM – SREBRNY**

od 12.12.2017 r.



Od ponad 100 lat siedziba Wodociągów Krakowskich mieści się przy ul. Senatorskiej.

Budowę obiektu ukończono w 1913 roku.

W 2016 r. do sieci wodociągowej wtłoczono ponad 56 mln m<sup>3</sup> wody.

Szacuje się, że ponad 99,5% mieszkańców Gminy Miejskiej Kraków posiada możliwość korzystania z istniejącej sieci wodociągowej.

**CZŁONEK WSPIERAJĄCY PTM – ZWYCZAJNY**

od 12.09.2017 r.



Merck Sp. z o.o. jest częścią międzynarodowej grupy Merck KGaA z siedzibą w Darmstadt, Niemcy i dostarcza na rynek polski od roku 1992 wysokiej jakości produkty farmaceutyczne i chemiczne, w tym podłoża mikrobiologiczne