POLSKIE TOWARZYSTWO MIKROBIOLOGÓW
POLISH SOCIETY OF MICROBIOLOGISTS

Polish Journal of Microbiology

2015

Polish Journal of Microbiology
formely Acta Microbiologica Polonica
2015, Vol. 64, No 1

CONTENTS

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 3–13

MINIREVIEW

Virus Like Particles as Immunogens and Universal Nanocarriers

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Submitted and accepted 5 February 2015

Abstract

Over the last two decades virus-like particles (VLPs) have become an important tool in biomedical research and medicine. VLPs are multiprotein structures that resemble viable virus particles in conformation but lack the viral genome. Consequently, they are non-infectious and non-replicative, but retain the ability to penetrate cells, making them useful for a vast spectrum of applications. Above all, VLPs mimicking genuine viruses in antigenic structure provide a safe alternative to attenuated and inactivated viruses in vaccine development. Moreover, due to their transducing proprieties, VLPs may efficiently deliver foreign nucleic acids, proteins, or conjugated compounds to the organism, or even to specific cell types. Additionally, VLPs are versatile nanovectors due to their flexibility in terms of composition and expression systems. In this review, different approaches for of virus-like particle synthesis and manipulation, as well as their potential applications, will be discussed.

K e y w o r d s: delivery platform, vaccines, virus like particles (VLPs)

Virus-like particles

Virus-like particles (VLPs) are empty multiprotein structures closely resembling natural virions. Due to the absence of the genetic material, VLPs are replication- and infection-incompetent. They are formed spontaneously by the self-assembly of viral proteins during infection or by *in vitro* protein expression, usually in a eukaryotic system. Therefore, VLPs can be classified depending on the original virus taxonomy (*e.g.*, adenovirus, coronavirus, *etc.*) or depending on the synthesis method (*e.g.*, animal, yeast, plant, or cell-free expression). Another classification of VLPs relies on their architecture and distinguishes enveloped and non-enveloped VLPs, as well as native or chimeric VLPs. Non-enveloped VLPs are typically composed of one or more viral structural proteins, whereas enveloped VLPs consist of the host cell membrane with viral proteins displayed on the outer surface (Fig. 1) (Kushnir *et al.*, 2012). Non-enveloped VLPs are easier to produce and purify, but enveloped VLPs are more flexible, as antigens from different pathogens can be integrated. However, they may also contain host's proteins, which may affect downstream applications (Buonaguro *et al.*, 2011).

Expression systems

Animal cells are the most convenient expression system for virus-like particles because of their ability to carry out the complex post-translational modifications that are required for proper protein folding. Genes encoding viral proteins of interest are introduced into the host cell by plasmid transfection or viral transduction (using retroviruses, lentiviruses, adenoviruses, or adeno-associated viruses for mammalian cells, or baculoviruses for insect cells). Depending on the method used, the transgene is integrated into the genome or replicates in an episomal form. The expressed viral proteins spontaneously self-assemble within the cell, as during viral infection. Mammalian (CHO, BHK, HEK-293T, COS-11 and Vero), avian (ELL-0) and insect (*Lepidoptera*: *Spodoptera frugiperda* and *Trichoplusia ni*) cells have been used for VLPs production in animal expression systems (Kushnir, *et al.*, 2012). Examples of VLPs produced in animal cells and other expression systems are provided in Table I. Despite the fact that enveloped and non-enveloped, native and chimeric VLPs are efficiently formed in mammalian cells, this expression system has some limitations, including high production costs, potential safety concerns, and

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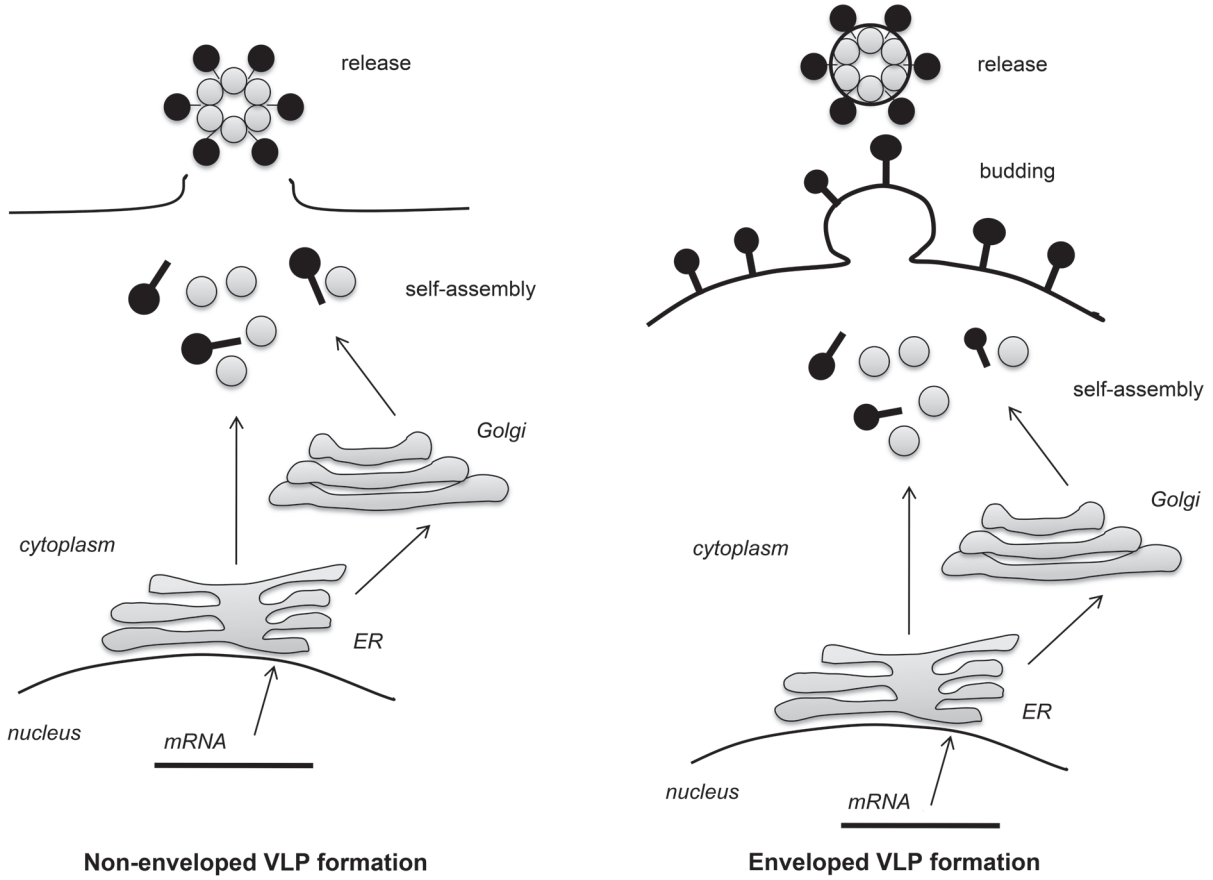

Fig. 1. Schematic representation of the formation of non-enveloped and enveloped VLPs.

difficulties with scaling up production. By contrast, insect cell expression system offers significant advantages for VLPs production. Not only is this system less expensive, but it is also easier to scale up and can be used for the simultaneous expression of many proteins, facilitating the assembly of VLPs (Liu *et al.*, 2013, Rychlowska *et al.*, 2011). Furthermore, insect cells have the ability to post-translationally process and modify the recombinant proteins, similarly to their mammalian counterparts. Lastly, insect cells can be cultured without supplements derived from mammalian organisms, and therefore the risk of contaminating of VLPs developed for human therapy with opportunistic pathogens is minimal (Roy and Noad, 2008). Importantly, baculoviruses infect only a small group of *Lepidoptera* and represent no threat to humans.

Yeast cells are a well-established system for the expression of recombinant proteins. It is, however, important to remember that this system has some drawbacks that are worth considering. Yeast cells differ from mammalian cells in their post-translational modification of expressed proteins, particularly protein glycosylation pattern. For this reason, yeast is used mostly for the production of non-enveloped VLPs. However, a number of studies have demonstrated successful self-assembly of VLPs in *Pichia pastoris* or *Saccharomyces cerevisiae* cells, and two popular vaccines licensed worldwide; against hepatitis B virus (HBV) and human papilloma virus (HPV) are based on VLPs produced in yeast. Interestingly, VLPs formation of HBV proteins has never been observed in yeast cells, but it is presumed that self-assembly takes place during the processing of the yeast biomass (Lünsdorf *et al.*, 2011).

Plant expression systems represent another option for VLP synthesis that is cost-effective, scalable, and free of mammalian pathogens. Expression of recombinant proteins in plants can be achieved either *via* stable or transient transfection of the transgene into the cell. Both enveloped and non-enveloped, as well as native and chimeric, plant-produced VLPs have progressed into clinical development (Scotti and Rybicki, 2013). Interestingly, plant viral vectors used for transformation such as Tobacco mosaic virus (TMV), Alfalfa mosaic virus (A1MV), Cowpea mosaic virus (CPMV), and Papaya mosaic virus (PapMV) may incorporate recombinant proteins (even if these are not of viral origin) to form chimeric VLPs. This strategy has been used to obtain prophylactic vaccine candidates against viruses HBV, influenza virus, respiratory syncytial virus (RSV), Norwalk virus (NV), bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus anthracis, and Yersinia pestis)* and protozoa (*Plasmodium falciparum)*

Table I
Different expression systems for diverse virus-like particles. Chimeric VLPs are denoted with an asterix*; the exterior protein is in the first position and the scaffold protein in the second.

Expression system	VLP	E/NE	Reference
Mammalian			
CHO:	HBsAg	NE	(Soulié *et al.*, 1991)
BHK:	Hantaan	E	(Betenbaugh *et al.*, 1995)
Vero:	Influenza	E	(Barrett *et al.*, 2009)
COS-1	DENV	E	(Chang *et al.*, 2003)
HEK:	Ebola	E	(Yamayoshi and Kawaoka, 2007)
	Marburg	E	(Kolesnikova *et al.*, 2004)
	Chikungunya	E	(Akahata *et al.*, 2010)
Avian			
Fibroblasts ELL-0:	Nipah-NDV	E	(McGinnes *et al.*, 2010)
Insect			
Spodoptera frugiperda:	Influenza	E	(Pushko *et al.*, 2005)
	RSV-Influenza M1 protein*	E	(Quan *et al.*, 2011)
	HIV	NE	(Gheysen *et al.*, 1989)
	NV	NE	(El-Kamary *et al.*, 2010)
	RV	E	(Zeng *et al.*, 1994)
	HCV	E	(Baumert *et al.*, 1998)
Trichoplusia ni:	HPV	NE	(Harper *et al.*, 2004)
	HEV	NE	(Li *et al.*, 2000)
Plant			
Nicotiana tabacum:	HBV (HBcAg)	NE	(Pniewski, 2012)
	HIV	NE	(Rosales-Mendoza *et al.*, 2012)
	Bacillus anthracis-CPMV*	NE	(Phelps, *et al.*, 2007)
Nicotiana benthamina	Influenza – TMV*	NE	(Petukhova *et al.*, 2014)
	Rabies – A1MV*	NE	(Yusibov *et al.*, 2002)
	FMDV – TMV*	NE	(Wu *et al.*, 2003)
Arabidopsis thaliana	HIV/HBV (HBsAg)*	NE	(Greco *et al.*, 2007)
Yeast			
Pichia pastoris	HBV (HBsAg)	NE	(Lünsdorf, *et al.*, 2011)
	NV	NE	(Tomé-Amat *et al.*, 2014)
	HCV	E	(Acosta-Rivero *et al.*, 2001)
	DENV (Dengue virus)	E	(Tang *et al.*, 2012)
Saccharomyces cerevisiae:	HPV	NE	(Woo *et al.*, 2008)
	HIV	NE	(Sakuragi *et al.*, 2002)
	RV	E	(Rodríguez-Limas *et al.*, 2011)
Hensuela polymorpha:	HBV (HBsAg)	NE	(Seo *et al.*, 2008)
Bacteria			
Escherichia coli:	HCV-PapMV*	NE	(Denis *et al.*, 2007)
	P. falciparum-HBcAg*	NE	(Sällberg *et al.*, 2002)
Lactobacillus casei:	HPV	NE	(Aires, *et al.*, 2006)

E – enveloped, NE – non-enveloped.

(Table I). Cultured cells originating from *Nicotiana benthamina* and *N. tabaccum* are widely used plant cells to generate VLPs (Scotti and Rybicki, 2013).

Bacteria, the most widely used expression system for recombinant proteins are not the host cell of choice for VLPs synthesis, due to the absence of post-translational modifications, imperfect disulfide bond formation, and problems with solubility (Zeltins, 2013). Nevertheless, bacteria are used to produce non-enveloped VLPs, often by means of a recombinant bacteriophage with

foreign epitopes fused to surface proteins (Tissot *et al.*, 2010). Another strategy relies on prokaryotic expression of viral protein in the form of insoluble inclusion bodies, followed by purification under denaturing conditions, refolding, and *in vitro* self-assembly (Sánchez-Rodríguez *et al.*, 2012). Even though *E. coli* remains the most commonly used bacterial host for VLP production, some groups have successfully produced VLPs in *Lactobacillus* (Aires *et al.*, 2006) and *Pseudomonas* (Phelps *et al.*, 2007) host species.

VLPs may also be formed by proteins produced in cell-free expression systems and assembled *in vitro*. Further modification of this approach is to incorporate expressed proteins into virosomes, which are also categorized as VLPs by some authors. Virosomes, defined as synthetic lipid or polypeptide scaffoldings decorated with viral antigens, were evaluated as vaccine candidates as early as in the 1970s (Almeida *et al.*, 1975, Morein *et al.*, 1979). Two examples of licensed human virosome vaccines are Epaxal and Inflexal, which are used for hepatitis A and influenza prophylaxis, respectively. The critical change in virosome production is that currently proteins of interest are expressed in a system of choice and then incorporated into a liposome or other carrier. Initially, viral antigens originated from infectious viruses, which were inactivated, dissociated, and fractionated before processing.

Applications

VLPs have a broad range of potential applications due to the diversity and multigenicity of viruses. As shown with the examples provided above, these particles may be formulated not only of proteins originating from one virus species, but also of proteins from unrelated viruses or even different pathogens, *i.e.,* bacteria and protozoa. Chimeric VLPs are of special interest as highly specific nanovectors for the delivery of antigens or therapeutic molecules.

VLPs as vaccines

Due to their virus-like appearance and repetitive surface structure, VLPs show high immunogenicity and antigenicity. Importantly, they interact with the immune system similarly to their parental pathogens, inducing not only humoral but also cellular response, which is not common for traditional, subunit vaccines. B cell activation and the production of high antibody titers have been shown to be dependent on the conformation and epitope abundance of symmetric VLPs (Buonaguro *et al.*, 2011). Moreover, VLPs, as exogenous antigens, are efficiently taken up by professional antigen-presenting cells (APC), particularly dendritic cells (DC), which is followed by antigen processing and presentation by MHC class II molecules, leading to stimulation of CD4+ T helper cells. On the other hand, VLPs, similarly to native viruses, are also processed in the cytosol of DC as endogenous antigens and presented by MHC class I molecules to cytotoxic CD 8+ T cells (CTLs) (Grgacic and Anderson, 2006, Groothuis and Neefjes, 2005). This cross-presentation mechanism ensures comprehensive and strong immunological responses. Additionally, numerous VLP vaccines candidates are known to possess "self-adjuvant" properties, probably due to their particulate structure and small size of (~100 nm), which favors uptake by APC (Keller *et al.*, 2010, Manolova *et al.*, 2008). Potentially phasing-out adjuvants in VLPs vaccine formulations is unquestionably beneficial as it would both simplify the production process and increase the safety of the vaccine.

Of the four VLP vaccines currently on the market, those for HBV and HPV are the best described (Buonaguro, *et al.*, 2011) (Table II). The HBV VLPs contains virus surface antigen (HBsAg) and was the first VLPs vaccine to be generated (in 1980s) (Zuckerman, 1980, Zuckerman, 1985). Its successful production was reported in mammalian (Aden *et al.*, 1979) yeast (Valenzuela *et al.*, 1982), insect (Takehara *et al.*, 1988), and plant (Mason *et al.*, 1992) expression systems. Afterwards, HBV VLPs based on the core antigen (HBcAg-VLPs) were also developed. However, these are still in the preclinical stage of evaluation and are mainly used as a platform for the presentation of foreign epitopes from heterologous pathogens or tumor antigens (Kazaks *et al.*, 2008).

For HPV prophylaxis, VLPs are formed with L1 HPV capsid protein overexpressed in *S. cervisiae* or *T. ni* is used to prepare the licensed vaccine, but HPV VLP formation was demonstrated also in *P. pastoris* (Hanumantha Rao *et al.*, 2011) and plant cells (Deleré *et al.*, 2014; Scotti and Rybicki, 2013). The HPV vaccine, which showed 98% efficacy in protecting individuals from HPV16/18 infections (which are responsible for more than 70% of invasive cervical cancers) became a milestone in cancer prevention (Deleré, *et al.*, 2014).

As for the influenza vaccine, which must be produced *de novo* each year, development, production, and approval times are critical. Conventional licensed influenza vaccines are prepared from embryonated chicken eggs, inoculated individually with virus strains selected for the season. Viruses are harvested from the allantoic fluid, chemically inactivated, concentrated, and purified to remove non-viral protein contaminants. The whole production process takes several months and is often hampered by the poor growth yield of some influenza strains (Reperant *et al.*, 2014). Therefore, the VLP-based

Table II
VLP vaccines and vaccine candidates.

VLP (protein)	Vaccine	Expression system	Development status
HBV (HBsAg)	Hepatitis B (*Engerix-B, Recombivax HB, GenHevacB, Euvax B, others…*)	Bacteria Yeast Insect Plant	On market (GlaxoSmithKline, Merck, Sanofi-Pasteur, LG Life Sciences)
HPV (L1)	Human papilloma virus (*Gardasil, Cervarix*)	Yeast Insect	On market (Merck, GlaxoSmithKline)
HEV (HE antigen)	Hepatitis E (*Hecolin*)	Bacteria	On market (Xiamen Innovax Biotech)
Influenza virus	Influenza virus (*Flublok*)	Insect	On market USA (Protein Science Corporation)
Influenza RSV Norovirus HIV Parvovirus B19 Rabies Malaria (chimeric)	 *Malarivax*	Plant, insect Insect Cell free, insect, plant Yeast Insect Plant Bacteria	 Clinical studies

vaccines which are faster, cheaper and safer to obtain, represent an attractive alternative (Fig. 2). An influenza VLPs vaccine produced in insect cells was approved in US in 2013, but other influenza VLP candidates, produced in plant cells, bacteria, or cell-free systems, demonstrated as effective and safe and are currently in clinical trials (Chroboczek *et al.*, 2014; Lee *et al.*, 2014).

Other VLP vaccines that are currently in clinical trials utilize proteins from different viruses, including respiratory syncytial virus (Glenn *et al.*, 2013), Norwalk virus (Herbst-Kralovetz *et al.*, 2010), human parvovirus B19 (Chandramouli *et al.*, 2013), and *P. falciparum* (chimeric VLP) (Agnandji *et al.*, 2012). VLPs vaccine candidates for human use, that were shown to be immunogenic in animal models but are still under preclinical investigation include: human immunodeficiency virus (HIV-1), severe acute respiratory syndrome coronavirus (SARS-CoV), hepatitis C virus (HCV), RSV, rotavirus (RV), dengue (DENV), rabies (Kushnir, *et al.*, 2012; Roldão *et al.*, 2010), and others.

Veterinary vaccinology is a distinct, broad field, which will not be discussed in this review, but VLPs

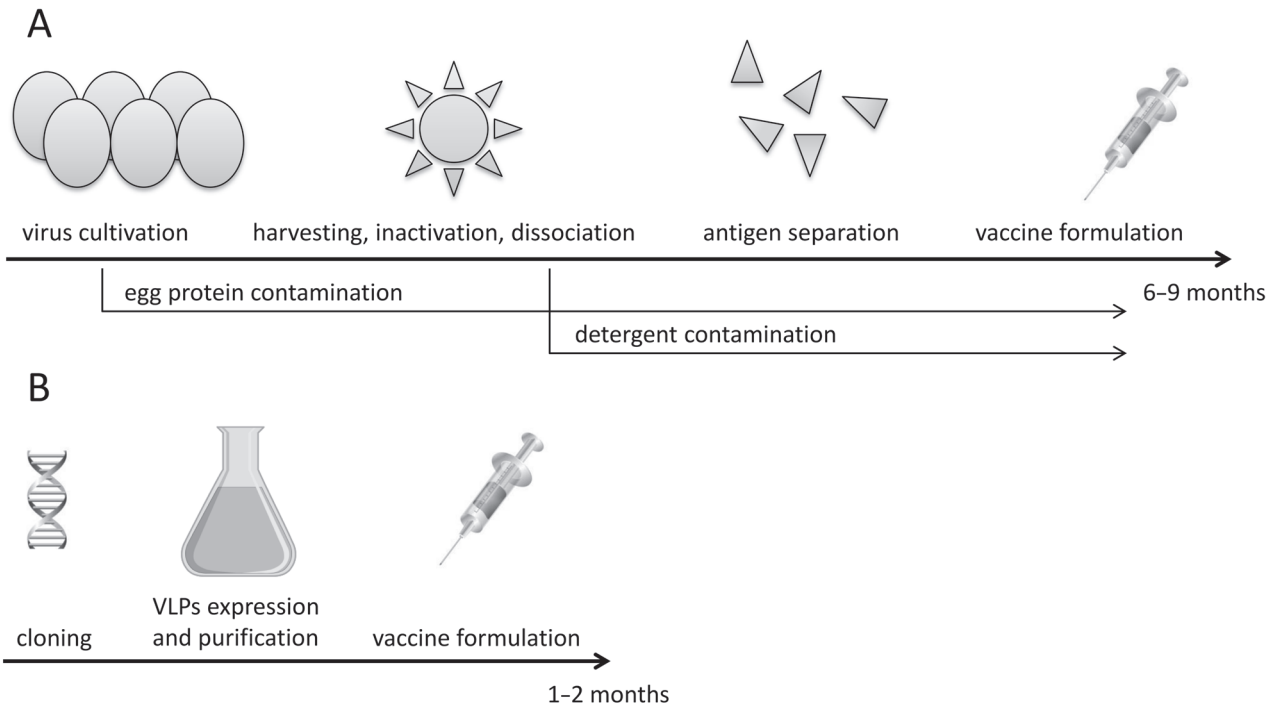

Fig. 2. Comparison of vaccine production using traditional virus cultures (panel A) and VLPs (panel B).

have also been investigated as vaccine candidates for animal use (Liu *et al.*, 2012).

As mentioned above, the plasticity of VLPs enables the formulation of chimeric vaccines loaded with heterologous antigens. This can be achieved either by co-expression of structural proteins of different viruses or by gene fusion, and results in foreign epitopes displayed on the external surface of the VLPs. The first strategy can be illustrated by the SARS-CoV VLP, where SARS spike protein is incorporated into an influenza matrix 1 protein scaffold, overcoming the problem of low yield in the production of the native SARS VLPs (Liu *et al.*, 2011). Using the second approach the synthesis of particles that do not form native VLPs at all, becomes feasible. An anti-malaria vaccine, currently in clinical trials, is an example of a VLP displaying a non-viral antigen (S protein of *P. falciparum*) that is expressed as a fusion partner with HBsAg (Agnandji *et al.*, 2012).

Alternatively, VLPs may be engineered to modulate the immune response by conjunction of adjuvants or stimulatory molecules such as Toll-like receptors (TLR) ligands and T-cell epitopes. For example, VLPs containing CpG motifs, a ligand for TLR-9, have significantly improved anti-HPV VLP humoral responses in the peripheral blood and in genital mucosal secretions in animal models (Gerber *et al.*, 2001; Hjelm *et al.*, 2014). Similarly, HIV VLPs equipped with cholera toxin subunit B showed increased stimulation of mucosal cellular response (Kang *et al.*, 2003; Ludwig and Wagner, 2007). Another VLP developed to co-deliver interleukin-2 resulted in robust cytotoxic T-lymphocyte activation (Juarez *et al.*, 2012; Oh *et al.*, 2004). In addition to enhancing the immune response, including immunity potentiators, improves the pharmaco-kinetic profile of, VLPs reducing nonspecific binding to serum proteins.

Vaccines for non-infectious diseases

Vaccines for non-infectious diseases are an interesting application for chimeric VLPs, which are designed to induce specific antibodies directed against self-antigens and thus boost humoral immunity. These VLPs carry antigens typical for cancer (melanoma), neurodegenerative diseases (Alzheimer's disease), autoimmune diseases (allergic rhinoconjunctivitis and asthma) and other disorders (Table III). Most therapeutic VLP vaccines are based on the bacteriophage Qβ, which is highly expressed in *E. coli*, enabling the development of an economical large-scale production process. Importantly, during self-assembly, bacteriophage Qβ packages host-derived RNA (the natural ligand for TLR7 and TLR8), thus activating antigen-specific IgG2a responses (Bachmann and Jennings, 2011).

VLPs as delivery platforms

The remarkable and unique advantage of VLPs as delivery vehicles is theirs specificity, which results from the receptor-mediated transduction mechanism. Similar to native viruses, most VLPs target and penetrate different cell types depending on receptors exposed at their surface. As a consequence, the diverse tropisms of parental viruses provide a range of natural targeting capabilities for VLPs, without the need for further modifications (Seow and Wood, 2009).

Decorating VLPs with foreign molecules may be achieved by various means, including genetic manipulation, non-covalent interactions, or chemical coupling of different compounds. Physical encapsulation of organic and non-organic moieties has been described as well and consists of the disassembly of purified VLPs (by osmotic shock, use of chelating agents, or varying the pH) and reassembly in the presence of the desired molecule. Table IV summarizes VLPs packaging methods and examples of cargo molecules that have been successfully delivered to target cells.

Delivering drugs into target cells is particularly important for anti-proliferation chemotherapeutics, where systemic administration causes undesired side-effects. Therefore, specific carriers, such as VLPs, are of significant interest in this field. Successful transfer of VLPs packaged with anticancer agents was described for bleomycin (BLM) and doxorubicin (DOX). Both conjugates were chemically coupled to VLPs (BLM to adenoviral VLPs and DOX to RV VLPs) and have dem-

Table III
Therapeutic VLP vaccines.

VLP	Therapeutic target	Status	Ref
Qβ-amyloid β epitope	Alzheimer disease	Phase 2	(Chackerian *et al.*, 2006)
Qβ-CpG oligodeoxynucleotides	Allergic rhinoconjunctivitis and asthma	Phase 2	(Senti *et al.*, 2009)
Qβ-IL1β protein	Diabetes mellitus type 2	Phase 2	(Bachmann and Jennings, 2011)
Qβ-angiotensin II epitope	hypertension	Phase 2	(Tissot *et al.*, 2008)
Qβ-melan A peptide	Malignant melanoma	Phase 2	(Braun *et al.*, 2012)
Qβ-nicotine	Nicotine addiction	Phase 2	(Maurer *et al.*, 2005)
HPV-TNFα	Arthritis	–	(Chackerian *et al.*, 2001)

Table IV
VLPs as delivery platforms for foreign molecules (PyV: Polyoma virus).

Cargo	VLP	Attachment	Ref.
Proteins			
Antibody	PyV	genetic fusion to VLP protein	(Gleiter and Lilie, 2003)
GFP	RV	genetic fusion to VLP protein	(Charpilienne *et al.*, 2001)
MBP tag	AdV	protein adaptor	(Garcel *et al.*, 2006)
Enzyme	AdV	biotin-streptavidin interaction	(Fender *et al.*, 2003)
Cytokine (IL-2)	HPV	encapsulation	(Oh, *et al.*, 2004)
Nucleic acids			
Plasmid DNA	HPV	encapsulation	(Malboeuf *et al.*, 2007)
RNAi	PyV	encapsulation	(Kimchi-Sarfaty, *et al.*, 2003)
Metal nanoparticles			
Gold	AdV	encapsulation	(Fuschiotti, *et al.*, 2006)
Magnetic nanoparticles	HBV	encapsulation	(Pushko *et al.*, 2013)
Fluorophore			
Carboxyfluorescein	HPV	chemical coupling	(Bergsdorf *et al.*, 2003)
Propidium iodode	PyV	chemical coupling	(Goldmann *et al.*, 2000)
Quantum dots	PyV	chemical coupling	(Wojta-Stremayr and Pickl, 2013)
Drugs			
Bleomycin	AdV	chemical coupling	(Zochowska, *et al.*, 2009)
Doxorubicin	RV	chemical coupling	(Zhao, *et al.*, 2011)

onstrated improved drug bioavailability and growth inhibition of targeted cancer cells (Zhao *et al.*, 2011; Zochowska *et al.*, 2009).

The ability to bind nucleic acids is common for viral structural proteins and is used to pack oligonucleotides and plasmids (as well as other negatively charged substances) into VLPs (Zeltins, 2013). DNA-loaded VLPs were developed for gene therapy and aroused great hope as potential specific carriers of nucleic acids (Petry *et al.*, 2003, Ramqvist *et al.*, 2007). Successful expression of β-galactosidase, encoded by a plasmid delivered by a polyomavirus VP1 VLP to different tissues, demonstrated the feasibility of the VLP-mediated gene therapy concept (May *et al.*, 2002). Up to 17 kb of DNA may be packaged into an SV40 polyoma VLP, as reported by Kimchi-Sarfaty (Kimchi-Sarfaty *et al.*, 2003), but the theoretical capacity of larger VLPs is probably higher. In subsequent years, the increasing interest in use of modified small interfering RNAs as therapeutics resulted in further studies on VLPs as delivery vessels for these molecules (Seow and Wood, 2009). For example, treatment of lupus-prone mice with bacteriophage MS2 VLPs loaded with miR-146a miRNA – a recognized gene silencer – inhibited the production of auto-antibodies and inflammatory cytokines (Pan *et al.*, 2012).

Protein delivery by VLPs has been described for heterologous antigens, antibodies, cytokines, enzymes, and reporter proteins such as green fluorescent protein (GFP) or maltose binding protein (MBP). Cargo proteins are usually tethered to the external surface of the VLP by genetic fusion to structural VLP protein or by exploiting natural interactions between the transported protein and structural proteins present in VLPs (antigen-antibody interactions, ligand-receptor interactions, inter-domain interactions *etc.*). Conjunction of Herceptin, an antibody directed against a mutated version of the tyrosine kinase receptor HER2 to the polyoma VLP is a good illustration of the potential use of VLPs in directed cancer immunotherapy (Gleiter and Lilie, 2003). Another example of a protein targeting ligand is epidermal growth factor (EGF), which was incorporated into SV40 polyoma VLPs, that selectively transduced cells overexpressing the EGF receptor (Kitai *et al.*, 2011).

Labeling VLPs with fluorophores is another way of tethering foreign substances to these nanovectors but also a powerful research tool. Fluorescent VLPs can be traced within host cell, tissue, or organism. Viral particles can be labeled by covalent or non-covalent chemical methods or by fusion of fluorophores to proteins integrated into the viral particle. Chemical methods consist of inserting fluorescent dyes into the viral membrane. Alternatively, attachment of streptavidin-coupled fluorophores can be achieved by biotinylating target structures of interest (Wojta-Stremayr and Pickl, 2013).

The feasibility of encapsulating metal nanoparticles within the VLP cavity was shown by packaging adenovirus (AdV) VLPs with nanogold molecules (Fuschiotti *et al.*, 2006). Recently, an interesting application

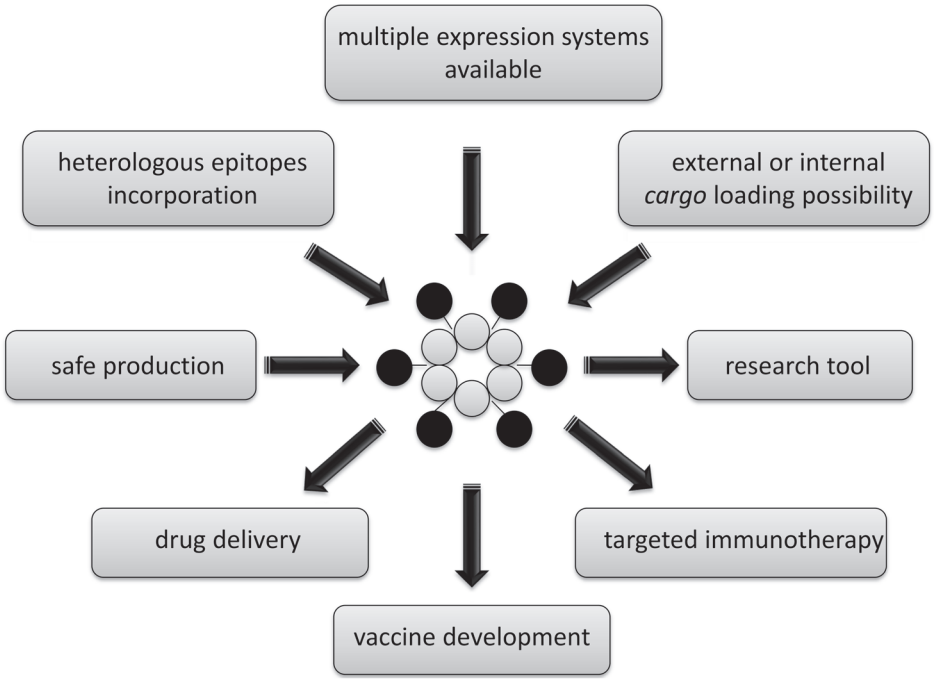

Fig. 3. Diagram showing possible VLP applications.

for another functionalized VLPs has been described providing proof of concept for the use of VLPs for diagnostic purposes. Cowpea chlorotic mottle virus (CCMV) VLP loaded with Gd^{3+} ions or HBcAg-VLPs loaded with Fe_3O_4 serve as paramagnetic high contrast agents for magnetic resonance imaging (Mateu, 2011, Shen *et al.*, 2014).

Even though VLPs seem to be universal delivery platforms, they do possess several limitations, especially to their use as gene or drug carriers. The most challenging problem concerns the pre-existing immunity in most individuals for common viruses. A solution proposed to overcome undesirable immunogenicity of VLPs consists of modifying them with immune masking agents, such as polyethylene glycol (PEG), which has been shown to suppress the primary antibody response (Jain and Jain, 2008). Another concern that must be kept in mind is that like any virus-based treatment, VLPs are not suitable for repetitive administration. The exception here is cancer immunotherapy, where the goal is to stimulate tumor recognition in an escalating manner. Initial enthusiasm for VLP-mediated gene therapy has been tempered by the lack of large-scale methods for adequate internalization and proper release of the desired nucleic acid, and further research will be necessary to overcome this problem (Pattenden *et al.*, 2005). It is also worth noting, that the different mechanism of assembly of non-enveloped and enveloped VLPs impede *in vitro* encapsulation of foreign molecules in the latter.

Concluding remarks

Virus-like particles have been produced for a wide range of taxonomically and structurally distinct viruses and have unique advantages in terms of construction flexibility. Moreover, their versatility relies on their unique structural diversity and tailorable functionality. Potential uses of VLPs include vaccine production, vectors for gene therapy and targeted drug delivery, and molecular imaging tools (Fig. 3). However, translation from preclinical research to licensed products requires extensive studies in animal models that remain to be completed. Firstly, effective and scalable loading of cargo onto VLPs vectors requires further investigation. Futhermore, careful evaluation of the toxicity, biodistribution, stability *in vivo*, and clearance of VLPs will be crucial in order for these nanocarriers to be considered for medical use.

Acknowledgments

This work was supported by a Fuga grant from the National Science Center (AN; UMO-2013/08/S/NZ6/00730) and a LIDER grant from the National Centre for Research and Development (KP; Lider/27/55/L-2/10/2011). The Faculty of Biochemistry, Biophysics and Biotechnology at Jagiellonian University is a beneficiary of structural funds from the European Union (grant no: POIG.02.01.00-12-064/08 – "Molecular Biotechnology for Health"). The Facu lty of Biochemistry, Biophysics and Biotechnology is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 15–21

ORIGINAL PAPER

Epidemiology and Genotyping of Patients with Chronic Hepatitis B: Genotype Shifting Observed in Patients from Central Europe

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Submitted 25 September 2014, revised 4 December 2014, accepted 5 December 2014

Abstract

Background: Knowledge on HBV prevalence and genotype distribution in Europe still is hampered by lack of coherent data sampling, small numbers of patients studied so far, and also modern times migration which influences both parameters in a quite dynamic manner. To find out whether HBV prevalence and genotype distribution has undergone any significant changes over the past decades, we have analyzed our cohort of HBV patients. Methods: Retrospective analysis of virological data and correlation with the epidemiological backgrounds of 408 chronically HBV-infected patients, followed in the year 2009 at Tübingen Virus Hepatitis Center, Germany. Results: A background of migration was found in more than 80% of our HBV patients, displaying an origin from 41 different countries. Analysis of the genotypes revealed that genotype A predominated only among patients from Central Europe with 55.8% while genotype D, known to be most common worldwide, was most prevalent in patients born in Eastern and Southern Europe, Central Asia and Middle East, exhibiting a range from 81% to 94%. In Central Europe, genotype A was particularly seen in older patients as compared to genotype D that predominated in the younger patients. Conclusions: These data suggest that Central Europe is straight on its way to switch from genotype A to genotype D. One reason for this significant shift may be related to the ongoing European and global migration flow.

Key words: chronic hepatitis B, epidemiology, genotype, HBV, migration background

Introduction

Worldwide, over 360 million people suffer from a chronic course of hepatitis B virus (HBV) infection. Beyond that, more than 2 billion people are estimated to have experienced contact with HBV (World Health Organization, 2009). Due to the potential risk of developing liver cirrhosis or hepatocellular carcinoma, chronic hepatitis B constitutes a serious health burden (World Health Organization, 2009; Robert Koch Institut, 2013a).

Interestingly, great differences in HBV prevalence and in HBV genotypes are observed worldwide: in African and Asian countries, a high rate of HBV prevalence (> 8%) is found, whereas much lower rates (< 2%) are documented for European and Northern American countries (World Health Organization, 2009; Robert Koch Institut, 2013a). Furthermore, distinct regions exhibit a dominance of distinct HBV genotypes: in Central Europe and also for a long time in North America countries, genotype A had been found to be predominant. In contrast, non A genotypes are predominant in the other world regions: genotypes B and C in Eastern and Southeastern Asia, genotype E in Western Africa. Furthermore, genotype D is spreading worldwide (Norder *et al*., 2004; Schaefer, 2005). Besides their epidemiological importance, HBV genotypes may also cause differences in the progression and the outcome of chronic hepatitis B (Schaefer, 2005; Lin and Kao, 2011; Araujo *et al*., 2011) and influence the choice of treatment regimes. In this respect, especially genotype A patients have been suggested to preferably benefit from the application of interferon α2 (Lin and Kao, 2011; Cornberg *et al*., 2011; European Association for the Study of the Liver, 2012; Flink *et al*., 2006).

One reason for low prevalence rates in Central Europe are recommendations from both the WHO and national organizations such as the STIKO (German Standing Committee on Vaccination) for immunization of children as early as in 1992 and 1995, respectively

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(Robert Koch Institut, 2012). These resulted in significantly increasing rates of HBV vaccination already in children entering school in Germany (2001: 57.2%, 2011: 86.2%) (Robert Koch Institut, 2013a; 2013b). However, it is surprising that despite this highly successful vaccination program the prevalence of chronic hepatitis B in Germany hardly decreased in recent years and still remains at a fairly high rate of 0.6% (Thierfelder *et al.*, 2001). Therefore, other reasons must contribute to this more or less steady state pattern of HBV prevalence in Germany.

To gain more insight in the epidemiology and the origin of chronic hepatitis B in Central Europe, as recently demanded by the German Robert Koch Institute (RKI) (Robert Koch Institut, 2013a), we performed a single center analysis in chronic hepatitis B infected patients, monitored 2009 at our Virus Hepatitis Center of the University Hospital Tübingen, Germany.

Experimental

Materials and Methods

In the year 2009, a total of 408 patients presented with chronic hepatitis B infection at our Virus Hepatitis Center at the University Hospital Tübingen, Germany. All 408 study cohort patients were analyzed retrospectively with respect to virological, epidemiological and geographical parameters. Data were collected after the respective outpatient contact using the patient files.

All countries of birth were geographically classified in 7 different areas: Central Europe (encompassing Germany, Poland, and the Czech Republic; please note: other Central European countries could not be taken into account due to a lack of patients from there in our study cohort), Southern Europe, Eastern Europe with Russia, Middle East with Turkey, Central Asia, Eastern and Southeastern Asia, and Africa.

In our study cohort, treatment-naïve hepatitis B viral load could be assessed retrospectively in 318 patients using the patient files. HBV genotypes could be determined retrospectively in 276 patients using the patient files. The genotyping was done either by genome sequencing or by DNA Line Probe Assay (INNO-LiPA HBV Genotyping, Innogenetics, Hannover, Germany) (Guirgis *et al.*, 2010). As expected, genotype analysis did not yield results in cases with very low viral loads (< 500 IU/mL).

To obtain evidence of age-related phenomena as a surrogate for changes over time, the 276 genotyped study cohort patients were assigned to three age groups: (i) < 30 years (n = 91), (ii) 30–45 years (n = 100), (iii) > 45 years (n = 85), respectively.

The non-parametric Mann-Whitney-Wilcoxon U test was performed to statistically analyze the age distribution in different patient groups. *P*-values < 0.05 were considered to be statistically significant.

Anonymised data were collected retrospectively, no parameter was determined in addition to the standard outpatient setting. According to the local ethics committee of the University Hospital Tübingen, Germany, no ethics approval is required for this retrospective analysis with cross sectional design.

Results

All 408 study patients, that presented at our Virus Hepatitis Center in the year 2009, were characterized as shown in Table I. Treatment-naïve hepatitis B viral load could be assessed retrospectively in 318 patients with a median viral load in HBeAg-positive patients of 1×10^6 IU/ml and a median viral load in HBeAg-negative patients of 2×10^3 IU/ml.

Table I
Characteristics of Tübingen chronic hepatitis B study cohort.

Tübingen study cohort	
Evaluated patients, total number [n]: Male [n]: Female [n]:	408 255 (62.5%) 153 (37.5%)
Age at first contact to Tübingen Virus Hepatitis Center All patients [years]: Male [years]: Female [years]:	 median 37.0 (range 15–74) median 37.0 (range 15–74) median 36.5 (range 16–71)
Country of birth, assignable [n]:	400
HBV genotype, determinable [n]:	276
HBV genotype & country of birth, definable [n]:	269

In our Tübingen study cohort of chronically HBV infected patients, countries of birth could be identified in 400 of the 408 patients. On this basis, 41 different countries of origin were identified and assigned to seven major geographic areas. Individual countries together with the respective number of patients and the assignment to our classified geographic areas are given in detail in Table II. Of note, 81% of our HBV patients were born outside of Germany. Additionally, also 18 of the 76 patients born in Germany were found to have a background of migration due to the foreign origin of their parents (second generation immigrants). Thus, an overall migration background was identified in more than 85% of our Tübingen study cohort patients.

In 276 of the 408 patients (67.6%) the HBV genotype could be identified respectively. Genotype D was found to be most frequent, genotype A was found to be the second most frequent one. In detail, the distribu-

Table II
Geographic origin of patients from Tübingen chronic hepatitis B study cohort [n = 400]

Geographic groups	Countries of birth	Patients [n]	Percentage [>1%]
Central Europe [n = 85]	Germany	76	19.0
	Poland	8	2.0
	Czech Republic	1	
Southern Europe [n = 82]	Greece	24	6.0
	Italy	22	5.5
	Kosovo	14	3.5
	Croatia	9	2.25
	Bosnia	6	1.5
	Serbia	4	
	Albania	3	
Eastern Europe with Russia [n = 45]	Russia	20	5.0
	Romania	16	4.0
	Ukraine	3	
	Byelorussia	2	
	Georgia	2	
	Moldova	2	
Middle East with Turkey [n = 81]	Turkey	77	19.25
	Iran	1	
	Lebanon	1	
	Palestine	1	
	Syria	1	
Central Asia [n = 60]	Kazakhstan	41	10.25
	Kyrgyzstan	9	2.25
	Uzbekistan	7	1.75
	Tajikistan	3	
Eastern and Southeastern Asia [n = 31]	Vietnam	13	3.25
	China	9	2.25
	Thailand	4	
	Malaysia	1	
	Philippines	1	
	South Korea	1	
	Sri Lanka	1	
	Taiwan	1	
Africa [n = 16]	Ghana	5	1.25
	Cameroon	3	
	Ethiopia	2	
	Nigeria	2	
	Congo	1	
	Sierra Leone	1	
	Somalia	1	
	Togo	1	

tion of the HBV genotypes in our cohort is shown in Table III. In the remaining 132 cases, the genotype was not determinable due to a low viral load.

In 269 of the 408 patients both origin (country of birth) as well as the respective genotype could be determined (Table IV). Our results demonstrate that the distribution of HBV genotypes is strictly related to the typology of immigration and goes along with the pattern of geographic origin. This is underlined by our finding that only in Central European patients genotype A was found to predominate (55.8%), whereas genotype D was second most frequent (38.5%). These

Table III
Distribution of HBV genotypes in the Tübingen study cohort [n = 276]

Genotype:	Patients		Age [median, range]
	[n]	[%]	
A	55	19.9%	39 years (15–67 years)
B	7	2.5%	43 years (16–52 years)
C	18	6.5%	33 years (18–47 years)
D	188	68.1%	35 years (15–74 years)
E	6	2.2%	31 years (23–47 years)
F	1	0.4%	n. d.
G	0	0%	n. d.
H	1	0.4%	n. d.

n.d.: not determined

type D [n = 20] (33 years, range 21–74 years) did not differ much from the median age of non Central European patients with genotype D [n = 168] (35.5 years, range 15–64 years) (Fig. 1). Patients born in Eastern Europe with Russia, Southern Europe, Central Asia, and Middle East with Turkey, were found to be predominantly infected with HBV genotype D, patients born in Eastern and Southeastern Asia were predominantly infected with HBV genotype C, and patients born in Africa were predominantly infected with HBV genotype E, as shown in Table IV.

In the next step of our data analysis, a more refined examination was conducted in our patients exhibiting the two most dominant genotypes A (n = 55) and D (n = 188). We specifically focused on the question

Table IV
Correlation of country of birth and HBV genotype [n = 269]. The regions were assigned as shown in Table II.

Region of birth	Patients [n]	Most frequent genotype [n, %]	Second most frequent genotype [n, %]
Central Europe	52	**A** 29 [55.8%]	D 20 [38.5%]
Eastern Europe / Russia	37	**D** 30 [81.1%]	A 7 [18.9%]
Southern Europe	53	**D** 45 [84.9%]	A 7 [13.2%]
Central Asia	41	**D** 37 [90.2%]	A 4 [9.7%]
Middle East / Turkey	52	**D** 49 [94.2%]	A 2 [3.8%]
Eastern / Southeastern Asia	25	**C** 16 [64.0%]	B 4 [16.0%]
Africa	9	**E** 6 [66.7%]	A 2 [22.2%]

genotype A patients from Central Europe [n = 29] (Table IV) exhibited a median age of 45 years (range 16–62 years) whereas genotype A patients with non Central European origin [n = 26] displayed a median age of only 30 years (range 15–67 years) (Fig. 1). Thus, Central European patients with genotype A were found to be significantly older than genotype A patients with a non Central European origin (p = 0.011). In contrast, the median age of Central European patients with geno-

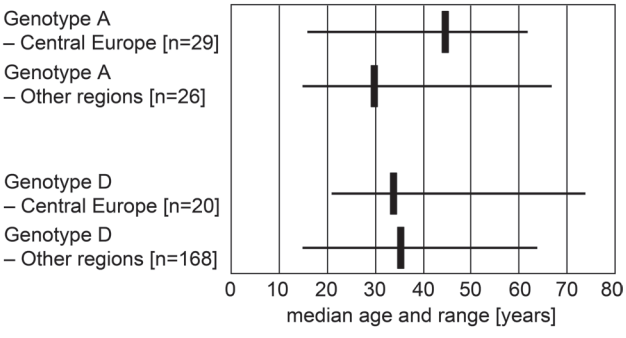

Fig. 1. Correlation of median age and origin of birth in patients with HBV genotypes A [n = 55] and D [n = 188]. Genotype A patients born in Central Europe were found to be significantly older then genotype A patients born in other regions (p = 0.011). In contrast, genotype D patients showed no difference in the median age concerning their region of origin. Other genotypes are not depicted because of low prevalences.

whether an "HBV genotype shifting" might have taken place over the last two decades potentially resulting from HBV vaccination programs being installed in Central European countries since the mid-1990s and from the ongoing European and global migration flow. For this purpose, we first assigned all genotyped study patients (n = 276) to our three age groups and the genotype A patients could be assigned as follows: (i) age group < 30 years: 15 patients (16.5% of all genotyped patients < 30 years), (ii) age group 30–45 years: 18 patients (18.0% of all genotyped patients 30–45 years), (iii) age group > 45 years: 22 patients (25.9% of all genotyped patients > 45 years).

We then also compared the frequency of genotypes A and D in patients born in Central and non Central European countries with respect to the assignment to our three age groups (Fig. 2). In Central European patients, genotype A patients (n = 29) were assigned as follows: (i) age group < 30 years: 3 patients (20.0% of 15 patients), (ii) age group 30–45 years: 11 patients (68.7% of 16 patients), (iii) age group > 45 years: 15 patients (71.4% of 21 patients) (Fig. 2, grey bars). In the non Central European genotype A patients (n = 26), age dependent distribution was: (i) age group < 30 years: 12 patients (15.8% of 76 patients), (ii) age group 30–45 years: 7 patients (8.3% of 84 patients), (iii) age

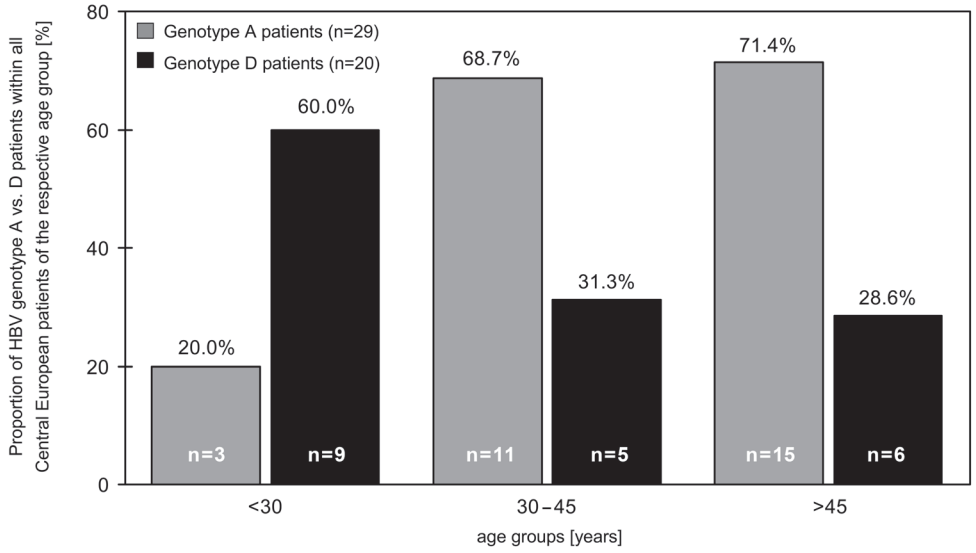

Fig. 2. Genotype shift in 52 patients born in Central Europe from genotype A to genotype D. Age-dependent decrease of the proportion of HBV genotype A [n = 29] versus increase of the proportion of HBV genotype D [n = 20]. The remaining 3 of the 52 patients had other genotypes and were younger than 30 years. Interestingly, an over proportional amount of Central European genotype A patients (in the range of 70%) was seen in the patients ≥ 30 years of age.

group > 45 years: 7 patients (11.1% of 63 patients) (data not depicted in Fig. 2).

Conversely to this decline in the proportion of genotype A in Central European patients with decreasing age, genotype D gained importance in patients with decreasing age born in Central Europe as demonstrated by the sharp rise from 28.6% (> 45 years) and 31.3% (45–30 years) to 60.0% (< 30 years) (Fig. 2, black bars). Beyond that, also other genotypes seem to come up in the age group < 30 years: two male patients with genotype C and one male patient with genotype F were identified in this patient group. Taken together, these data indicate a significant genotype shifting in Central European patients with chronic hepatitis B.

Discussion

We present here the data of our single center analysis on a large cohort of chronic HBV infected patients who presented in 2009 at our Virus Hepatitis Center at the University Hospital Tübingen, Germany.

Importantly, our monocentric Tübingen data first of all reflect the well-documented migration flow into many developed countries in Central Europe and elsewhere, especially coming from high endemic areas for HBV (Robert Koch Institut, 2013a): more than 80% of our patients were identified as foreigners and more than 85% exhibited a background of migration (first plus second generation immigrants). As compared with two other recent German studies (analyzing either 250 patients in the German Ruhr area (2001–2006) (Niederau, 2007) or 1,535 patients with a viral load > 2,000 IU/ml (2004–2007) (Fischer *et al.*, 2012)), the migration background has increased considerably from 63.1% (Fischer *et al.*, 2012) and 67.6% (Niederau, 2007) to our Tübingen study cohort rate of now more than 85% (Table II). Especially the number of immigrants from the former Soviet Union and its satellite states increased remarkably from low levels of 11.2% (Niederau, 2007) and 17.2% (Fischer *et al.*, 2012) to a percentage as high as 37.5% in our study cohort (Table II). Concomitantly, the number of patients with a Turkish background decreased from 34.8% (Niederau, 2007) and 22.4% (Fischer *et al.*, 2012) to 19.3% in our study cohort (Table II).

Our data further imply that in most instances HBV infection has been acquired in the respective native countries of the immigrants. Thus, genotype D, being known as the most frequent genotype worldwide (Norder *et al.*, 2004), was also found to be highly prominent in our Tübingen patients, representing 68.1% of our study cohort (Table III). Genotype A was present in the subgroup with Central European origin in a high percentage (55.8%), while patients born in Eastern or Southeastern Asia mostly exhibited genotypes B or C. In the patients born in Africa, genotype E predominated (Table IV). These findings match similar data being obtained in the respective countries and regions (Norder *et al.*, 2004; Schaefer, 2005).

When correlating the distribution of genotypes A and D with different age groups of our study cohort, an interesting age-dependent shift was observed: genotype A patients born in Central Europe were significantly older than the respective genotype A patients born in other regions (Fig. 1). Additionally, for the

whole study group a decrease in the proportion of genotype A patients was observed age-related so that more and more younger patients had to be identified as "non genotype A" patients. This could be referred to the fact, that genotype A was overrepresented in older patients born in Central Europe and thus the genotype A predominated only in the patients born in Central Europe in the age groups of 30 years and older (Fig. 2, grey bars to the right).

An overall decline in HBV prevalence in the last decades and thus specifically also in genotype A prevalence is supposed to be due to the introduction of perioperative HBV diagnostics and the availability of a first vaccine in 1982 which later on led to the introduction of routine HBsAg screening in pregnancy in 1994 in Germany with consecutive active and passive immunization of infants (Mutterschafts-Richtlinien, 2012), and due to the general recommendation for HBV immunization of children in Germany in 1995 (Robert Koch Institut, 2012) (in response to the WHO resolution imposed in 1992).

Our data regarding the median age as well as the proportion of the distinct genotype subgroups provide some evidence for the hypothesis that young Central European patients are less frequently infected with genotype A. Thus, our data indicate that the autochthonous European genotype A infection probably will be displaced over time, while genotype D concurrently will close this gap. In younger Central European patients, a dominance of genotype D already is in place (Fig. 2, black bar to the left). As an obvious cause, this phenomenon seems to be due to (i) European HBV specific measures (diagnostics and vaccinations), sharply cutting down all autochthonous cases including the predominant genotype A, and due to (ii) the spread of imported genotypes other than A, reflecting the opening of Central European societies in an increasingly globalized world.

These data point out that the proportion of genotype A patients in our Tübingen study cohort might be constantly decreasing over time, irrespective of the origin of birth of our study patients. Thus, the proportion of genotype A patients was found to have decreased from a high level in the Central European patients in an above average manner (Fig. 2, grey bars), whereas in the non Central European patients the proportion of genotype A varied only little and remained at low rates (in the range of about 10%).

A similar replacement of the formerly most frequent genotype A by genotypes B and C already can be observed in Northern American countries, there being fueled mainly by the considerable migration inflow from Asian countries (exhibiting strong association with ethnicity), as recently published by Congly *et al.*, (2013). Thus, the global migratory flow may significantly modify also the Central European geographic distribution of HBV genotypes in the near future. Taking into account, that antiviral therapy with pegylated interferon α2 is most promising in patients with genotype A infection (Lin and Kao, 2011; Cornberg *et al.*, 2011; European Association for the Study of the Liver, 2012), the assumed shift of genotypes in Central Europe will possibly influence the proportion of interferon usage in patients with chronic hepatitis B infection.

However, the specific contribution of the diverse routes of HBV transmission for this shifting towards non A genotypes remain unclear. One possible explanation could be that infections with non A genotype hepatitis B viruses of unvaccinated take place preferably in adolescence (due to changing sexual and travel behaviors). A second explanation could be a transmission already in infancy (due to contacts with chronically infected playfellows, *e. g.* originating from abroad). A third way is the vertical mother to child transmission especially in cases born in Germany with a background of familial migration. In this context, it also has to be noted, that the rate of chronicity of HBV infections decreases with increasing age (World Health Organization, 2009). Thus, it is evident that a consequent and timely immunization of children and adult migrants as well as especially the treatment of highly viremic women in pregnancy (Cornberg *et al.*, 2011; European Association for the Study of the Liver, 2012) is of utmost importance to achieve an assured closing of these routes of HBV transmission. Screening for hepatitis B should be carried out even more consistently, particularly aiming at risk groups such as individuals with migration background, as recommended by the German Standing Committee on Vaccination, STIKO (Robert Koch Institut, 2012). In seronegative and susceptible individuals HBV immunization should be propagated consequently.

Competing Interest

The authors declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' Contributions

ALB was the treating physician, ALB and CF collected the data. ALB and CPB designed the study, analyzed the research quality and wrote the manuscript. CRW, UML and NPM critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 23–28

ORIGINAL PAPER

Species-Specific Identification of Human Adenoviruses in Sewage

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Submitted 21 August 2014, revised 10 December 2014, accepted 23 December 2014

Abstract

Human adenovirus (HAdV) diversity in sewage was assessed by species-specific molecular methods. Samples of raw sewage were collected in 14 sewage disposal systems from January to December 2011, in Poland. HAdVs were detected in 92.1% of the analysed sewage samples and was significantly higher at cities of over 100 000 inhabitants. HAdV DNA was detected in sewage during all seasons. The most abundant species identified were HAdV-F (average 89.6%) and -A (average 19.6%), which are associated with intestine infections. Adenoviruses from B species were not detected. The result of the present study demonstrate that human adenoviruses are consistently present in sewage in Poland, demonstrating the importance of an adequate treatment before the disposal in the environment. Multiple HAdV species identified in raw sewage provide new information about HAdV circulation in the Polish population.

Key words: adenoviruses' detection, sewage, species-specific identification

Introduction

Human adenoviruses (HAdVs) are members of the *Adenoviridae* family, group of medium-sized viruses characterized by a nonenveloped icosahedral nucleocapsid and a double stranded DNA genome. The *Adenoviridae* family consists of five genera, of which the genus *Mastadenovirus* includes human viruses classified into 57 serotypes clustered into 7 subgroups (A-G). Human adenoviruses have different organ tropisms, causing a wide variety of clinical manifestations including respiratory tract infections, acute conjunctivitis, cystitis, gastroenteritis and systemic infections in immunocompromised patients. Subgroup A, F and G species primarily infect the gastroenteric tract, but only types 40 and 41 (subgroup F) have been strongly associated with gastroenteritis. Enteric adenoviruses multiply in the gastrointestinal tract, and are excreted in large numbers in the faeces of infected persons (even more than 10^8 copies/g of stool). The main source of adenoviruses in the environment is human faecal matter. Viruses cannot replicate outside their host's tissues and therefore cannot multiply in the environment; however, they can survive in the environment for extended periods of time and have good tolerance for changing environmental conditions. HAdVs are extremely common in wastewater throughout the year, with concentrations between 10^3 and 10^7 genome copies/L reported in municipal wastewater from different geographical areas (Katayama *et al*., 2008; Fong *et al*., 2010; Kishida *et al*., 2012; Rodriguez *et al*., 2013;). In seawater, the enteric adenoviruses have been shown to be substantially more stable than either polio 1 or hepatitis A virus (Enriquez and Gerba, 1995). Many studies have suggested that HAdV is a good candidate as a fecal pollution indicator because of its known stability and persistence in aquatic environments compared to other enteric viruses (Pina *et al*., 1998; Hundesa *et al*., 2006; Albinana-Gimenez *et al*., 2009; Fong *et al*., 2010; Hewitt *et al*., 2013).

During the last years, more attention has been focused on the sewage virological quality, the risk of virus-associated waterborne illness, the need for routine monitoring viral contamination and the environmental surveillance through the analysis of sewage. The presence of enteric viruses in sewage and hence in environmental surface waters reflects the infectious status of the population and constitutes a public health risk. The aim of this study was species-specific identification of human adenoviruses in sewage from Poland. Sewage samples from 14 sewage disposal systems in Poland (located in 13 towns) were examined for the presence of adenoviruses by molecular methods. Predominant adenovirus species in sewage were determined.

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Experimental

Materials and Methods

Sewage samples. Samples of raw sewage were collected in 14 sewage disposal systems from January to December 2011, one sample a month. A total of 163 sewage samples were processed according to the protocol described earlier (Zurbriggen *et al*., 2008). To describe the process briefly, $AlCl_3$ (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 µl of a SiO_2 slurry, the samples were stirred for 30 min., followed by centrifugation at room temperature and 1500 × g for 5 min. to pellet the 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 × g, the concentrates were used to DNA extraction.

DNA extraction and PCR. Viral DNA was extracted from 100 µl of concentrated sewage using spin columns (QIAamp DNA stool kit – Qiagen) following the manufacturer's instructions. PCR was carried out using human adenovirus group-specific primers complementary to regions of the hexon gene (ADV-F, ADV-R) and HAdV species-specific primers (Table I). PCR amplification was performed in 25 µl volumes containing 23 µl of reaction mixture (Platinum PCR SuperMix with additions of 200 nM of each primer) and 2 µl of DNA extract. Amplification with group-specific starters started with an initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Reaction mixtures were then held at 4°C. Amplification with species-specific starters started with an initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 56°C for 60 s, extension at 72°C for 120 s., and final extension at 72°C for 5 min. Reaction mixtures were then held at 4°C. Amplification products were analysed in 1% agarose gels, GelRed-stained and viewed with the Molecular Imager Gel Doc system (BioRad Laboratories Inc.).

Statistical analysis. To identify statistical significance, a Student's *t* test analysis was performed online (http://www.physics.csbsju.edu/stats/). Results with p values of less than 0.05 were considered significant.

Results

Sewage samples were collected each month from each sampling site. In total, 163 samples of raw sewage were investigated by molecular methods. Out of the 163 samples analysed, 150 were positive for human adenoviruses using HAdV group-specific primers (92.1%). The percentage of PCR-positive samples in different sampling months was 76.9% (October) to 100% (February, March, June, July, August, November) (Fig. 1). The percentage of PCR-positive samples at the different sampling sites range between 75% and 100% and was significantly higher at cities of over 100 000 inhabitants ($p = 0.027$) (Fig. 2).

A total of 163 sewage samples were examined for the presence of A, B, C, D, E and F species of human adenoviruses by molecular methods using species-specific primers. Adenoviruses from species A were found in 32 specimens (19.6%). The percentage of positive samples in different sampling months was 7.1% (June, July, September) to 57.1% (March) (Fig. 1), with the highest

Table I
Oligonucleotide primers used in this study.

Target	Name	Sequence (5'→3')	Amplicon size (bp)	References
Human adenovirus	ADV-F	GCCACGGTGGGGTTTCTAAACTT	131	Gunson *et al*., 2009
	ADV-R	GCCCCAGTGGTCTTACATGCACATC		
HAdV species A	AdA1	GCTGAAGAAMCWGAAGAAAATGA	1444–1537	Xu *et al*., 2000
	AdA2	CRTTTGGTCTAGGGTAAGCAC		
HAdV species B	AdB1	TSTACCCYTATGAAGATGAAAGC	670–772	Xu *et al*., 2000
	AdB2	GGATAAGCTGTAGTRCTKGGCAT		
HAdV species C	AdC1	TATTCAGCATCACCTCCTTTCC	1988–2000	Xu *et al*., 2000
	AdC2	AAGCTATGTGGTGGTGGGGC		
HAdV species D	AdD1	GATGTCAAATTCCTGGTCCAC	1205–1221	Xu *et al*., 2000
	AdD2	TACCCGTGCTGGTGTAAAAATC		
HAdV species E	AdE1	TCCCTACGATGCAGACAACG	967	Xu *et al*., 2000
	AdE2	AGTGCCATCTATGCTATCTCC		
HAdV species F	AdF1	ACTTAATGCTGACACGGGCAC	541–586	Xu *et al*., 2000
	AdF2	TAATGTTTGTGTTACTCCGCTC		

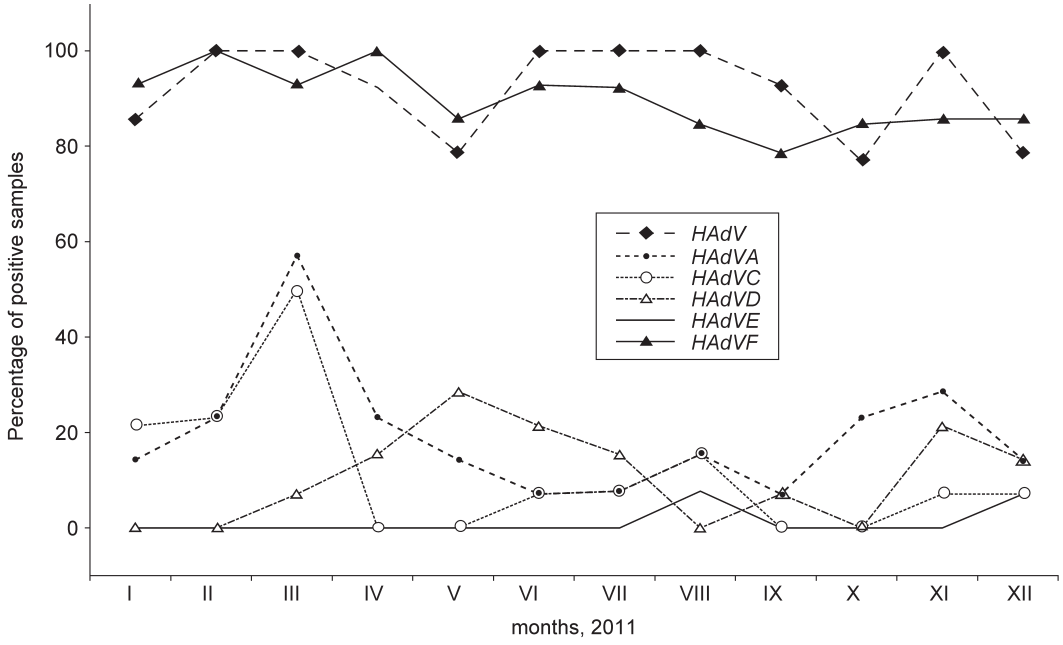

Fig. 1. Adenovirus positive sewage samples depending on season.

detection rate in the winter and early spring. The percentage of samples positive at the different sampling sites ranged between 0% and 58.3% and was higher at cities of over 100 000 inhabitants (not significantly different). Adenoviruses from B species were not detected in sewage. Adenoviruses from species C were found in 19 specimens (11.6%). The percentage of positive samples in different sampling months was 0% (April, May, September, October) to 50.0% (March) (Fig. 1). The percentage of samples positive at the different sampling sites ranged between 0% and 33.3% and was higher at cities of over 100 000 inhabitants, this difference is not significant. Adenoviruses from species D were found in 18 specimens (11.0%). The percentage of positive samples in different sampling months was 0% (January, February, August, October) to 28.6% (May) (Fig. 1). The percentage of samples positive at the different sampling sites ranged between 0% and 37.5% and was higher at cities of over 100 000 inhabitants (not significantly different). Adenoviruses from species E were found only in two specimens (1.2%) collected in August and December from two different cities. Adenoviruses from species F were the most prominent species detected in sewage, they were found in 146 specimens (89.6%). The percentage of positive samples in different sampling months was 78.6% (September) to 100% (February, April) (Fig. 1). The percentage of samples positive at the different sampling sites ranged between 50% and 100% and was significantly higher at cities of over 100 000 inhabitants ($p = 0.017$) (Fig. 2).

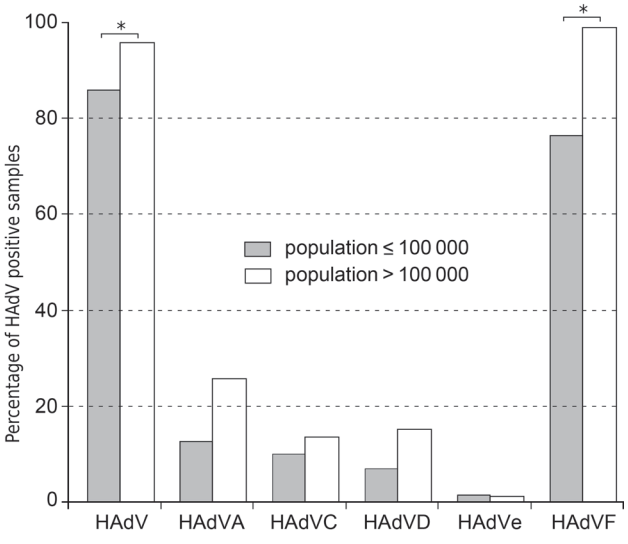

Fig. 2. Percentage of adenovirus positive sewage samples at cities with a population over and below 100 000 inhabitants. The asterisk indicates a statistically significant difference ($p < 0.05$).

Discussion

Adenoviruses have been detected in sewage worldwide. Recent metagenomic studies have shown that multiple types of viruses can be found in raw sewage (Cantalupo *et al.*, 2011; Ng *et al.*, 2012). In this study, sewage samples were used to detect of diversity of HAdVs in wastewater. Samples of sewage were collected from several locations around Poland. Samples were evaluated by PCR assay for the presence of human adenoviruses. Our investigation confirmed that sewage is a rich source of adenoviruses. HAdVs were detected in 92.1% of the analysed sewage samples and was significantly higher at cities of over 100 000 inhabitants.

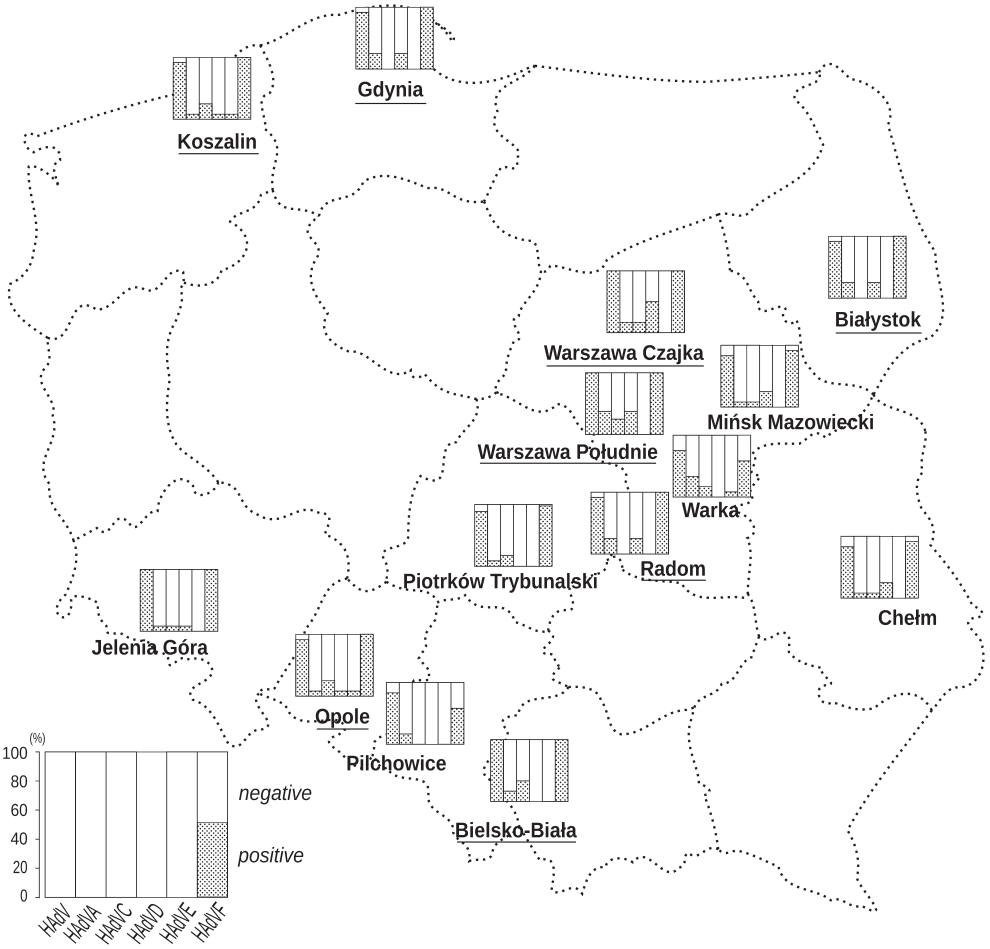

Fig. 3. Proportion of adenovirus positive and negative samples depending on sampling site (names of cities with population over 100 000 are underlined).

Our results are in agreement with Puig *et al.* (1994), reporting detection of HAdVs in 100% of sewage. In other studies, HAdVs were detected in 56 and 20% of sewage and treated effluent samples, respectively (Pusch *et al.*, 2005). Katayama *et al.* (2008) detected HAdVs in 100 and 99% sewage and treated effluent, respectively. Adenoviruses were detected in sewage during all seasons. Adenovirus infections have been observed to occur throughout the year with little or no seasonal variation in shedding. After infection, HAdV excretion by the host can last from months to years (Jiang, 2006) and the consequent lack of a seasonal pattern for this viruses in sewage has been confirmed by several studies (Jiang, 2006; Katayama *et al.*, 2008).

All samples were subjected to species-specific PCR for detection human adenovirus species A, B, C, D, E, and F. Species F adenoviruses mainly, but also A, C, D, G cause diseases of intestine. HAdV-A was identified as the etiologic agent of a diarrhea outbreak in a hematology hospital ward in London (Jalal *et al.*, 2005). Recently, HAdV-D and -C were detected in faeces of children with diarrhea in Bangladesh, Kenya and Brazil (Jarecki-Khan *et al.*, 1993; Filho *et al.*, 2007; Magwalivha *et al.*, 2010). In this study, species F was identified as the predominant in sewage (89.6%), followed by species A, C, D and E. We detected all species of human adenoviruses associated with intestine infections. Seasonal differences in detection were observed for species A, C (peak in March) and D (peak in May). Probability of detection of species F in sewage was determined by population size. Adenoviruses from species F were detected in 99% samples at cites over 100 000 inhabitants. This trends agrees with results from other environmental studies. Barrella *et al.* (2009) detected the presence of human adenovirus from species F in 82% of sewage samples. Fong *et al.* (2010) isolated adenoviruses from species F (63%), A (29%), B (3%) and C (3%) from raw sewage and primary effluents. Santos *et al.* (2004) isolated adenoviruses 40 and 41 (species F) from 62 of 69 sewage and surface water samples collected in San Paulo, Brazil, over a 3-year period. In South Africa, species F, D and C (serotype 2) were isolated from treated drinking water and river water, human adenovirus species D isolates were predominant in treated drinking water (van Heerden *et al.*, 2005). Adenovirus species F has been identified as one of the most prevalent

viruses globally in the etiology of childhood gastroenteritis. The HAdV serotypes 40 and 41 (species F) have long been recognized as the main etiological agents of 1–20% of acute viral gastroenteritis in children (Cruz *et al*., 1990; Jothikumar *et al*., 2005; Shimizu *et al*., 2007). Shimizu *et al.* state that 50% of all adenoviruses found in stool specimens are types 40 and 41 (species F). HAdV-40 is known to be widespread in the European population, where it can cause outbreaks of gastroenteritis, mostly in children during winter. After infection, HAdV types 40 and 41 can cause mortalities as much as 50% in immunocompromised individuals (Echavarria, 2008). Considering that these viruses are shed for extended periods in faeces, urine, and respiratory secretions of infected persons (Jiang *et al.,* 2006), their high prevalence in this study suggests a high incidence of species F infections in the host population. Several authors have suggested that the enteric serotypes 40 and 41 (genus F) dominate overall HAdV serotypes in sewage. Although some recent studies have demonstrated (Sinbanda and Okoh, 2012; Bibby and Peccia, 2013) that HAdV-C were more frequent detected in sewage and environmental samples than HAdV-F. In the present work HAdV-C were detected in 11.6 % of the sewage samples, but study results differed depending on sampling site (from 0 to 33%) and sampling month (from 0 to 50%). Bibby and Peccia (2013) identified species C in 78% of sewage samples. These results do not seem to be divergent taking under consideration that Bibby and Peccia collected ten samples in winter months from five wastewater treatment plants, served population ranging from 100 000 to 1 000 000 people.

In the present study, human adenoviruses in sewage were detected by direct nucleic acid amplification from sewage concentrates by PCR. A number of studies have demonstrated that PCR is more rapid and sensitive than cell culture for adenovirus detection and species-specific identification by PCR is a popular method used to conduct adenovirus characterization (Xu *et al*., 2000). Nevertheless serotyping of adenoviruses on the basis of sequencing of PCR products is potentially more informative. Methods used for the identification of human adenoviruses in raw sewage have many limitations, mainly due to the nature of the samples examined. Raw sewage usually contains organic compounds at high concentrations, which may inhibit PCR reaction.

In conclusion, this research demonstrates that human adenoviruses are consistently present in sewage in Poland. Human adenovirus species F was the most prominent adenovirus species detected in sewage, but molecular analyses indicated the presence of additional adenovirus species. HAdV species A, C, D and E were found in sewage samples, demonstrating the occurrence of multiple HAdV species in sewage. The presence of adenovirus species in sewage may represent a public health risk particularly taking in account immunocompromised individuals. Raw wastewater represent a significant source of pathogens that has a potential to contaminate aquatic environments and very important is adequate treatment before the disposal in the environment.

Acknowledgments

This research was undertaken as part of 14/EM.1/2014. The authors thank Tobiasz Wieczorek for his help with the figures.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 29–36

ORIGINAL PAPER

Effect of DNA Extraction Methods on the Apparent Structure of Yak Rumen Microbial Communities as Revealed by 16S rDNA Sequencing

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Submitted 27 July 2014, reviewed 27 November 2014, accepted 27 November 2014

Abstract

To more efficiently identify the microbial community of the yak rumen, the standardization of DNA extraction is key to ensure fidelity while studying environmental microbial communities. In this study, we systematically compared the efficiency of several extraction methods based on DNA yield, purity, and 16S rDNA sequencing to determine the optimal DNA extraction methods whose DNA products reflect complete bacterial communities. The results indicate that method 6 (hexadecyltrimethylammomium bromide-lysozyme-physical lysis by bead beating) is recommended for the DNA isolation of the rumen microbial community due to its high yield, operational taxonomic unit, bacterial diversity, and excellent cell-breaking capability. The results also indicate that the bead-beating step is necessary to effectively break down the cell walls of all of the microbes, especially Gram-positive bacteria. Another aim of this study was to preliminarily analyze the bacterial community *via* 16S rDNA sequencing. The microbial community spanned approximately 21 phyla, 35 classes, 75 families, and 112 genera. A comparative analysis showed some variations in the microbial community between yaks and cattle that may be attributed to diet and environmental differences. Interestingly, numerous uncultured or unclassified bacteria were found in yak rumen, suggesting that further research is required to determine the specific functional and ecological roles of these bacteria in yak rumen. In summary, the investigation of the optimal DNA extraction methods and the preliminary evaluation of the bacterial community composition of yak rumen support further identification of the specificity of the rumen microbial community in yak and the discovery of distinct gene resources.

Key words: 16S rDNA sequencing, bacterial community, comparative analysis, DNA extraction methods, yak rumen

Introduction

As the first chamber of the ruminant stomach, the rumen contains abundant symbiotic microbes that perform important functions in the digestion of complex cellulolytic biomass and that supply nutrients to the host in the form of short-chain fatty acids and microbial proteins. Rumen microbes have been recently extensively studied due to their association with environmentally and economically important traits, such as feed conversion efficiency (Guan *et al*., 2008; Petri *et al*., 2013), methane production (Zhou *et al*., 2009), and the discovery of microbes or enzymes that facilitate the fermentation of biomass for biofuel production.

Rumen microbial community could be studied by culture-based or some molecular techniques (DGGE and ribosomal RNA clone libraries). Given the low sequencing depth of these approaches, previous community analyses represent a mere snapshot of the diverse community (Bibby *et al*., 2010; Fouts *et al*., 2012; Zhang *et al*., 2012). With the advent of next-generation sequencing technologies, conducting in-depth sequencing on samples that are derived from any environment of choice, including the complex rumen microbial community at a deeper level than previously performed is feasible (Bergmann *et al*., 2010; Cuív *et al*., 2011; McOrist *et al*., 2002). Moreover, this technique is an effective tool for analyzing the bacterial community structure based on detailed taxonomic information. Notably, however, extracting DNA with a sufficient yield and high quality is a prerequisite to fully identify the members of the bacterial community with fidelity. Several studies reported that DNA extraction methods significantly impact the microbial community in samples from different habitats, including the rumen (Bergmann *et al*., 2010; Cuív *et al*., 2011; McOrist *et al*., 2002;Yu and Morrison 2004; Guo and Zhang, 2013). To enable a direct comparison of the rumen community structure from different individual samples, standardizing DNA extraction methods is crucial. Commercial DNA extraction kits for rumen content are currently unavailable, but kits that are designed for other samples

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are often used. Thus, the effectiveness of these cross-use kits should also be evaluated. Recently, several studies have evaluated DNA extraction methods for rumen content samples (Henderson *et al.*, 2013; Villegas-Rivera *et al.*, 2013). Here, we want to systematically compare the effectiveness of a variety of microbial DNA extraction methods for rumen based on the integrity, yield, purity, and sequencing results of extracted DNA.

Given the variation in the diet and inhabiting environment, different kinds of ruminants could harbor a distinct population of rumen microbes. Yak (*Bos grunniens*) lives exclusively on the Qinghai-Tibetan Plateau, China, at an average height of 3000 masl in a full-grazing style with coarse grasses as the only food due to poor living conditions (Qiu *et al.*, 2012). As a result, special microbe species may exist in the yak rumen compared to those of other ruminants in plains. Moreover, with the development of modern breeding technologies and the abuse of antibiotics, the rumen microbial community has been subjected to change. Therefore, yaks in native areas (*i.e.*, untouched by these modern advances) have a relatively complete and unbroken microbial community (An *et al.*, 2005; Huang *et al.*, 2012). Therefore, yaks can be considered an ideal animal model to study the microbial community and discover new microbes or valuable genes. For yaks, abundant humic substances and silt exist in the rumen, making DNA extraction difficult and maybe influencing subsequent sequencing. In the light of these aspects, this study aimed at selecting a method for DNA extraction from the yak rumen and preliminary analysis of the basic bacterial community structure in the yak rumen.

Experimental

Materials and Methods

Ethics statement and sample collection. Yaks were supplied by the Hongyuan Yak Breeding Farm (N32°47′35.50′ latitude, E102°32′34.32′ longitude, average 3600 masl), Sichuan, China. The experimental procedures and the collection of rumen samples that were used for this study were approved according to the animal care and use of laboratory animals of the Institutional Animal Care and Use Committee of the Southwest University for Nationalities. Samples of the rumen contents were collected immediately after death and transported in an airtight container full of dry ice to the laboratory within 30 min. Then, the samples were stored at –80°C.

Acquisition of microbial pellets. One gram of rumen content from each yak was mixed to form a sample pool (total amount 3.0 g). For DNA extraction, 2.0 g of rumen content was thawed on ice and transferred to a 50-ml tube. A 20-ml portion of PBS buffer was added to the tube to wash the rumen content, and the mixture was vortexed thoroughly for 2 min. After centrifugation (2 min, 200 g), the supernatant was collected. The samples were washed 3 times, and the total mixed supernatant was then centrifuged (2 min, 12,000 g) to obtain microbial pellets for DNA extraction.

Extraction of DNA from the rumen content. Based on the existing DNA extraction methods that were designed for other samples and on the characteristics of the yak rumen (Nettmann *et al.*, 2008; Willner *et al.*, 2012), we summarized and improved several extraction methods and further compared their DNA extraction efficiency. The specific parameters of every method are listed in Table I.

Table I
Parameters of 10 improved DNA extraction methods

	DNA extraction methods									
Parameter	1	2	3	4	5	6	7	8	9	10
CTAB[a]	+	+	+	+	+	+	–	–	–	–
SDS[b]	+	+	+	+	+	+	+	+	+	+
Proteinase K[c]	+	+	+	+	+	+	+	+	+	+
Lysozyme[d]	+	+	+	–	–	–	+	+	+	–
Freeze-thaw	–	+	–	–	+	–	–	+	–	–
Bead beating	–	–	+	–	–	+	–	–	+	–
Silica column	–	–	–	–	–	–	–	–	–	+

a: Treatment with 5% CTAB (w/v).
b: Treatment with 1% SDS (w/v).
c: Treatment with 0.3 mg/ml proteinase K.
d: Treatment with 0.3 mg/ml lysozyme.

Method 1 (CTAB-SDS-Lysozyme):Total genomic DNA was isolated by the method of Nettmann, *et al.* (2008) which includes an enzymatic cell lysis with lysozyme and two chemical lysis with SDS and CTAB as detergent, respectively. The DNA was purified with chloroform-isoamyl alcohol, precipitated with isopropanol and dissolved in 30 µl of nuclease-free water.

Method 2 (CTAB-SDS-Lysozyme- physical lysis by freeze-thaw): According to the protocol 1, there exists some modification: after the CTAB solution was added to cell suspension, three cycles of freezing in –80°C refrigerator for 10 min. and heating in a water bath at 65°C was performed until the samples was completely thawed.

Method 3 (CTAB-SDS-Lysozyme- physical lysis by bead-beating): Referring to method 1, there are some changes in method 3, after treating the cell suspension with lysozyme (0.3 mg/mL), 100 mg of zirconium-silica beads (0.1 mm diameter) was added to the mixture. Next, the mixture was vortexed thoroughly for 10 min.

Method 4 (CTAB-Lysozyme): For chemical cell lysis by CTAB and enzymatic lysis by lysozyme, the DNA isolation protocol of Nettmann, *et al.* (2008) was used.

Method 5 (CTAB-Lysozyme- physical lysis by freeze-thaw): The protocol combined the chemical enzymatic cell lysis and physical lysis by freeze-thaw, referring to methods 2 and 4, the specific process was designed.

Method 6 (CTAB-Lysozyme- physical lysis by bead-beating): The protocol combined the chemical enzymatic cell lysis and physical lysis by beads, referring to methods 3 and 4, the specific process was designed.

Method 7 (SDS-lysozyme): Firstly, the cell pellet was lysed with lysozyme (0.3 mg/mL), the samples were incubated at 37°C for 60 min., followed by an chemical cell lysis using SDS an detergent.

Method 8 (SDS-lysozyme- physical lysis by freeze-thaw): For protocol 8, the DNA was obtained by combined freeze-thaw lysis and chemical cell lysis (refer to methods 2 and 7).

Method 9 (SDS-lysozyme-physical lysis by bead-beating): For protocol 9, the DNA was obtained by combined bead-beating lysis and chemical cell lysis (refer to methods 3 and 7).

Method 10 (DNA Extraction Kit): The procedure was performed according to the instructions of QIAamp DNA Stool Extraction Kit (Qiagen, Germany).

Determination of the DNA yield, purity, and fragment distribution. The DNA yields of the different methods were calculated using the NanoDrop ND-3300 spectrometer. The purity of the extracted DNA was assessed via the values of OD260/OD280 and OD260/OD230. The size of the isolated DNA fragments was determined by agarose gel electrophoresis using the λ-Hind III Marker as a reference.

PCR amplification for pyrosequencing. The V3 to V4 regions of 16S rDNA genes from bacteria were amplified using the bar-coded primer set of 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR products were examined on an agarose gel, and the DNA bands were excised and purified. In addition, the concentration and qualities of the DNA were determined using the TBS-380 fluorophotometer. DNA pool was prepared by mixing together equal quantities of DNA samples of three DNA samples per treatment and then sent to BGI (Shenzhen, China) for sequencing on the Illumina MiSeq PE300 platform (Illumina, USA).

Sequence processing. Prior to taxonomic analysis, the DNA sequences were subjected to the following filtering steps: (1) sequences containing windows of 50 consecutive base pairs with an average quality score of less than 20 were truncated at the start of the low-quality region; (2) pair-end reads were combined into one sequence, and the length of overlap was longer than 10 bp; and (3) the mismatch rate for the overlap region of assembled sequences was less than 0.2; otherwise, the sequences were removed. Furthermore, those sequences with a 97% identity cutoff were labeled as an operational taxonomic unit (OTU). After the above filtering, RDP (Ribosomal Database Project) Classifier was used to assign the sequences to different taxonomy levels at a bootstrap cutoff of 30%. The pyrosequencing reads were then aligned using Infernal based on the bacterial alignment model in the Align module of RDP. By applying Complete Linkage Clustering, the sequences in each sample were assigned to phylotype clusters of 97% identity. Based on these clusters, a Rarefaction curve (Colwell and Coddington, 1994; Schloss and Handelsman, 2005), ACE richness estimations, and Shannon diversities (Gotelli, 2002) were generated using RDP software.

Results

Extraction efficiency, purity, and integrity. The length of all of the fragments appeared to be at about 20 kb. The yield and the OD260/OD280 ratio are shown in Table II. Methods 3 and 6, which included a bead-beating step, produced highest yield and purest DNA compared to the other protocols, suggesting that bead beating is necessary in rumen DNA extraction. Methods 7, 8, and 9, which did not use CTAB (hexadecyltrimethylammomium bromide) as a detergent to disrupt the cell and yielded low DNA, demonstrating that the chemical lysis of CTAB is also required for DNA extraction from rumen content samples.

Table II
Yield and purity of DNA extracted using 10 methods

Sample	Yield (ng/μl)	OD260/280
1	336.61 ± 7.22	1.60 ± 0.08
2	455.30 ± 6.55	1.53 ± 0.12
3	527.48 ± 5.62	1.85 ± 0.13
4	324.12 ± 6.88	1.72 ± 0.12
5	384.81 ± 7.82	1.79 ± 0.21
6	525.55 ± 11.22	1.88 ± 0.11
7	59.71 ± 12.11	1.82 ± 0.21
8	39.03 ± 0.23	1.65 ± 0.12
9	49.64 ± 0.75	1.81 ± 0.15
10	62.05 ± 0.24	1.81 ± 0.15

Sequencing results. Considering the requirements of DNA quantity and quality for sequencing, DNA that was extracted using Methods 2 and 8 could not be successfully analyzed. A total of 191,349 raw sequences from all of the samples were generated. After trimming, sorting, and quality control, 171,231 or 89.5% of the sequences were used in our analysis. Specific sequence information for each method is included in Table III. Consistent with the quantitative results, the number of

Table III
Specific sequence information of each method

Sample	Sequences	Bases (bp)	Average Length (bp)
1	25 465	10 022 673	393.59
3	26 080	10 270 236	393.8
4	23 216	9 134 524	393.46
5	20 325	7 997 392	393.48
6	29 763	11 722 005	393.84
7	10 446	4 114 615	393.89
9	15 781	6 211 301	393.59
10	20 155	7 944 212	394.16

Table IV
Specific OTUs and OTU-based diversity index of different extraction treatments

Sample	Diversity index (0.97)		
	OTU	Ace	Shannon
1	937	1069	5.24
3	942	1093	5.24
4	953	1084	5.22
5	929	1055	5.4
6	1026	1126	5.46
7	824	983	5.47
9	914	1055	5.44
10	829	898	5.48

raw sequences of methods 3 and 6 were also larger than those of other methods.

OUT-based analysis. Fig. 1 displays the rarefaction plots for each sample, in which at least 800 OTUs were observed, indicating that the rumen microbial community is complex. Table IV lists the number of OTUs and the diversity index of each DNA extraction treatment. The ACE index was usually used to calculate the richness of the output, and the Shannon index could reflect the diversity of microorganisms. A larger value of Shannon indicates more-abundant species in a sample. The treatments with the bead-beating step had evidently higher numbers of OTUs and a higher diversity index than the others, suggesting that robust bead beating is necessary for cell lysis. In contrast, the QIAamp kit had fewer OTUs but a higher diversity index, indicating that this kit was efficient in cell lysis but lost much DNA during the subsequent purification steps.

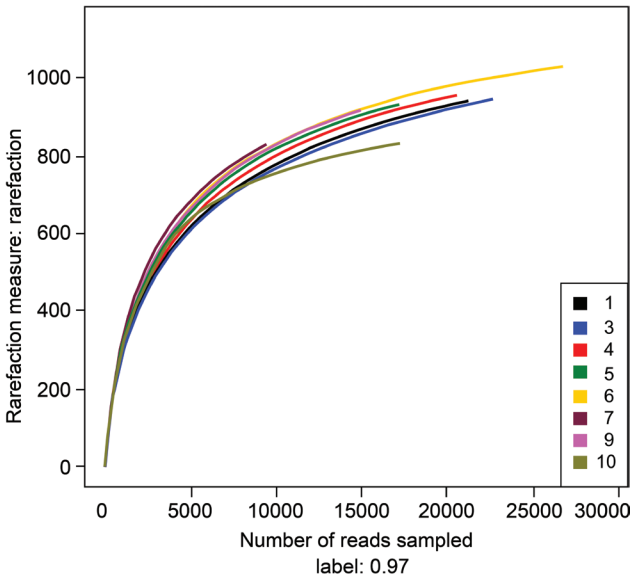

Fig. 1. Rarefaction curves of the rumen microbial community based on 16S rRNA gene sequencing (at the 0.97 identity level).

Abundant genera in each sample were selected and compared *via* their abundance in other samples, as shown in Fig. 2. In the heat map, the lower number of the blue blocks (indicating low abundance) in the total OTUs and the greater abundance of dominant genera translate into a more efficient method for DNA extraction from different kinds of microorganisms. Therefore, we speculated that methods 6 and 9 were more representative than other methods. In addition, a cluster analysis indicated that methods 7, 8, and 9 clustered together and other traditional methods were included in another cluster, suggesting that bead beating was determinative in DNA extraction.

Comparison of Gram-positive bacterial abundance. As shown in Fig. 3., the abundances of the top eight Gram-positive genera in each sample were investigated to further compare the efficiencies of cell lysis for different treatments. Among these methods, method 6 exhibited the best capability for cell lysis; methods 1 and 3 also performed well. Moreover, for three Gram-positive genera with lower abundances (*Ruminococcus*, *Acetitomaculum*, and *Mogibacterium*), the treatments with bead beating evidently had a higher abundance than did the other methods. This observation indicates that robust mechanical homogenization is required to efficiently disrupt Gram-positive bacteria. When the cells were lysed gently, certain rare Gram-negative phyla, including *Fibrobacteres*, *Proteobacteria*, and *Tenericutes*, were actually overestimated.

Taxonomy-based analysis of rumen microbes. Based on the above results, method 6 was deemed as effective for extracting complete DNA from all of the microbes. A taxonomy-based analysis of sample 6 showed that the rumen bacterial community consisted of 21 phyla, 35 classes, 52 orders, 75 families, and 112 genera. At the phylum level, the community structure is shown in Fig. 4. Within the bacterial population, 21 phyla were found across the rumen contents. The bacterial composition of the rumen consisted mainly of the *Firmicutes* and *Bacteroidetes* phyla, at 64% and 20% of the total reads on average, respectively. The remaining microbes consisted of low-abundance phyla (< 10% of the total reads), such as *Fibrobacter*, *Spirochaeta*, and

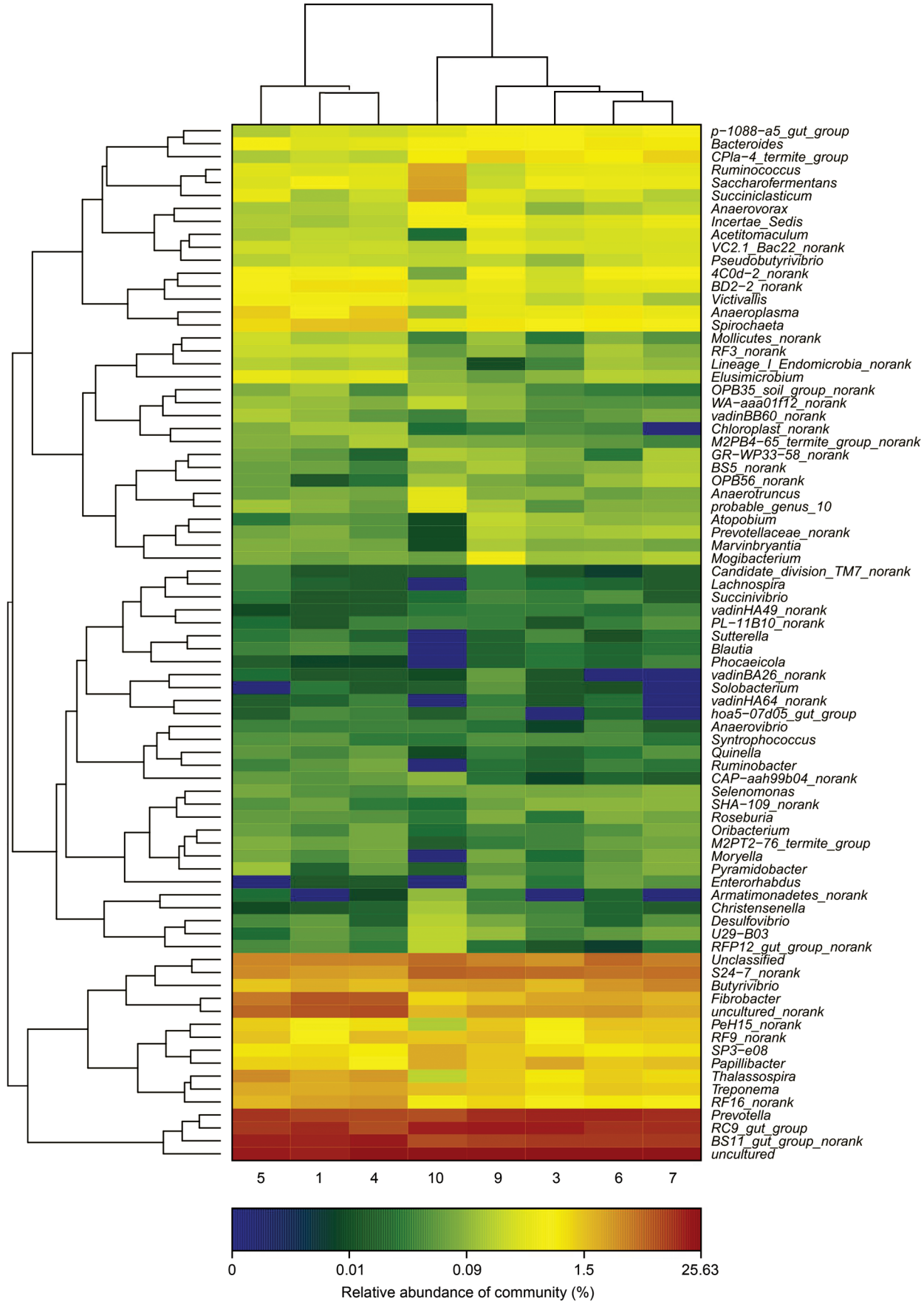

Fig. 2. Heat map analysis of the bacterial community based on the top 50 genera. The top 50 most-abundant genera in each sample were selected (a total of 80 genera for all 8 treatments) and compared with their abundance in other samples. The color intensity in each panel shows the percentage of a genus in a sample, referring to the color key at the bottom.

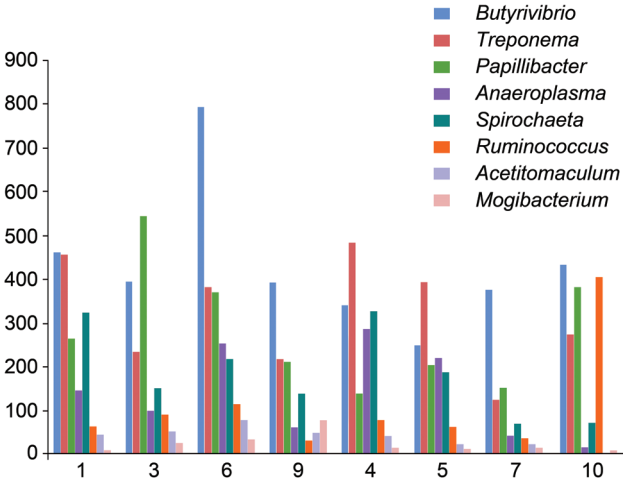

Fig. 3. The abundance of the top eight Gram-positive genera in the different treatments.

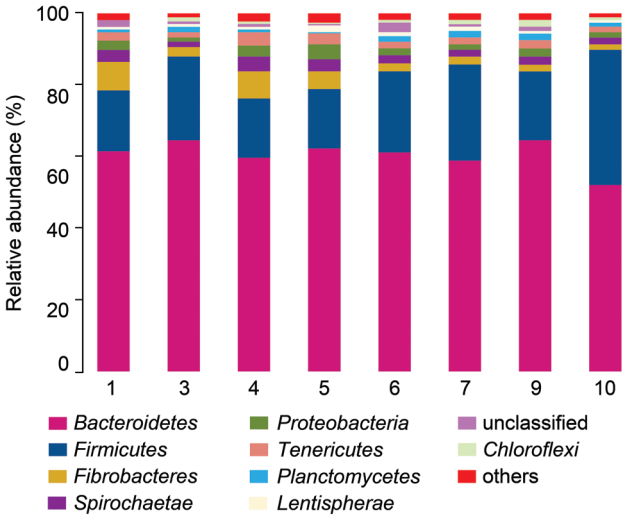

Fig. 4. Microbial community structures of the samples of yak rumen. The relative abundance of bacterial 16S rRNA genes was estimated through classification at the phylum level.

Proteobacteria, which represented approximately 1.7%, 2.3%, and 1.8%, respectively. Notably, the five most-abundant phyla in the yak rumen accounted for 90% of the analyzed sequences, and the phylum distribution was similar to those of other ruminants. At the genus level, *Prevotella*, *Butyrivibrio*, *Fibrobacter*, the uncultured, and two poorly described genera (*RC9_gut_group* and *BS11_gut_group_norank)* were identified as the dominant genera in the rumen bacterial community.

Discussion

Comparison of the DNA extraction methods. With the advent of next-generation sequencing technologies, conducting in-depth sequencing and data analysis on the rumen microbial community and the exploration of uncultured microorganisms are feasible. However, effective DNA extraction with high quality is the premise. An increasing number of reports exist regarding the biases of different methods during DNA extraction as revealed by 16S rDNA sequencing. However, information about the yak rumen content is scarce. Compared to other ruminants in plains, yaks live in a free-ranging pattern; therefore, the yak rumen usually contains much silt and many humic substances that complicate DNA extraction. The aim of the current study was to select one optimal method to extract DNA from yak rumen microbes as evaluated by 16S rDNA sequencing. The factor that primarily affects DNA yield is the efficiency of cell lysis. The methods with bead beating produced more DNA than did the other methods, indicating that bead-beating is efficient for cell lysis. Method 9, which included the bead-beating step, produced less DNA, suggesting the importance of CTAB in cell lysis.

For PCR-based community analysis, the quantity of DNA is not the key factor because trace DNA (> 500 ng) is adequate for PCR amplification and subsequent sequencing. As shown in our results, both protocols 7 and 9 produced DNA of low quantity and quality, but the OTU-based analysis generated an almost fair profile of the bacterial community. Interestingly, method 2 produced a relatively higher amount of DNA. However, the DNA did not meet the requirements for 16S rDNA sequencing This exclusion may be due to numerous DNA fragments with small segment sizes as a result of robust shear force during the freeze-thaw and chemical cleavage processes, prohibiting the DNA from serving as a template for PCR amplification. Moreover, the PCR reaction would also be affected if certain impurities (*i.e.*, humic acid) existed in the DNA content. For sample 8, the DNA quantity did not meet the further analysis requirements, resulting in analytical failure.

The total OTU number and the relevant diversity indexes can be used to evaluate the efficiencies of the DNA extraction methods. In general, more OTUs and higher diversity indexes may represent more species within samples. Therefore, the methods with bead-beating steps are significantly better than are other methods that use chemical lysis or freeze-thaw. The kit effectively lysed the cell walls of bacteria but lost much DNA during following steps, resulting in larger diversity index but fewer OTUs. Due to the thick cell well and spore formation, the Gram-positive bacteria are relatively resistant to both detergents and mechanical lysis. Therefore, we believe that more Gram-positive bacteria detected indicated more-efficient DNA extraction methods. For ruminants, *Firmicutes* is a relatively dominant phylum in the rumen, performing essential functions in energy conversion. *Actinobacteria* is also an important Gram-positive bacterium that regulates

polymer degradation, glycogen accumulation, and polyphosphate accumulation. In terms of these two phyla, method 6 was the most efficient because more Gram-positive bacteria were detected.

Microbial community of yak rumen. Given the significant differences in their diet and habitats, yaks could harbor a distinct population of rumen bacteria compared to that of other ruminants. Currently, the rumen microbes of yak have not received adequate attention. Therefore, another aim of the present study was to preliminarily analyze the bacterial community *via* 16S rRNA high-throughput sequencing. Previously, An *et al.* (2005) compared the prokaryote diversity in the rumen between yak and cattle as estimated by 16S rDNA clone library analysis. Given the limitations of the sequencing depth, An *et al.* sequenced fewer fragments than in their study (An *et al*., 2005). 16S rDNA sequencing is an efficient tool to fully explore valuable gene resources and to understand the specificity of the bacterial community from any sample, including yak rumen content. Huang *et al.* (2012) compared the methanogen diversity from "energy-saving" yak and cattle based on 16S rDNA sequencing to explain why yaks produce less methane than do cattle. This previous study revealed that the methanogen community structure of yak was significantly different from that of cattle, ultimately resulting in less methane production and indicating the effectiveness of the technique in analyzing the microbial community. Unfortunately, the results cannot truly reflect the microbial community due to the limited capacity of the QIAamp DNA Stool Kit for rumen DNA extraction. In this study, the 16S rDNA sequencing results demonstrated that the bacterial community of the yak rumen included approximately 21 phyla, 35 classes, 75 families, and 112 genera. Compared with cattle, the percentage of taxa in yak is significantly different in relative abundance, ranging from 10.5% at a phyla level to 105.5% at a genus level (Jami and Mizrahi 2012; Omoniyi *et al*., 2014; Ross *et al*., 2012). The sequencing results revealed that, at the phylum level, the microbial community of the yak rumen was dominated by *Bacteroidetes* and *Firmicutes*, and the microbial distribution of the major phyla is similar to that of cattle (Zened *et al*., 2013), thereby suggesting the importance of the two phyla in ruminal metabolism (Turnbaugh *et al*., 2008).

The sequencing results also indicate that uncultured or unclassified species in the yak rumen comprise a large proportion of the bacterial community. For example, *RC9_gut_group* and *BS11_gut_group_norank* accounted for a large percentage of the total bacteria (13.12% and 10.10%, respectively). Moreover, numerous rare, undescribed genera, such as C*AP-aah99b04-norank*, *SHA-109-norank*, and *M2PT2-76_termite_group*, were also detected in the present study. Similarly, An *et al*. (2005) found that a higher ratio of uncultured microbial species was also identified by clone library analysis in the yak rumen content. We can speculate, therefore, these bacteria may possess important and yet unrecognized ecological functions and occupy a special ecological niche in the rumen.

Regarding ruminants, *Fibrobacter* and *Ruminococcus* are well-known fibrolytic species in rumen. Yaks are predicted to harbor more fibrolytic microorganisms in the rumen because their feed mainly consists of fiber-containing grasses. The results show that *Fibrobacter* in the rumen represent approximately 2.5% of all of the reads, which is similar to that of cattle. Interestingly, the percentage of *Ruminococcus* in yak (0.4%) is significantly less than that in cattle (5%) (Jami and Mizrahi, 2012), and the significant variance in the abundance is an important subject for further research. One explanation could be that other dominant fibrolytic microorganisms exist in yak.

The species of the genus *Prevotella* consist of a large group of bacteria with functional diversity, including promoting initial dietary protein breakdown and acting synergistically with cellulolytic species to improve the ruminal cellulolytic capacity. The genus *Prevotella* is the core bacterial genus, with approximately 40% to 50% percentage of all bacteria in adult dairy cattle or cattle. However, in the present study, the *Prevotella* in yak rumen only represents 15%. The significant variation may be attributed to differences in the available nutrients; dairy cattle and cattle are fed with high-quality forage and grain, whereas yak mainly feed on coarse grasses. Several studies have found that the identified diet impacts the bacterial community. Petri *et al*. (2013) reported that the percent of *Prevotella* varied significantly based on the dietary composition and accounted for approximately 8.9%, 12.8%, and 31.6% in cattle that were fed with forage, mixed forage, and high grain, respectively (Petri *et al*., 2013). In summary, the diet has a significant influence on the bacterial community in the yak rumen.

Acknowledgements

This work was supported by the Veterinary Medicine Discipline Program of the Southwest University for Nationalities (2014XWD-S0906), Key Natural Science Project of Education Department in Sichan province (15ZB0486), and National Science & technology Pillar Program of China (2012BAD13B06).

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 37–45

ORIGINAL PAPER

Bacterial Diversity in Çamaltı Saltern, Turkey

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Submitted 24 March 2014, revised 17 November 2014, accepted 20 November 2014

Abstract

A combination of culture-dependent and culture-independent approaches was employed to identify the bacterial diversity of Çamaltı solar saltern in Turkey. The bacterial communities of Çamaltı Saltern were analyzed by molecular techniques that included denaturing gradient gel electrophoresis of 16S rRNA gene fragments PCR amplified from DNA extracted from the water samples of the saltern and 16S rRNA gene library analysis. A total of 42 isolates were identified at the genus/species level and 17 of them were found to belong to the *Bacteria* domain. All bacterial isolates were phylogenetically related to *Halobacillus, Virgibacillus* and *Halomonas* genus. A total of 50 clones from 16S rRNA gene library were analyzed by ARDRA. 16S rRNA sequence analysisof these clones revealed that most (85%) of the bacterial clones were related to *Salinibacter* genus members of the *Bacteroidetes*. The sequences of DGGE bands were related to the uncultured *Salinibacter*, uncultured halophilic bacterium and *Halomonas* sp. This work highlights the halophilic bacterial diversity of Çamaltı marine solar saltern.

Key words: bacterial diversity, halophilic bacteria, solar salterns

Introduction

Salts are necessary for all organisms but halophiles require high salt concentrations for growth and thus thrive in saline environments (DasSarma and DasSarma, 2012). Microorganisms adapted to life at high salt concentrations are widespread, both within the bacterial and the archaeal domain. As a result, highly diverse prokaryote communities can be found at all salt concentrations, from seawater up to about 340–350 g/liter (brines saturated with NaCl), in both thalassohaline and athalassohaline environments (Oren, 2006). The crystallizer ponds of solar salterns are reflecting characteristics of thalassic environment. Despite the prevailing extreme environment, a great diversity of microbial life has been observed in hypersaline areas of greater than 3.5 mol/l NaCl, a point at which only a few extreme halophiles can grow (DasSarma and DasSarma, 2012). These extreme halophiles grow best at the highest salinities (3.4–5 mol/L NaCl), forming dense blooms, and resulting in the red colour of many salterns (Guixa-Boixereu *et al*., 1996). There are three major groups of organisms in brines containing more than 12% salt. These include the halophilic (salt loving) members of the domain Bacteria which generally have a broad salt tolerance and the halophilic Archaea, the salt-loving prokaryotes, with a requirement for highly elevated concentrations of salt, often up to 10 times the salinity of seawater (Litchfield *et al*., 2001).

Çamaltı Saltern is the biggest artificial marine solar saltern in Turkey. It is a multipond system consisting of 182 ponds covering 58 km^2 and located about 38°35′N and 26°57′E on the east cost of the Aegean sea (Fig. 1). Sea salt extraction has been carried out in the area since 1863. It is divided into several evaporation ponds connected by pipes and channels along a 18 km seacoast. Çamaltı Saltern have been functioning with the system of successive evaporation basins (Tıraş, 2007). In this saltern, solar irradiance and wind are main factors contributing to water evaporation and salt crystallization. The brines originate by evaporation of seawater (so-called thalassohaline brines) and reflect the ionic composition of the sea which Na^+ is the predominant cation, Cl^- the main anion, followed by SO_4^{2-} (Oren, 2006).

Microbial diversity of the different salterns around the world have been examined both by culture-independent and culture-dependent techniques. These techniques have been used to analyse the microbial diversity of Salterns in Santa Pola, Spain (Anton *et al*., 1999; Anton *et al*., 2000), and coastal Australia (Burns *et al*., 2004a), Peru (Maturrano *et al*., 2006), Turkey (Mutlu *et al*., 2008), Croatia (Pašić *et al*., 2007), Korea (Park *et al*., 2006) and Tunisia (Hedi *et al*., 2009). Litchfield *et al*. (2001) examined and compared whole metabolic diversity of two different solar salterns. Polar lipids and pigments were also used as biomarkers to study microbial communities of solar salterns (Litchfield and Oren, 2001).The Çamaltı Saltern, the largest saltern in

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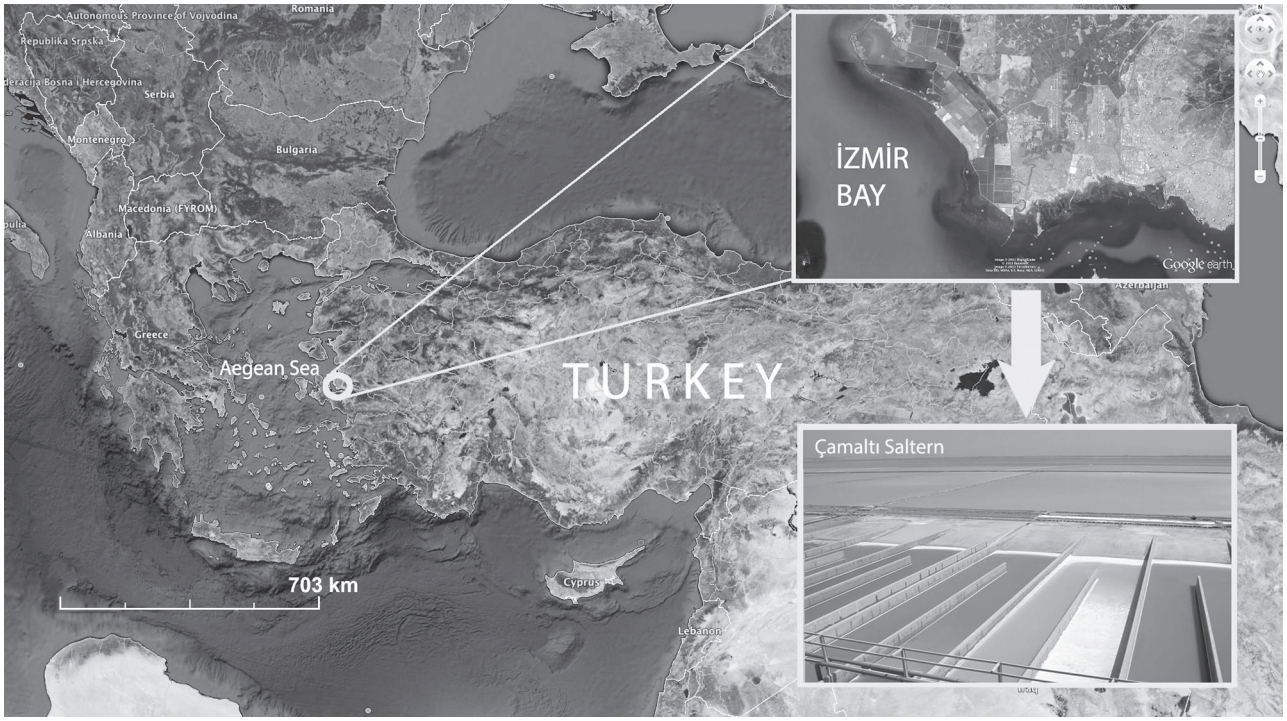

Fig. 1. Detailed map showing Çamaltı Saltern, in Turkey. Sampling points were shown with circle on the map. GPS coordinates of the sampling points were 38°28′47N–26°56′11E; 38°29'57N–26°53'37E. (Satellite imagery: Google/Google Earth).

Turkey, is an important source of salt for food. Two culture independent techniques namely, real time PCR and fluorescence *in-situ* hybridization (FISH) were used in a preliminary study which gave us some perspective to reveal the prokaryotic diversity of this hypersaline area (Mutlu and Guven, 2011). Given it's economic value for the region as a salt source, we have proposed to conduct a survey to gain better knowledge of the prokaryotic diversity thriving in this extreme ecosystem not only at a domain level, but also at genus, as well as species level. In a previous study, we determined PAH degrading archaeal isolates from the Çamaltı saltern (Erdogmuş *et al*., 2013) and *Haloferax* sp., *Halorubrum* sp., *Halobacterium* sp., and *Haloarcula* sp. were seen as dominant genera. Therefore, we only present the bacterial populations of largest Turkish saltern using both culture-dependent and culture-independent approach that includes denaturing gradient gel electrophoresis of PCR-amplified fragments of 16S rRNA gene from DNA extracted from the saltern in this study.

Experimental

Materials and Methods

Sample collection. Brine samples were taken from 10 different locations (38°28′47N–2656′11E; 38°29'57N–26°53'37E) of the Çamaltı Saltern in July 2007. A circle containing the sampling area is indicated on the map in Fig. 1. The total salt concentration of each sample was determined *in situ* with a hand refractometer (Eclipse) and the pH was measured with TOA WQC water analyser at the sampling point. The total salt concentration of these samples were measured between 6% and 32% and the pH values were between 6.5 and 7.5.

Isolation and selection of microorganisms by ARDRA (Amplified Ribosomal DNA Restriction Analysis). The following medium was used for isolation: to a liter of a solution of salts, named as 25% Sea Water (SW) and containing ($g\,l^{-1}$): NaBr 0.65, $NaHCO_3$ 0.17, KCl 5, $CaCl_2$ 0.72, $MgSO_4$_$7H_2O$ 49.5, $MgCl_2$_$6H_2O$ 34.6, NaCl 195, 1 g yeast extract, and 20 g of agar were added. Several dilutions (from 10^{-1} to 10^{-5}) of the original water sample were used to inoculate the plates by a plate spread technique. Two hundred µl of water samples were plated in duplicate onto 25% Sea Water Medium. Samples were incubated at 37°C for 3–4 weeks. Selected colonies were analyzed by 16S rRNA gene PCR amplification. Isolates were screened for redundancies by ARDRA (Amplified Ribosomal DNA Restriction Analysis) with the enzymes *Hinf*I and *Mbo*I (Vaneechoutte *et al*., 1992). Enzymatic digestions were performed by incubating 10 µl of the PCR product with 5U of enzyme and the corresponding enzyme buffer. The digestion products were analyzed in 2% agarose gels in 0.5X Tris-boric acid-EDTA (TBE) buffer. The gels were stained using ethidium bromide (0.2 µg/ml) and visualized and photographed under a UV transilluminator.

Identification bacterial isolates by 16S rRNA gene sequencing. Pure cultures were lysed in 100 µl MQ water and boiled for 10 min. Cell debris were pelleted by centrifugation at 13 000 × g for 10 min. One µl cell lysate was used in a PCR reaction containing (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20), 0.2 mM dNTPs, 3 mM $MgCl_2$, 20 pmol forward primer, 20 pmol reverse primer, 2.5 U Taq polymerase and MQ water to a final volume of 50 µl. To amplify the 16S rRNA genes, bacteria domain specific primer sets were used. The sequence of the forward primer was 27f (5'-AGAGTTTGATCATGGCTCAG-3'). The reverse primer was 1492r 5'-GTTACCTTGTTACGACTT-3' (Lane *et al*., 1985). The following conditions were used for amplification: a cycle of 94°C for 3 min, 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min; plus an extension step of 7 min at 72°C. Negative controls were included with no addition of template DNA. Five µl of PCR product was loaded onto 1% agarose gel in 1X Tris Acetic acid-EDTA (TAE) buffer. The gel was stained using ethidium bromide (0.2 µg/ml), visualized and photographed under a UV transilluminator. PCR products were purified using the Wizard PCR and Gel Purification Kit (Promega) and stored at –20°C until required. DNA sequencing was performed by Beckman CEQ 8000 genetic analyser. The DNA sequences were analyzed using the BLASTN homology search program, which is available at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) to identify close matches (Altschul *et al*., 1990). Multiple gene alignments were performed using MUSCLE 3.7 and Gblocks 0.91 b modules of "Phylogeny.fr". Phylogenetic analysis was performed using PhyML 3.0 a LRT and cladograms were generated using TreeDyn 198.3 module of Phylogeny.fr. All the above software and modules are freely available at http://www.phylogeny.fr/ (Dereeper *et al*., 2008).

Nucleic acid extraction. Microorganisms were collected by filtration of 50 ml of a brine sample on a 0.22-µm pore size GV filter (Durapore, Millipore). The filter was cut into small pieces with sterile scissors, and placed in RNAse- and DNAse-free 2-mL cryotubes containing 600 µl of extraction buffer (100 mM Tris-HCl, 100 mM EDTA pH 8.0). Six µl of lysozyme (3 mg ml^{-1}) was added and incubated at 37°C for 15 min. Then, 9 µl of proteinase K (150 mg ml^{-1}) and 60 µL of 10% sodium dodecyl sulfate (SDS) were added to the tubes and incubated at 37°C for 30 more minutes. After the addition of 120 µl 5M NaCl and 90 µl CTAB solution (10% CTAB, 0.7 M NaCl), the tubes were incubated at 65°C for 10 min., immersed into liquid nitrogen for 2 min., and incubated again for 2 min. at 65°C. The freeze-and-thaw steps were repeated three times. Nine hundred µl of phenol:chloroform:isoamylalcohol (25:24:1) (PCI) was added, mixed, and centrifuged at 16 000 g for 5 min. at 4°C. The aqueous phase was transferredto a new tube and one volume of PCI was added, vortexed, and centrifuged again (two to three times) until a clear interphase between the aqueous and the organic phases was observed. Finally, nucleic acids were precipitated with ethanol and resuspended in 50 µl of sterile deionized water. To check the quality of nucleic acids, they were run in 1% agarose (LE, FMC Products, Rockland, ME) gel and visualized under UV light after ethidium bromide staining. Extracts were stored at –85°C until used.

Cloning of PCR products. Ligation of the PCR products with the pCRII-TOPO vector, transformation of *Escherichia coli* TOP10, and selection of the transformants were carried out using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's protocol. 16S rRNA gene library was generated with the pooled products of at least three independent PCR reactions. Clones were screened for redundancies by amplified rDNA restriction analysis (ARDRA) (Vaneechoutte *et al*., 1992) with the enzymes *Hinf*I and *Mbo*I (New England Biolabs) as described before. Clones representing the different restriction patterns were selected for sequencing.

DGGE analysis. 16S rRNA gene fragments were PCR amplified from pooled samples for DGGE analysis with the following primer sets: 341F-GC (5'-GCclamp-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTRAGTTT-3') (Muyzer *et al*., 1993). The forward primer was supplied with a GC-clamp (CGCCCGCCGCGCGCG-GCGGGCGGGGCGGGGGCACGG GGGG) at the 5' end. The PCR program was: 94°C for 5 min., 65°C 1 min., 72°C 3 min., and nine touchdown cycles of: 94°C for 1 min., 65°C (with the decreasing 1°C each cycle) 1 min., 72°C 3 min., followed by 20 cycles of: 94°C for 1 min., 55°C 1 min., and 72°C 3 min. During the final cycle, the length of the extension step was increased to 10 min.

The PCR products were separated by DGGE on a Ingeny system. Two stock solutions were prepared, representing 0 and 100% denaturing agent, respectively. The 0% solution consisted of 10% (wt/vol) acrylamide-bisacrylamide (37.5:1) in 1X Tris-acetic acid-EDTA buffer (TAE), and the 100% solution consisted of 10% (wt/vol) acrylamide-bisacrylamide, 420 g of urea per liter, and 400 ml of formamide per liter in 1X TAE. The DGGE gels were cast by using mixtures of these stock solutions in linear denaturing gradients with 40% denaturing agent in the top and 70% in the bottom of the gels. The wells in each gel were loaded with 15 µl of PCR products, and the gels were run for 18 h at 70 V and 60°C. The gels were stained for 30 min. in ethidium bromide solution and evaluated on a transilluminator (Uvitec). Individual bands were excised and

Table I.
Bacterial isolates and their closest GenBank Matches.

ARDRA Pattern[a]	Selected Isolate	No. of Isolates	Closest GenBank Match		
			% Identity	Taxon	Accession no.
IB	C12	2	99%	*Halobacillus* sp.	AB189301
IIB	C13	1	99%	*Halomonas halophila*	042050
IIIB	C15	2	97%	*Virgibacillus marismortui*	GU213159
VIB	C17	2	94%	*Halobacillus* sp.	FM992846
VIIB	C18	2	97%	*Halomonas halophila*	FN257740
VIIIB	C20	3	98%	*Halomonas halophila*	FN257740
IVB	C22	2	99%	*Halobacillus* sp.	AB189301
VB	C25	3	99%	*Halobacillus* sp.	JX992844

[a] The ARDRA pattern is indicated by a roman numeral followed by B (Bacterial).

resuspend in 20 microliters Milli Q water and incubated at 4°C overnight. An aliquot of the supernatant was used for PCR reamplification with the original primer set, and 50 ng of reamplified PCR products were used for the sequencing reaction.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers KF863788 to KF863800 and KF938670 to KF938672.

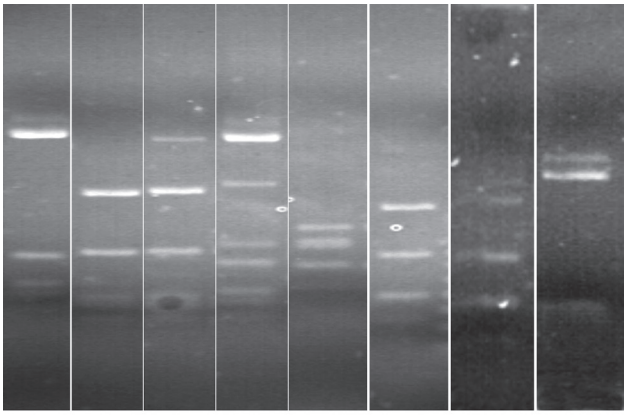

Fig. 2. ARDRA profiles of the Çamaltı Saltern isolates. *Hinf* I restriction products of *16S rRNA* gene PCR amplified DNA obtained from the isolates.

Results

Selection of bacterial isolates by ARDRA (Amplified Ribosomal DNA Restriction Analysis) and their identification. Fourty two colonies were selected according to their morphological characteristics such as size, shape and colour. Seventeen of them were found to be belonging to the *Bacteria* domain by PCR using *Eubacteria* specific primers. They were then characterized by 16S rRNA gene analysis. ARDRA profiles showed that these 17 Bacterial colonies yielded 8 different patterns (Table I). Fig. 2 shows *Hinf* I restriction products of 16S rRNA gene PCR amplified DNA obtained from the isolates. ARDRA profiles of isolates and their closest genbank matches were shown in Table I. High similarities to previously cultured halophilic bacteria, such as *Halobacillus* (99%), *Virgibacillus* (97%) and *Halomonas* (98%) were obtained in Gene-Bank. The sequences of isolates C12 and C25 were 99% identities with the genus *Halobacillus* sp. isolated from the Sahara Desert in Tunisia (Hua and Naganuma, 2007), and sea water in China (Acc. Number JX992844) respectively. The sequence of C17 was 94% identity with the genus *Halobacillus* sp. isolated from sediment in Greece (Gärtner *et al*., 2011). The sequence of the isolate C15 was related (97%) to *Virgibacillus* sp. isolated from Ebro Delta microbial mat in Spain (Villanueva *et al*., 2010). The sequences of the isolates C13, C18 and C20 were afiliated to the genus *Halomonas* with 99%, 97% and 98% similarity, respectively (Table I). Phylo-

Table II.
Clones and their closest GenBank Matches.

Selected Clone	No. of Clones	Closest GenBank Match		
		% Identity	Taxon	Accession no.
1A1	32	99%	*Salinibacter* sp. 5Sm6	AY987851.1
1A4	11	98%	*Salinibacter ruber* strain POLA 18	AF323502.1
1A5	7	92%	Uncultured bacterium clone 4–48B	EF459714.1

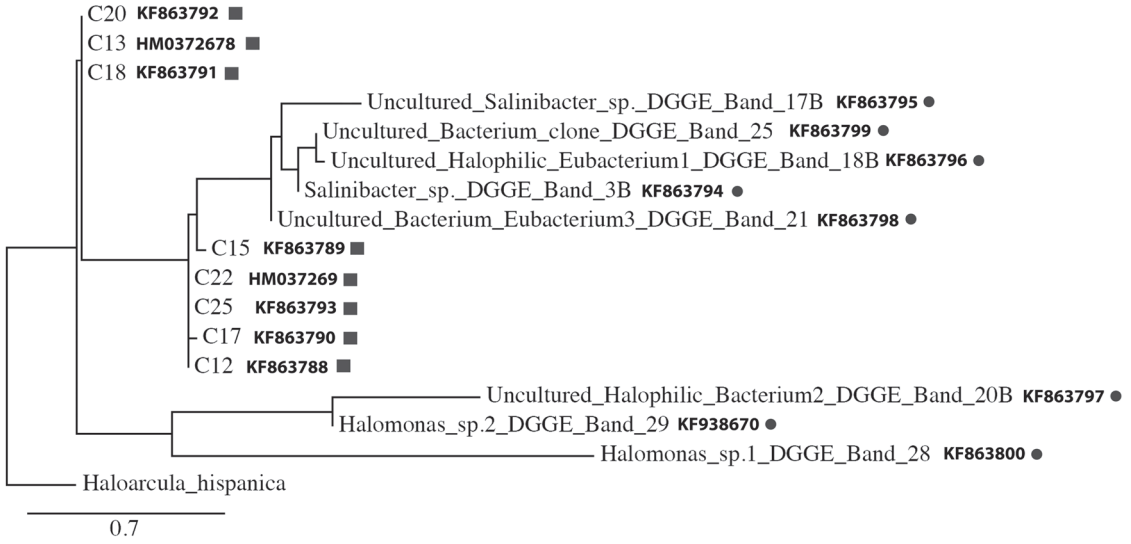

Fig. 3. Phylogenetic inferences based on *16S rRNA* gene sequences from isolates (indicated by green circle) and DGGE bands (indicated by blue circle) belonging to the halophilic bacteria.

genetic inferences based on 16S rRNA gene sequences from isolates (indicated by green circles) belonging to the halophilic bacteria were given in Fig. 3.

16S rRNA gene library construction. A total of 50 clones were analyzed by ARDRA, which yielded a total of three different patterns for Bacteria. At least one clone per restriction pattern was chosen for partial sequencing. The best match with the sequences in databases was obtained by BLAST analysis of the selected clones (Table II). Most (85%) of the bacterial clones were related to *Salinibacter* genus (*Bacteroidetes*).

DGGE fingerprint analyses. A total of 7 bands were identified using DGGE (Fig. 4). Most of the DGGE bands halted at between 50% and 60% denaturant concentrations. The sequences obtained from the bands yielded similarities to uncultured halophilic bacterium (bands 20B and 21B in Fig. 4) with high percentages of similarity (95% and 93% respectively) and three bands were closely related to the uncultured bacterium clone (bands 17B, 18B and 25B (72%, 98% and 97% respectively) (Fig. 4). Two bands yieldedsimilarity to *Halomonas* sp. and their sequences (28B and 29B) shared (83% and 99% respectively) sequence identity with the *Halomonas* sp. (Tsiamis *et al*., 2008) that was isolated from a Greek solar saltern. The sequencefrom Band 3B was affiliated to the genus *Salinibacter* and displayed relatedness (88%) to the sequence retrieved from the North Armof Great Salt Lake, USA (Acc. Number KF569484). The sequences from Band 17B and Band 18B were affiliated to the uncultured *Salinibacter* clone from Chula Vista Saltern California in USA (Zhaxybayeva *et al*., 2013) and uncultured bacterium clone from Guerrero Negro Solar Saltern in Mexico (Acc. Number KF741593) (82% and 98% similarity respectively). The sequence of the band 25B shared

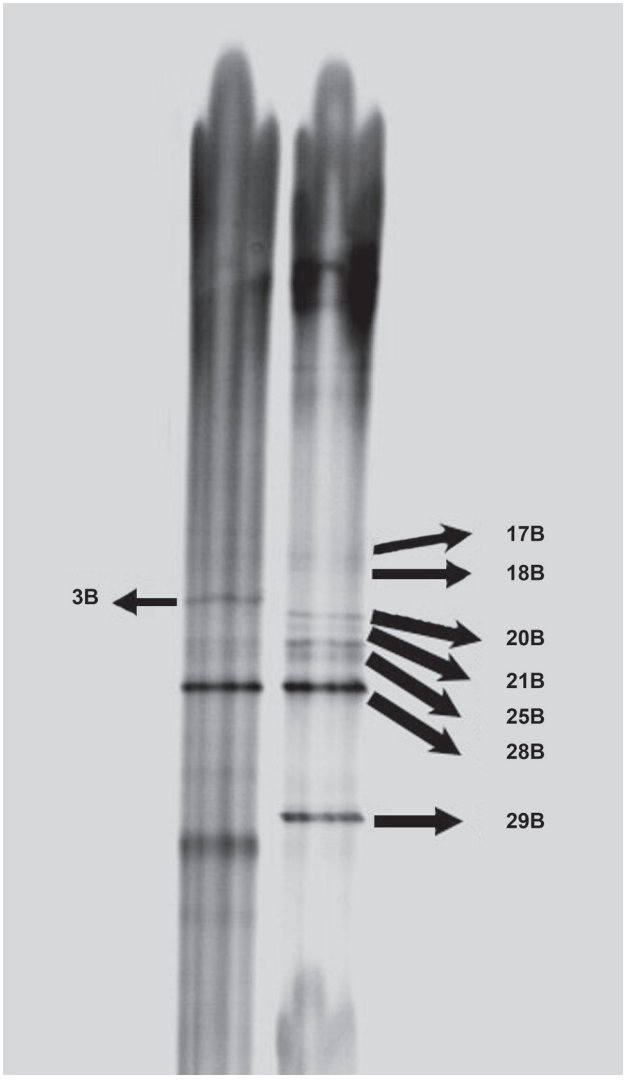

Fig. 4. DGGE analyses of partial *16S rRNA* gene fragments amplified with the general bacterial DGGE primer set, as described in the text. Sequenced bands are marked.

Table III
Obtained DGGE bands and their closest relatives in GenBank.

DGGE bands	% Similarity Closest relative in BLAST search of Gen Bank
Bacteria	
3B	88% KF569486 *Salinibacter* sp.
17B	82% JN839857 uncultured *Salinibacter* clone
18B	98% KF234381 uncultured bacterium clone
20B	95% FN994933 uncultured halophilic bacterium
21B	93% FN994932 uncultured halophilic bacterium
25B	92% JX881795 uncultured bacterium clone
28B	83% DQ873739 *Halomonas* sp.
29B	99% EU308363 *Halomonas* sp.

92% identity with the uncultured bacterium clone from Lake Tyrrel in Victoria Australia (Podell *et al.*, 2013). The sequences of Band 20B and Band 21B were 95% and 93 related respectively to the uncultured halophilic bacterium from Solar Saltern in Tunus (Baati *et al.*, 2008) (Table III). Phylogenetic inferences based on 16S rRNA gene sequences from DGGE bands (indicated by blue circles) belonging to the halophilic bacteria were given in Fig. 3.

Discussion

Multi-pond solar salterns represent ideal candidate model systems due to their managed nature, in which salt concentrations are kept relatively constant over time, in contrast to natural systems which are more susceptible to external variables such as climatic variation. Additionally, salterns exist around the world, albeit under somewhat different conditions. This provides a greater degree of international comparability than most natural systems (Burns *et al.*, 2007). This work contributes to our knowledge of prokaryotic communities of Çamaltı solar saltern located in the Aegean region of Turkey together with our previous studies (Mutlu and Güven 2011; Erdogmus *et al.*, 2013).

Several studies examined solar salterns by comparisons of polar lipid and pigment profiles (Litchfield *et al.*, 2001); comparisons of metabolic properties (Litchfield *et al.*, 2001); 16S rDNA sequencing from both denaturing gradient gel electrophoresis (DGGE) and clone libraries and FISH technique (Anton *et al.*, 1999; Anton *et al.*, 2000; Casamayor *et al.*, 2002; Burns *et al.*, 2004ab; Pašić *et al.*, 2007; Mutlu *et al.*, 2008; Hedi *et al.*, 2009). There have been many investigations of the archaeal, bacterial, and eukaryal inhabitants in these environments using both culture and culture-independent techniques (Javor *et al.*, 1982; Javor, 1984; Diez *et al.*, 2000; Benlloch *et al.*, 2002; Casamayor *et al.*, 2002; Litchfield and Gillevet, 2002; Ovreas *et al.*, 2003; Burns *et al.*, 2004ab; Maturrano *et al.*, 2006; Mutlu *et al.*, 2008; Rossello-Mora *et al.*, 2008).

The analysis of microbial diversity has shifted in the last two decades from cultivation-dependent approaches to 16S rDNA-based cultivation-independent approaches, which led to the discovery of many novel microbial taxa. Nevertheless, this approach also has important limitations and is often confined to naming 16S rDNA clones through sequence similarity and speculation on their ecophysiology on the grounds of this similarity. Therefore, cultivation is still the method of choice to understand fully the physiology and complex ecological interactions in which microorganisms engage (Gunde-Cimmerman *et al.*, 2005). Litchfield *et al.* (2009) reported that the microbial community in the waters of a solar saltern is variable and represantatives of the *Archaea* and *Bacteria* domains can be found throughout the saltworks.

The prokaryotic community of Çamaltı saltern was already investigated by two culture-independent methods, fluorescence*in-situ* hybridization (FISH) and Real Time PCR (Mutlu and Güven, 2011). DAPI counts of the samples fell in the range of $1.21–3.2 \times 10^7$ cells mL^{-1} indicating a variety of morphologies of cells (straight rods, curved rods, and cocci) and high salinity samples contained higher numbers of *Archaea*. FISH indicated that cells hybridized with the *Eubacteria* specific probe (EUB338) ranged from 48% to 67% of all DAPI-stained cells, and from 33% to 57% of all DAPI stained cells hybridized with the ARC915 probe (*Archaea* spesific probe) in the Çamaltı samples (Mutlu and Güven, 2011). However, limited archaeal diversity, *Haloferax* sp., *Halorubrum* sp., *Halobacterium* sp., and *Haloarcula* sp., were observed in culture-dependent assay (Erdogmus *et al.*, 2013).

The bacterial diversity described in Çamaltı saltern is similar to that described in other coastal solar salterns in the world. Since the saline water of solar salterns were found to be thalassohaline Lim *et al.* (2004), Tsiamis *et al.* (2008) and Hedi *et al.* (2009) detected *Halobacillus* sp. and *Halomonas* sp. in their samples from solar saltern in Korea, Greece and Tunisia respectively.

In this study, nine out of seventeen of the bacterial strains grown as pure colonies were related to *Halobacillus* sp., six out of seventeen were *Halomonas* sp. and only two strains were related to *Virgibacillus* sp. known as extreme halophiles growing in the presence of 10 to 30% total salts. The sequence of our isolate C17 had 94% identity with the genus *Halobacillus* sp. isolated from sediment in Greece (Gärtner *et al.*, 2011). If a threshold value of 16S rDNA similarity considered to be 90% for assignment to the genus *Halobacillus*, 16S rDNA sequence similarity (94%) of C17 indicate that this strain might be a novel species of the genus. There-

fore, further studies *e.g.* DNA:DNA homology should be carried out with different *Halobacillus* species.

Banding patterns identified in DGGE provide good insights in understanding the composition change of microbial communities. It is well known that most of the Bacteroidetes group is predominantly aerobic and is generally found in natural environments such as water and soil (Benlloch *et al*., 2002). In this study, most of the sequences obtained from DGGE for *Bacteria* and clone library yielded similarities to uncultured species of *Salinibacter* sp. which is a member of the Bacteroidetes group. However, we did not succeed incultivating any *Salinibacter*. *Salinibacter* representatives have been detected in saline environment using different techniques, with different levels of sensitivity (Anton *et al*., 2008). Contradictory results of same samples were obtained when analyzing the bacterial community inhabiting the hypersaline Tuz Lake in central Anatolia, Turkey. Although FISH counts gave very low numbers, sequences related to *Salinibacter* dominated bacterial 16S rRNA gene clone libraries and DGGE profiles (Mutlu *et al*., 2008).

It was reported that certain haloarchaea (*e.g. Halorubrum*) can inhibit *Salinibacter* growth (Anton *et al*., 2008). In a previous study (Erdogmuş *et al*., 2013), *Haloferax* sp., *Halorubrum* sp., *Halobacterium* sp. and *Haloarcula* sp. strains have been cultivated in Çamaltı Saltern and detected as dominant archaeal genera may have antogonistic effect on the cultivation of *Salinibacter* in this study.

More diversity among the bacterial isolates (*Halobacillus* sp., *Halomonas* sp., *Virgicacillus* sp.) than among the clones (*Salinibacter* sp.) was obtained in this study, supporting the observation that has been made previously for hypersaline environments by Benlloch *et al.* (2002) and Maturrano *et al.* (2006).

It is known that the microbiology of saltern systems in various parts of the world suggests a high degree of similarity (Oren, 1993) but some differences must occur as the result of changes in incident radiation, temperature, nutrient availability, residence time in the ponds (Litchfield *et al*., 2001). Tsiamis *et al.* (2008) observed microbial diversity by using a high-density oligonucleotide microarray (PhyloChip) as the part of their culture-independent studies of a Greek solar saltern located on the Agean coast just opposite of Çamaltı saltern at the same latitude. Similar to our results, most of the prokaryotic isolates recovered from hypersaline water (26% salinity) were extremely halophilic bacteria which were phylogenetically related to *Actinobacteria*, *Firmicutes* and δ-*Proteobacteria*in their study.

The genus *Halomonas* consists currently of 82 species (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=2745) although Vreeland *et al.* (1980) originally described the genus *Halomonas* with only one species as *H. elongata*.

In this study, extremely low diversity in terms of genera was obtained by culture independent assay results, whereas a high number of different species within the single genus may occur as it was reported by Benlloch *et al.* (2001).

The recent development of sequencing technologies generating massive amount of bioinformatic data has enabled us to assess much deeper layers of microbial communities at lower costs (Kim *et al*., 2013). Whole-genome molecular techniques offer a more comprehensive view of genetic diversity compared to PCR-based molecular approaches that target only a single or few genes. These techniques attempt to analyze all the genetic information present in total DNA extracted from an environmental sample or pure culture (Rastogi and Sani, 2011). New approaches such as pyrosequencing which is considered as PCR and cloning bias-free method have contributed significantly to the development of microbial ecology. Ghai *et al.* (2011) recently described the microbiota of two hypersaline saltern ponds, one of intermediate salinity (19%) and a NaCl saturated crystallizer pond (37%) using pyrosequencing. The analyses of these metagenomes (nearly 784 Mb) reaffirmed the vast dominance of *Haloquadratum walsbyi* but also revealed novel, abundant and previously unsuspected microbial groups such as of low GC *Actinobacteria*. Metagenomic assembly revealed three new abundant microbes: a low-GC euryarchaeon with the lowest GC content described for any euryarchaeon, a high-GC euryarchaeon and a gammaproteobacterium related to *Alkalilimnicola* and *Nitrococcus*. These discoveries showed the combined power of an unbiased metagenomic and single cell genomic approach (Ghai *et al*., 2011). Therefore these new PCR and cloning bias-free technique could be used to reveal prokaryotic communities in Çamaltı Saltern in future studies.

This is the first study in which both culture-dependent and culture-independent techniques have been used simultaneously to target unique regions of the 16S rRNA gene in samples obtained from Çamaltı solar saltern and *Halobacillus* sp. *Halomonas* sp. and *Virgibacillus* sp. isolates.

Since, halophilic prokaryotes have great potential in industrial use such as production of compatible solutes, biopolymers, and bioremediation processes (Ventosa *et al*., 1998; Margesin and Schinner, 2001; Mellado nad Ventosa, 2003) and aromatic hydrocarbon degradation by halophilic archaea (Erdogmus *et al*., 2013) prompts us to screen our collection of halophilic bacteria isolated from Çamaltı saltern in future studies of biodegration and other biotechnological applications.

Acknowledgements

This study wassupported by Anadolu University Scientific Research Projects Commission under the grant no: 071018.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 47–54

ORIGINAL PAPER

Sulfur Removal from Dibenzothiophene by Newly Isolated *Paenibacillus validus* Strain PD2 and Process Optimization in Aqueous and Biphasic (Model-Oil) Systems

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Submitted 6 August 2014, revised 20 December 2014, accepted 20 December 2014

Abstract

Dibenzothiophene (DBT) is an organic sulfur compound which remains in oil after hydrodesulfurization (HDS) process and can be removed by biodesulfurization (BDS). A new strain of *Paenibacillus validus* (strain PD2) was isolated from oil contaminated soils that is able to desulfurize DBT. HPLC analysis and Gibb's assay showed that this strain was capable to convert DBT to 2-Hydroxybiphenyl (2-HBP) as final product. The presence of *dsz*C gene confirmed that DBT desulfurization occurred through the 4S pathway. Maximum growth and the highest induction in *dsz* operon obtained in the presence of dimethyl sulfoxide (DMSO) as sole sulfur source. DBT concentration, temperature and pH were optimized statistically for growing and resting cells by using Response Surface Methodology (RSM). All parameters in growing cells had a significant effect on 2-HBP production during BDS of DBT by *P. validus* PD2, although in resting cells temperature in range of 20–40°C was not a significant factor. Maximum BDS for growing cells was obtained at 0.41 mM DBT concentration, pH 6.92 and temperature 31.23°C. For resting cells, optimum pH, temperature and DBT concentration were 6.62, 27.73°C and 7.86 mM respectively. The results of this study showed that high concentrations of DBT could be desulfurized by *P. validus* strain PD2 in model-oil. Thus, the isolated strain could be introduced as a proper candidate for biodesulfurization of organic sulfur in the oil industry.

Key words: *Paenibacillus validus*, 2-hydroxybiphenyl (2-HBP), dibenzothiophene (DBT), Response Surface Methodology (RSM)

Introduction

Sulfur is the third most common element in the crude oil composition after carbon and hydrogen which causes severe environmental pollutions. For example, burning the petroleum based fuels containing sulfur components will release SO_x gases to the atmosphere leading to acid rain and corrosion problems. Consequently, deep desulfurization of the crude oil is compulsory to prevent the production of such pollutants (Derikvand *et al*., 2014).

Hydrodesulfurization (HDS) process which has been usually employed in oil refineries, works at high temperatures and pressures (more than 300°C and 100 atm) (Ma *et al*., 2002) and is effective in removing a large amount of inorganic and a part of organic sulfur from the crude oil. However, the major part of organic sulfur content is resistant to HDS and remains in the oil composition after this treatment (Borgne and Quintero, 2003). As a remedy, biodesulfurization (BDS) process has been suggested to remove the recalcitrant organic sulfur compounds like dibenzothiophene (DBT) which is often used as a model of heterocyclic sulfuric compounds in biodesulfurization studies. In fact, BDS has several advantages which make it a potentially alternative or at least a complementary process to the HDS. For instance, BDS is more specific and cost effective and takes place at ambient temperature and pressure (Monticello, 2000). In addition, the combustion value of the oil would not be affected in the BDS process because the effective bacterial strains (such as *Rhodococcus* and *Gordonia*) can remove the sulfur through the 4S pathway, which attacks the C-S bounds selectively (Gou *et al*., 2002).

BDS through 4S pathway has been studied using different bacterial species such as *Gordonia* (Rhee *et al*., 1998), *Bacillus* (Kirimura *et al*., 2001), *Lysinibacillus* (Bahuguna *et al*., 2011) and *Rhodococcus* (Derikvand *et al*., 2014). Generally, there are four enzymes involved in the 4S pathway: first, DBT monooxygenase (DszC) oxidizes DBT to DBT-sulfone ($DBTO_2$). In the second step, flavomonooxygenase (DszA) catalyzes

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transformation of $DBTO_2$ to 2-2'-hydroxyphenyl benzene sulfinate (HPBS) and in the final step, HPBS is desulfinated by DszB to produce 2-hydroxybiphenyl (2-HBP) as the final product (Yan *et al*., 2000). Three catabolic genes, *dsz*A, B, C, are clustered in the *dsz* operon. The fourth enzyme (DszD) prepares $FMNH_2$ required for oxygenase reactions and is a chromosomal gene (Derikvand *et al*., 2013). Clearly, performance and activity of the enzymes in the 4S pathway is dependent on their operating conditions and a high BDS efficiency could be achieved in the optimum level of the effective parameters. Although BDS process has been studied for physiology of microorganisms and their gene modifications, metabolic pathways and kinetics of desulfurization in both model and diesel oils, the significance of operational factors and their optimization through statistical methods are rare. Response Surface Methodology (RSM) is one of the well known statistical methods which is utilized to find a relationship between a few effective variables and one or more responses in a system. In this approach, analysis of variance (ANOVA) and regression techniques have been employed to estimate a low degree polynomial model for optimization of the levels of significant explanatory variables in a limited number of experiments (Khurana *et al*., 2007).

In the present study, *Paenibacillus validus* PD2 which was isolated from an oil contaminated soil sample was employed to investigate the effects of important operational conditions such as initial DBT concentration, temperature and pH on the BDS efficiency. Box-Behnken RSM was used to determine the optimum value of these factors in BDS process for both growing and resting cells conditions in aqueous and biphasic (aqueous / model-oil) systems, respectively. Finally, the BDS efficiency was determined in the obtained optimum conditions.

Experimental

Materials and Methods

Chemicals. All chemicals were analytical grade and commercially available.

Enrichment, isolation and identification of DBT desulfurizing bacteria. Double deionized water was used to prepare sulfur free basal salt medium (BSM). The BSM contains the following composition (g/l): $Na_2HPO_4 7H_2O$ 8.0, KH_2PO_4 4.0, NH_4Cl 2.0, $MgCl_2$ 0.2, $FeCl_3$ 0.001 and $CaCl_2$ 0.001. More than 100 oil contaminated soil samples were collected from various areas in Iran. One gram of each soil sample was suspended in 50 ml of BSM supplemented with 10 g/l glucose and 0.3 mM DBT as the sulfur source in a 250 ml flask. After incubation for 3 days at 30°C and 180 rpm on a rotary shaker, 5 ml of supernatant was inoculated in 45 ml of fresh BSM supplemented with 0.3 mM DBT and 10 g/l glucose. Subculturing was repeated with 1% v/v inoculums five times. Afterwards, streak culture was done onto the same medium with 9 g/l agarose. Finally, a single colony was isolated and its 2-HBP production was detected by Gibb's assay. One of the Gibb's positive isolated strains named PD2, was genetically identified by partial 16S rDNA gene sequencing using DG74 and RW01general primers.

Detection of *dsz*C gene from *dsz* operon. Briefly, chromosomal DNA of *P. validus* was extracted (by DNA extraction kit, Fermentas) and separated on 1% agarose gel with 1X TAE buffer (Promega, Germany) at 80 V for 40 min. The extracted chromosomal DNA was amplified by PCR with applying specific *dsz*C primers as forward 5'-GACACTGTCACCTGAAA-3' and reverse 5'-CTCAGGAGGTGAAGCCG-3' primers. The amplified gene separated on 1% agarose gel with 1X TAE buffer (promega, Germany) at 80 V for 40 min. The amplified gene was purified using gel extraction kit (Promega Germany). The quantity and quality of the purified gene was assessed by optical density at 260 nm and 280 nm and by electrophoresis in 1% agarose gel (Promega, Madison, WI, USA). The correct orientation and nucleotide sequence of the gene was verified by double-strand sequencing.

Characterization of growth and biodesulfurization. Growth rate, DBT consumption and 2-HBP production by isolated *P. validus* PD2 were measured by turbidimetry (600 nm), UV spectrophotometry (323.8 nm) and Gibb's assay (610 nm wavelength) respectively for 4 days incubation period. The culture media was BSM supplemented with 0.3 mM DBT and 15 g/l glucose incubated at 30°C on a rotary shaker.

Biodesulfurization in the liquid media by growing cells. For growing cells, the strain was inoculated in BSM supplemented with different amounts of DBT and 10 g/l glucose and 2-HBP production was measured after 48 h incubation time.

Biodesulfurization in the biphasic media by resting cells. The biphasic media consist of BSM (aqueous phase), n-tetradecane (organic phase) and different amount of DBT as a sulfur source, were prepared to monitor the BDS activity by the resting cells. The ratio of aqueous to oil phase volume was 2:1. 2-HBP production was determined after 20 h.

Effect of different sulfur sources on growth and BDS rate. Several sulfur sources such as DBT, thiophene, dimethyl sulfoxide (DMSO) and $MgSO_4$ were added to 50 ml BSM in concentrations equal to 0.3 mM, 1 µl/ml, 200 mg/l and 200 mg/l respectively. The carbon source was 10 g/l glucose. 0.5 ml of cell suspension with $OD_{600} = 1$ was inoculated to each medium and growth rate and 2-HBP production were determined after 30 h.

Table I
Coded values of experimental variables in growing and resting cells of PD2 strain

Independent variables	Growing cells			Resting cells		
	–1	0	+1	–1	0	+1
X_1: DBT concentration (mM)	0.2	0.5	0.8	2	6	10
X_2: Temperature (˚C)	25	30	35	20	30	40
X_3: pH	6	7.5	9	5	7	9

Table II
Response surface Box-Behnken design (BBD) and the rate of produced 2-hydroxybiphenyl (2-HBP) in aqueous system by growing cells and biphasic system by resting cells.

Run	A*			B**			2-HBP (mM) Growing cells	2-HBP (mM) Resting cells
	X_1	X_2	X_3	X_1	X_2	X_3		
1	0	1	–1	0	1	1	0.151	0.41
2	1	1	0	1	0	–1	0.112	0.66
3	0	–1	1	0	–1	–1	0.111	0.51
4	1	0	–1	1	1	0	0.113	0.55
5	0	–1	–1	1	–1	0	0.118	0.51
6	0	0	0	1	0	1	0.198	0.59
7	1	0	1	–1	0	1	0.097	0.44
8	–1	–1	0	0	0	–1	0.121	0.88
9	0	1	1	–1	–1	0	0.119	0.45
10	–1	**0**	**1**	**0**	**1**	–1	0.133	0.49
11	1	–1	0	0	0	0	0.085	0.84
12	–1	0	–1	–1	0	–1	0.158	0.51
13	0	0	0	0	0	0	0.192	0.89
14	0	0	0	0	–1	1	0.201	0.42
15	–1	1	0	–1	1	0	0.146	0.34

*A: growing cell; **B: resting cell

Experimental design for biodesulfurization by growing and resting cells. DBT concentration (X_1), temperature (X_2) and pH (X_3) were considered as the important factors in biodesulfurization activity of growing and resting cells. A 3-factor and 3-level Box-Behnken design (BBD) was used to determine the optimum level of the important factors and to study their relationship to the BDS efficiency. Factors and their levels are shown in Table I. For resting cells design, we increased the range between high and low levels of factors (Table I) to better illustrate the impact of variables. All factors at middle (0) level constitute the central points while combination of factors consisting of one at its lowest level (–1) or highest level (+1). A 0.05 significance level was assumed to perform ANOVA for the model coefficients. A total of 15 experimental runs of three factors in different combinations were carried out in duplicates (Table II). All experimental design and data analysis were performed using Design Expert software package version 8.0.1.

Analytical methods. Cell density was measured by spectrophotometry at 600 nm (OD_{600}) (spectronic 21D Milton Roy). In the growing cells system, BDS activity was monitored using the Gibb's reagent (2,6-dichloroquinone-4-chloroimide). To measure the DBT consumption in the growing cells samples, their pH was reduced to 2 before mixing with equal volume of ethyl acetate. After centrifuging at 3000 rpm for 5 min., dissolved DBT in the organic phase was measured by UV spectrophotometry at 328 nm. In the resting cells system, HPLC was used to determine the amounts of DBT (retention time = 5.29 min.) and 2-HBP (retention time = 3.16 min.) in n-tetradecane phase. HPLC was performed on a KNAUER advanced scientific instruments (Germany) equipped with an MZ-analysentechnic C18 column (5 µ–250 mm) and a UV detector (Smartline 2600) set at 254 nm. The mobile phase was methanol-water (90:10, v/v) with a flow rate of 1.5 ml/min.

For all of the analytical methods standard curves of DBT and 2-HBP were prepared.

Results and Discussion

Identification of the strain PD2. The numerous bacterial colonies isolated from diesel contaminated soils were checked for 2-HBP production from DBT as sole sulfur source. The Gibb's analysis showed that a strain designated as PD2 was able to produce 2-HBP as a final product. Since 2-HBP production indicates selective cleavage of carbon-sulfur bond, further characterization and optimization was performed on this strain. Gram- and acid-fast staining, colony morphology and biochemical activities revealed the PD2 strain as Gram-positive, spore-forming and non-acid fast bacillus. Partial nucleotide sequence analysis of 16S rRNA gene implied 99% homology with *P. validus* SB3263 (NCBI Gene Bank Accession No. GU191921). Therefore, PD2 was identified as a novel strain *P. validus* PD2 and deposited in NCBI (NCBI Gene Bank Accession No. KC161368).

By now, several aerobic DBT-desulfurizing bacteria have been reported to date that are able to desulfurize DBT *via* 4S pathway. In particular, *Rhodococcus erythropolis* IGTS8, the first strain found able to desulfurize DBT through 4S pathway, has been widely used for BDS and the genes related to this pathway have been studied (Denome *et al*., 1993). However, according to our knowledge, no studies have ever been reported using *P. validus* cells for DBT biodesulfurization *via* 4S pathway. Therefore, strain PD2 is the first *P. validus* strain reported to be capable of DBT desulfurization, without carbon skeleton cleavage.

Characterization of growth and biodesulfurization. The growth curve of *P. validus* PD2 has been shown in Fig. 1. The growth was continued for 96 h, simultaneously with the consumption of DBT and the production of 2-HBP. As shown in Fig. 1, desulfurization of DBT to 2-HBP in the induced cells of PD2 was detected after 8 hours, afterwards, the concentration of 2-HBP in the medium increased up to 0.27 mM at 50 hours (initiation of stationary phase). The production of 2-HBP is less than DBT consumption which as previously suggested by Caro *et al*. (Caro *et al*., 2007), this is due to accumulation of 2-HBP and other 4S pathway compounds inside and on the surface of the cells.

Detection of *dszC* gene in *P. validus* PD2. The 1300 bp PCR product of amplified *dsz*C gene by specific primers (Fig. 2) purified from the gel and sequenced by double-strand sequencing. The length of sequenced fragment was 1153 bp. Sequenced DNA was blasted in NCBI GenBank. The blast result showed that *dsz*C in *P. validus* PD2 had 98% similarity with *dsz*C gene in *R. erythropolis* FMF. This sequence was submitted in NCBI by accession number KF056797. The presence of *dsz*C gene confirmed that DBT desulfurization takes place through the 4S pathway which produced 2-HBP as final product without cleavage of carbon ring; therefore, the strain PD2 could be used for BDS of oil without decrease in octane rate.

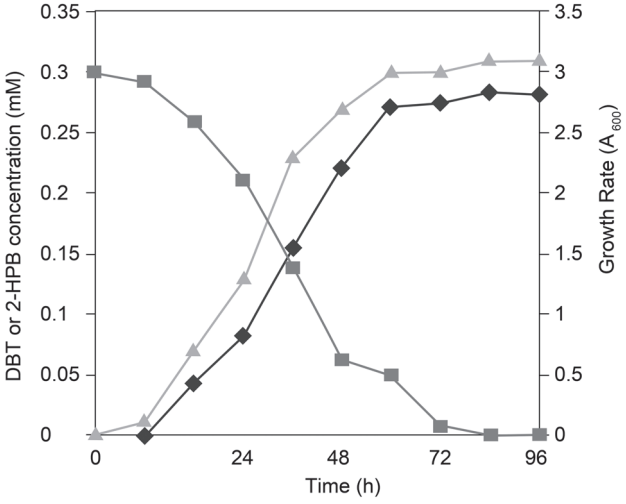

Fig. 1 Growth of *Paenibacillus validus* PD2, DBT utilization and 2-HBP production during cultivation at 30°C and 180 rpm shaking. The medium contained 0.3 mM DBT as sole sulfur source and glucose as carbon source. Symbols: ■, DBT concentration (mM); ♦, 2-HBP concentration (mM); ▲, growth (OD_{600nm}).

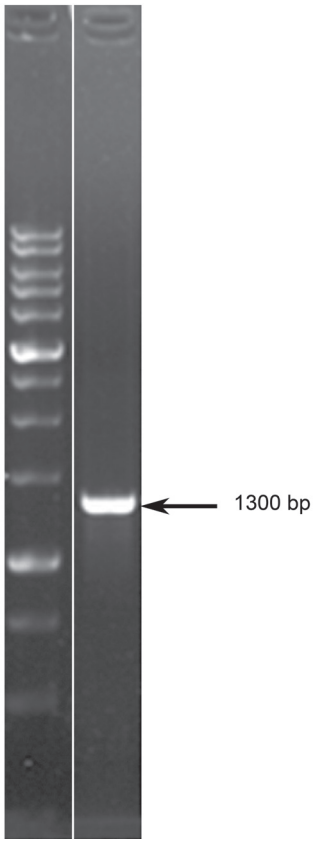

Fig. 2. PCR amplification of 1300 bp fragment of *dsz*C gene from isolated *Paenibacillus validus* PD2. M: GeneRuler 1 kb DNA ladder (Fermentas).

Effect of different sulfur sources on growth and biodesulfurization rate. *P. validus* PD2 was cultured in

Table III
Growth rate of isolated strain PD2 in the presence of different sulfur sources and 2-HBP production from DBT after cultivating of induced cells in 0.3 mM DBT.

Sulfur source	Growth (A_{600})	2-HBP (mM)
DBT	*2.39*	0.21
Thiophene	*0.83*	0.11
DMSO	*3.94*	0.25
Sulfate	*3.39*	0.09

the presence of different sulfur compounds. As shown in Table III, a higher cell density was obtained when DMSO was used as the sole sulfur source, because of more easily metabolization of DMSO than others sulfur sources (Bustos-Jaimes *et al*., 2003). Moreover, the growth rate can be inhibited by production of 2-HBP when DBT is used as the sulfur source (Setti *et al*., 2003). In fact, induction of *dsz* operon by DMSO in *Rhodococcus* sp. 1AWQ has been shown to be similar to DBT (Ma *et al*., 2006) while Mohebali *et al*. (2008) showed that 2-HBP production of resting cells grown on DMSO was higher than that of DBT. The results indicated that the cell density in the presence of $MgSO_4$ was more than DBT and thiophene. This occurs due to the lower water solubility of DBT and thiophene. Although the *dsz* operon can be repressed by $MgSO_4$ as a sole sulfur source during the growth (Li *et al*., 1996), PD2 grown on sulfate showed low BDS activity that could be explained by re-expression of *dsz* operon. Finally, the maximum BDS activity of the PD2 strain resting cells was achieved when they were grown on DMSO.

Statistical analysis. Growing cells: A quadratic polynomial equation was estimated to describe the relationship between 2-HBP production of growing cells and variables based on the experimental results of BBD (Table II). All of the main and interactive effects can be simultaneously investigated by response surface model. The model of coded units is calculated using follow:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} \beta_{ij} X_i X_j$$

where Y is the predicted response, X_i is the variable, β_0 is constant, β_i is the linear effect, β_{ii} is the quadratic effect, and β_{ij} is the interaction effect.

In the growing cells system, the empirical model of coded variables can be expressed as the equation 1 after removing non significant parameters:

Equation 1: $Y = 1.97 - 0.19X_1 + 0.12X_2 - 0.100X_3 - 0.063X_2X_3 - 0.40X_1^2 - 0.41X_2^2 - 0.32X_3^2$

Where Y is the response value (mM), X_1 is the initial DBT concentration (mM), X_2 is temperature (°C) and X_3 is pH. Positive and negative sign before each term indicates synergistic and antagonistic effects respectively (Tan *et al*., 2010).

The ANOVA results for 2-HBP production by growing cells have been shown in Table IV. The Model F-value was 142.23 that imply its significance and there is only a 0.01% chance that a Model F-value this large could occur due to the noise factors. Also values of Prob > F (P value) less than 0.05 indicate model terms are significant. In this case, X_1, X_2, X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant model terms (P values < 0.05). The proposed model was proved to be adequate through calculation of its Lack of Fit equal to 0.49 and adjusted determination factor (A*dj* R^2) equal to 0.9891. This

Table IV
Analysis of variance (ANOVA)

Source of variance	df	Growing cells			Resting cells		
		Mean square	F value	P value	Mean square	F value	P value
Model	9	0.21	142.23	<0.0001	0.048	130.21	< 0.0001
X_1	1	0.29	195.88	<0.0001	0.041	111.27	0.0001
X_2	1	0.11	74.30	0.0003	1.250E-003	3.42	0.1235
X_3	1	0.080	54.98	0.0007	0.012	32.91	0.0023
X_1X_2	1	1.000E-004	0.069	0.8037	5.625E-003	15.41	0.0111
X_1X_3	1	2.025E-003	1.39	0.2912	0.000	0.000	1.0000
X_2X_3	1	0.016	10.74	0.0220	2.500E-005	0.068	0.8040
X_1^2	1	0.60	411.12	< 0.0001	0.092	250.94	< 0.0001
X_2^2	1	0.61	421.40	< 0.0001	0.23	632.24	< 0.0001
X_3^2	1	0.37	251.80	< 0.0001	0.098	267.12	< 0.0001
Residual	5	1.455E-003			3.650E-004		
Lack of fit	3	1.025E-003	0.49	0.7252	1.417E-004	0.20	0.8876
Pure error	2	2.100E-003			7.000E-004		

Growing cells: R2 = 0

means that the Lack of Fit is not significant relative to the pure error and the model predictions were well fitted the experimental results. According to the present model, all of the main factors were significant in the interval of variation and temperature has significant interactions with pH.

Resting cells: The relationship between the 2-HBP production of resting cells and the studied variables after removing non significant parameters stated as equation 2.

Equation 2: $Y = 0.87 + 0.071X_1 - 0.039X_3 - 0.038X_1X_2 - 0.16X_1^2 - 0.16X_3^2$

In equation 2, all of the coded variables are defined similar to equation 1 in the previous section. The ANOVA results for 2-HBP production by resting cells have been shown in Table IV. P-value of the model was obtained equal to < 0.0001, which warrantees the model significance. In this case, X_1, X_3, X_1X_2, X_1^2 and X_3^2 were significant model terms. The Lack of Fit value of 0.2 indicated the Lack of Fit was not significant relative to the pure error and the adjusted coefficient of determination ($AdjR^2 = 0.9881$) confirmed the significance of the model. DBT initial concentration, pH and also the interactions between DBT concentration and temperature were significant in the BDS by resting cells (Table IV).

RSM analysis. The response surface and its contour plots at the base can represent the regression model developed to investigate the interaction between factors and specify the optimum level of each factor. The interaction of two independent factors can be shown by each response surface with a contour plot, while another factor is fixed at the level of zero. The fitted surface and contour plots between DBT concentration and temperature, DBT concentration and pH, temperature and pH are presented in Fig. 3 and Fig. 4 for growing and resting cells systems, respectively.

Effects of DBT initial concentration: Results indicated that an increase in the initial DBT concentration would make DBT more available to the cells and led to an enhancement in BDS. However, at higher initial concentrations of DBT, bacterial growth and BDS activity would be inhibited, presumably because of the toxicity of DBT at high concentrations beyond the tolerating level of the bacteria (Ansari *et al*., 2007). Such inhibitory effect of DBT has been reported formerly (Ohshiro *et al*., 1995). It is known that only water soluble compounds can play an inhibitory role on the cell activity. DBT has an extremely low solubility in water and expected to precipitate in aqueous medium or fully dissolved in the organic phase of biphasic medium and thus, the initial concentration of DBT expected to have an insignificant effect on the cell or enzyme activity. However, most bacterial species with DBT biodesulfurization activity secrete some biosurfactants to solubilize and enhance the bioavailability of DBT (Kim *et al*., 2004). Also, recombinant *Pseudomonas* strains with high production of biosurfactant have been developed, which could enhance biodesulfurization activity (Gallado *et al*., 1997). Therefore, by secretion of biosurfactants inhibitory effect of DBT would not be limited. Fig. 3B shows that the optimum concentration of DBT in growing medium for PD2 strain was 0.41 mM and BDS activity was reduced by both the increasing initial DBT concentration up to 0.8 mM or decreasing it to 0.2 mM due to the limit in the growth rate. Also in biphasic medium and resting cells system, the optimum concentration was determined at 7.86 mM and as can be seen in Fig. 4B, the BDS activity was reduced by going away from the optimal point. For the resting cells, the optimum point for the initial DBT concentration was far more than the growing cells. In fact, in the biphasic medium, DBT can be dissolved in n-tetradecane (organic phase) which reduces its toxic effect on bacteria.

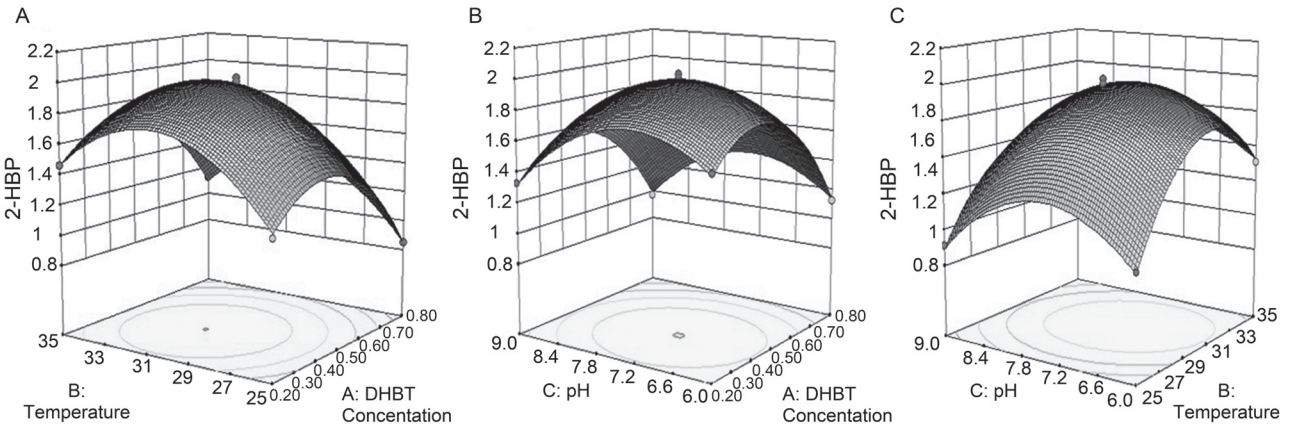

Fig. 3. The response surface and contour plot of 2-HBP production (mM) by growing cells of *Paenibacillus validus* PD2 in aquatic system. A: optimum temperature (°C), B: DBT concentration (mM), C: effect of pH on 2-HBP production.

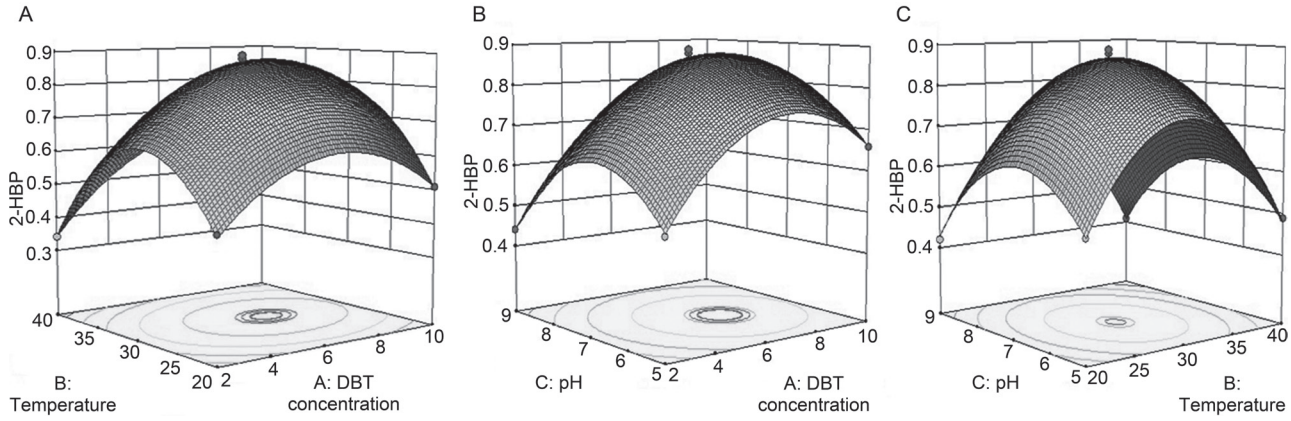

Fig. 4. The response surface and contour plot of 2-HBP production (mM) by resting cells of *Paenibacillus validus* PD2 in biphasic system (tetradecane-water). A: optimum temperature (°C), B: DBT concentration (mM), C: pH effect on 2-HBP production.

Effects of temperature: Temperature is a potentially limiting factor like essential chemical elements and organic substrates. In particular, temperature should be studied as an interactive factor, because it affects all chemicals and biochemical processes (Ratkowsky *et al*., 1982). *P. validus* PD2 is a mesophilic bacteria and its optimum temperature for BDS of DBT in growing medium was determined at 31.23°C. The surface and contour plot in Fig. 3A indicates that in high and low temperatures, 2-HBP production was low because of the limiting influence of temperature on the growth rate and enzyme activity. In resting cells, the optimum temperature for the 2-HBP production was 27.73°C (Fig. 4A). Although the growth rate limiting effect was absent in resting cells, the activity of enzymes can be reduced at the higher or lower temperatures. It has been indicated that in biodesulfurization by *Mycobacterium phlei* WU-F1, degradation of DBT to $DBTO_2$ stops at high temperatures while degradation of $DBTO_2$ and other intermediate compounds maintain at this condition. It can be concluded that the activity of the first enzyme oxidizing DBT to $DBTO_2$ is sensitive to temperature changes (Furuya *et al*., 2001). In fact, Furuya *et al*. suggested that the first and third enzymes in 4S pathway (DszC and DszB) are more sensitive to temperature changes in comparison with the other enzymes and are considered as the BDS rate-limiting enzymes.

Effects of pH: The pH, like other factors in culture medium such as temperature and carbon source, is an effective parameter that controls the bacterial growth rate (Madigan *et al*., 2012). In addition, enzymes are affected by the pH variations in the system because of the dependence of the 3-D shape of enzymes on the pH. The impact of pH variation is not devoted to only the shape of enzymes. However, it may also affect the shape or electrical charge properties of the substrate in such a way that the substrate cannot bind to the enzyme active site or it cannot undergo catalysis (Berg *et al*., 2007). Previous studies showed that the highest DBT biodesulfurization by the bacterial strains was obtained at pH near to neutral (Kirimura *et al*., 2001; Etemadifar *et al*., 2008; Ardakani *et al*., 2010). Also, it has been indicated that the purified 4S pathway enzymes such as flavin reductase are active only at the pH 6–8 (Matsubara *et al*., 2001). Figure 3C and Fig. 4C shows the surface and contour plots of pH for 2-HBP production in growing and resting cells systems, respectively. The optimum pH in growing condition was 6.92 and as shown in Fig. 3C, by a change in pH, 2-HBP production was reduced. In resting cells BDS of model oil, the optimum pH was equal to 6.62 (Fig. 4C).

In conclusion, this study demonstrated that the newly isolated strain *P. validus* PD2 can catalyze the conversion of DBT to 2-HBP in aqueous and biphasic systems *via* the 4S pathway. The response surface methodology showed that the optimum DBT concentration in aqueous and biphasic system was 0.41 and 7.86 mM respectively. Also in both media, the optimum temperature was mesophilic and the optimum pH was near the neutral. Therefore *P. validus* PD2 can be efficiently used for oil BDS under ordinary conditions and high activity can be obtained by setting the effective parameters at the optimum levels.

Acknowledgments

We appreciate the financial support for this work from the University of Isfahan .

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 55–59

SHORT COMMUNICATION

Recombinant MAG1 Protein of *Toxoplasma gondii* as a Diagnostic Antigen

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Submitted 8 August 2014, revised 24 December 2014, accepted 24 December 2014

Abstract

The aim of this study was to evaluate the potential diagnostic usefulness of the full-length recombinant *Toxoplasma gondii* MAG1 protein by determining the levels of specific IgM and IgG antibodies in mouse and human sera obtained from individuals with acute and chronic toxoplasmosis. The obtained results revealed that IgG antibodies against MAG1 are a sensitive and specific marker of *T. gondii* infection since the protein was recognized by both mouse and human sera, 100% and 94.3%, respectively, rendering the full-length rMAG1 a prospective alternative for the polyvalent native antigen (TLA).

Key words: *Toxoplasma gondii*, diagnostics of toxoplasmosis, ELISA, MAG1 antigen

Clinical diagnosis of *Toxoplasma gondii* infection, one of the most common parasitic zoonoses worldwide, relies primarily on a two-step procedure involving detection of specific antibodies (mostly IgM and IgG) and consecutive estimation of IgG avidity to distinguish between acute and chronic invasion in individuals with circulating anti-*Toxoplasm*a IgM immunoglobulins. Despite certain disadvantages, serological tests, in fact simple and relatively inexpensive, remain the main method for laboratory recognition of toxoplasmosis (Montoya, 2002). Most commercial tests use the *Toxoplasm*a lysate antigen (TLA) obtained from *in vivo* or *in vitro* cultured tachyzoites, which is costly and differs from batch to batch. These drawbacks may be overcome with the use of recombinant proteins offering an efficient, well-defined and standardized diagnostic tool enabling the comparison of results from different laboratories, reliable estimation of specific antibodies in samples taken from one patient at long time intervals and, possibly, discrimination between acute and chronic invasion. To date, several *T. gondii* recombinant proteins have been developed and assessed for their diagnostic utility (Kotresha and Noordin, 2010; Holec-Gąsior, 2013).

The 65 kDA MAG1 antigen of *T. gondii* is localized mainly in the matrix and the wall of tissue cysts and is considered a bradyzoite marker, however, it is also synthetized in tachyzoites (Ferguson and Parmley, 2002). Previously this antigen was tested individually, as a part of an antigen mixture or even as a chimeric protein revealing discrepancies which may have resulted from: different test models (humans and animals), applying various fragments of the amino acid sequence and the estimation of only one class of specific antibodies (Di Cristina *et al*., 2004; Holec-Gąsior *et al*., 2012, 2014; Xiao *et al*., 2013). Thus, the objective of the present study was to evaluate the diagnostic value of the full-length rMAG1 antigen expressed in the *Escherichia coli* system to detect both IgM and IgG antibodies, using concurrently human and mouse immune sera, the latter isolated at a defined time point of an experimental infection.

For the cloning of the recombinant MAG1 antigen standard molecular biology protocols were used (Sambrook and Russell, 2001). Briefly, the 3' fragment of *mag1* gene (U09029) of *T. gondii* encoding 424 amino acids (from 33aa to 452aa) and including 3 amino acids of exon 2 and the whole exon 3 was PCR amplified using the *T. gondii* RH strain DNA as a template and cloned initially into a pJET1.2/blunt vector (CloneJET PCR Cloning Kit, Fermentas), verified by sequencing and finally cloned into the pHis expression vector

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using *BamHI* and *HindIII* restriction enzymes since the restriction enzyme recognition sites were incorporated into a sequence of primers (underlined sequences) (5'-GGATCCGCTGAGCCAAAGGGTGCCAGAGCTAC-CAGAAGTG; 5'- AAGCTTTCAAGCTGCCTGTTC-CGCTAAGATC). The resultant vector pHis-mag1 was introduced into *E. coli* BL21 (DE3) cells to express the recombinant protein containing a 6-His tag within an extra 28 amino acids at the N-terminus. The expression and purification of rMAG1 were performed using a protocol described previously (Dziadek *et al.*, 2011). Briefly, transformed *E. coli* BL21(DE3) cells were grown in LB broth containing ampicillin and IPTG as an inductor and next they were harvested and lysed. The recombinant MAG1 protein, present in inclusion bodies, was isolated using the His-Bind Buffer Kit (Novagen) containing 6 M of urea, under denaturing conditions, and then purified by Ni(2+) affinity chromatography on His-Bind columns (Novagen), according to the manufacturer's procedures. The efficacy of purification was confirmed by 12% SDS-PAGE, staining with Imperial Protein Stain (Pierce) and Western-blot using anti-His antibodies (Novagen) (Fig. 1). Although two bands corresponding to the theoretical mass of 49.7 kDa were detected, the main portion of the protein formed a band of approximately twice the predicted mass, as noted previously (Hiszczyńska-Sawicka *et al.*, 2010). The amount of recombinant MAG1 was evaluated by the Bradford technique, yielding approximately 30 mg per liter of culture.

To obtain mouse immune sera 10–12 week-old male C57BL/6 (naturally more susceptible to *T. gondii* infection) and BALB/c (naturally more resistant to *T. gondii* infection) inbred mice, were inoculated intraperitoneally with 5 low virulent *T. gondii* DX strain cysts isolated from the brain of a latently infected C57BL/6 mouse by mechanical homogenization of tissues and gradient separation, as described before (Gatkowska *et al.*, 2006). Serum samples were collected from uninfected animals (negative controls) and mice with acute (3 weeks post inoculation) and chronic (6–8 weeks post inoculation) toxoplasmosis. All experimental groups consisted of 10 animals and each procedure was approved by 9. Local Ethics Commission in Łódź. Human serum samples were obtained from routine diagnostic laboratories and were classified as positive or negative based on the levels of specific IgM and IgG antibodies according to the diagnostic test description. Furthermore, all *T. gondii* positive sera were divided based on their IgG avidity value (determined either by the diagnostic laboratories or in our laboratory with commercially available NovaLisa™ *T. gondii* IgG Avidity Test, NovaTec Immundiagnostica GmbH) into an acute (n = 33, IgM^+ and IgG^+) and a chronic (n = 72: 34 IgM^+ and IgG^+; 38 IgM^- and IgG^+) group. Sera considered seronegative were used as controls (n = 21).

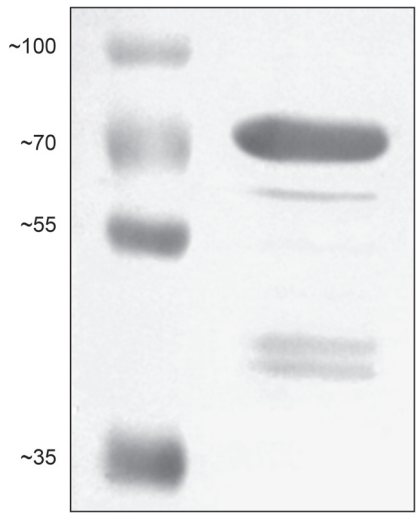

Fig. 1. Western blot analysis of the rMAG1 protein. Recombinant protein was detected using specific anti-His antibodies (line 2) and compared to the protein marker (line 1).

To determine the amount of specific anti-MAG1 antibodies in the test sera, the immunoenzymatic test was used according to the procedure described previously (Gatkowska *et al.*, 2006). Briefly, the serological MaxiSorp plates (NUNC) were coated with the recombinant MAG1 antigen, at the optimal concentration of 0.5 μg/well, established in the preliminary experiments. The immune and control sera were diluted 1:100 and the immunoenzymatic reaction was developed with goat anti-human and anti-mouse IgM or IgG antibodies (Jackson ImmunoResearch) labeled with horseradish peroxidase-HRP, the chromogene – ABTS (Sigma-Aldrich) and H_2O_2 as a substrate for HRP (Sigma-Aldrich). From the absorbance values obtained for negative control sera, both human and mouse, the cut-off values were calculated as the mean absorbance + 2 standard deviations. Immune sera reactivity with the recombinant MAG1 antigen was considered positive if the OD exceeded the cut-off value. The statistical analysis of the results was performed with the U Mann-Whitney test and differences were significant for $p < 0.05$.

Since the aim of this study was to evaluate whether the full-length recombinant MAG1 antigen may allow both detecting *T. gondii* invasion and discerning its phase, the levels of specific anti-MAG1 IgM and IgG antibodies were determined in human and mouse sera. The results obtained in this work revealed that both mouse strains, regardless of their genetic background, in the course of natural *T. gondii* invasion produced both IgM and IgG antibodies reacting with the recombinant MAG1 protein. As presented in Fig. 2, the response to MAG1 was detected as early as in acute infection in all subjects and it was represented by IgM and high levels of IgG antibodies ($p < 0.001$, compared

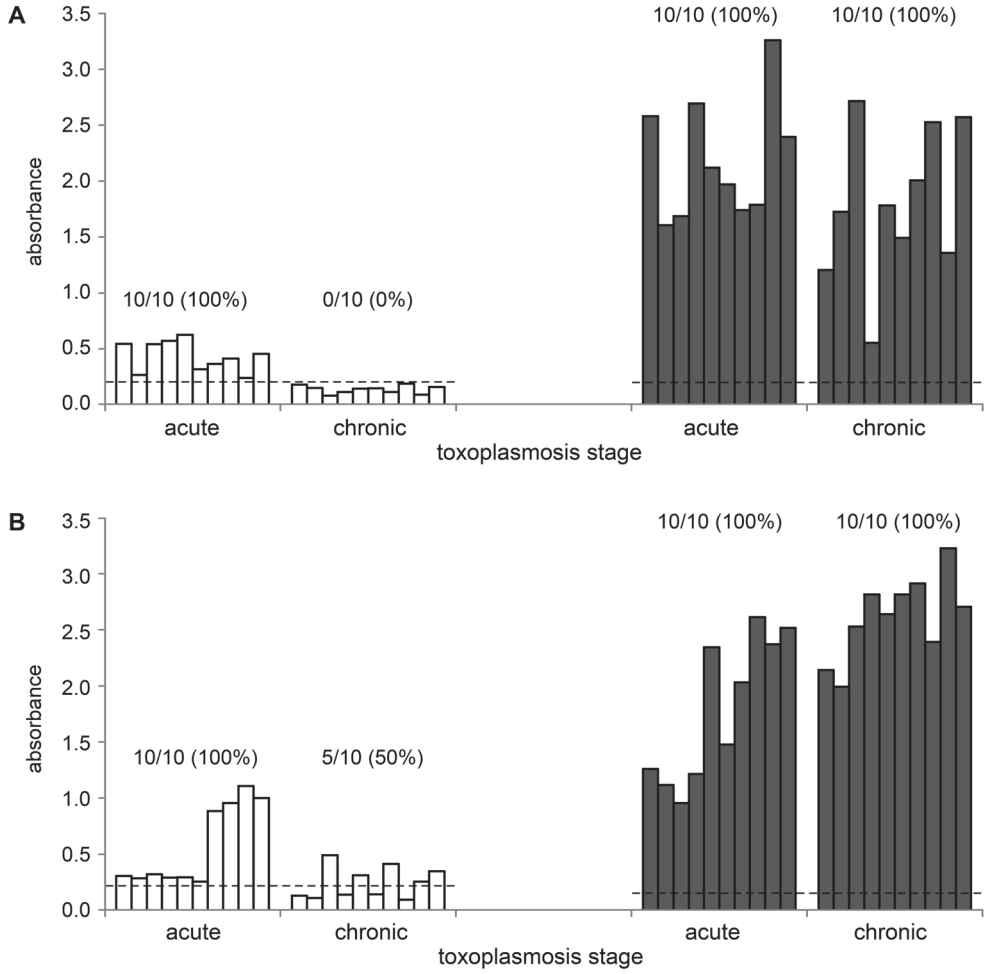

Fig. 2. Levels of IgM (□) and IgG (■) antibodies in individual C57BL/6 (A) and BALB/c (B) mouse sera recognizing rMAG1 antigen during acute and chronic toxoplasmosis, ------ cut-off values.

to negative controls in both antibody classes and mouse strains). However, during the chronic phase of toxoplasmosis the IgM level fell below the cut-off value in all C57BL/6 mice and in 50% of BALB/c mice, and statistical analysis showed no differences between infected and uninfected groups ($p = 0{,}427$ for C57BL/6 and $p = 0.734$ for BALB/c) suggesting that IgM antibodies produced in response to the native MAG1 antigen are rather short-lived. On the other hand, the IgG levels either remained stable (C57BL/6 mice, $p = 0.212$) or even rose slightly over time (BALB/c mice, $p = 0.005$). The results were further confirmed in tests with human serum samples. Although people represent a much more heterogeneous population compared to laboratory inbred mice, the sensitivity of the test detecting anti-MAG1 IgG antibodies was again high both in acute and chronic *T. gondii* infection, 97.0% and 93.1% respectively (Table I). Noteworthy, the lowest sensitivity was obtained with the serum samples from chronic *T. gondii* infection stage that did not contain specific IgM antibodies (89.5%). IgM anti-MAG1 antibodies appeared in the majority (63.6%) of subjects

Table I
The percentage of MAG1-positive human samples taken from patients with acute or chronic *T. gondii* infection.

Serum sample group	IgM		IgG	
	No. of reactive serum samples	% of reactive serum samples	No. of reactive serum samples	% of reactive serum samples
Acute phase IgM^+ IgG^+ sera	21/33	63.6	32/33	97.0
Chronic phase IgM^+ IgG^+ sera	5/34	14.7	33/34	97.1
Chronic phase IgM^- IgG^+ sera	1/38	2.6	34/38	89.5
Total	27/105	25.7	99/105	94.3

classified as acutely infected and only 14.7% of chronically infected individuals with circulating IgM anti-*T. gondii* antibodies (determined by commercial tests). Sera from chronic stage of toxoplasmosis classified as IgM^- IgG^+ generally did not contain specific IgM antibodies reacting with the rMAG1 protein, however, one false positive result was recorded.

The outcome of serological tests applied commonly in the laboratory recognition of toxoplasmosis depends heavily on the diagnostic antigen used. Most commercial tests use TLA (*Toxoplasma* lysate antigen) obtained from parasite tachyzoites, which in fact has a few drawbacks, such as high costs, culturing of invasive parasites and lack of standardization. All these disadvantages may now be eliminated by the use of DNA technology enabling a production of recombinant proteins. The present study focused on the diagnostic potential of the full-length rMAG1 protein tested concurrently in laboratory mice and humans. Based on a strictly defined experimental model (murine toxoplasmosis), we confirmed that the native MAG1 is highly immunogenic. In our study the full-length protein proved to be a sensitive marker of *T. gondii* invasion, in both acute and chronic stages, reaching the overall IgG test sensitivity of 94.3 % in humans and 100% in laboratory mice.

There is a discrepancy between our results and those reported by other authors. Holec *et al.* (2007) found that the rMAG1 antigen was much more frequently recognized by specific IgG antibodies present in human sera derived from the acute stage (97.3%) than by those from the chronic stage (only 7.5%) of toxoplasmosis. However, a much shorter fragment of the rMAG1 protein comprising a sequence from 30 to 222 amino acids was applied. This short rMAG1 fragment was excluded from a further study at the stage of preliminary experiments due to its unsatisfactory reactivity with ovine sera (Holec-Gąsior *et al*., 2014) and it exhibited high IgG sensitivity (90.8%) in tests with both acute and chronic human serum samples when accompanied by a fragment of another *T. gondii* antigen (MIC1) in the chimeric protein (Holec-Gąsior *et al*., 2012). Similarly to our observations, authors (Holec-Gąsior *et al*., 2012) also noted the lowest sensitivities of IgG tests in samples from chronic toxoplasmosis containing low concentration of specific antibodies. Di Cristina *et al.* (2004) analyzed the immunoreactivity of the glutathione S-transferase fusion protein containing MAG1 fragment with sera from *T. gondii*-seropositive women. Results revealed that IgG antibodies from 73% of tested individuals reacted with the rMAG1 antigen indicating broad recognition of bradyzoite antigens by human B-cells. Furthermore, the rMAG1 antigen (30–452 amino acid residues) was also used in a recombinant "line assay" performed on nitrocellulose, but the sensitivity of IgG detection in sera from individuals with acute infection was low (31.8%), compared to our results, with specific antibodies arising over time (Pfrepper *et al*., 2005).

Noteworthy, it has been found recently that the MAG1 of *Neospora caninum* (NcMAG1) is 54% identical to the MAG1 of *T. gondii* (TgMAG1) with the conserved C-terminal regions exhibiting 66% identity and the variable N-terminal about 32% suggesting that these variable regions possess unique antigenic characteristics (Guionaud *et al*., 2010).

To summarize the MAG1 protein offers a promising alternative to replace a TLA preparations in diagnostics due to its high sensitivity in specific IgG detection. Moreover, a combination of protein fragments differing in their length may be useful in discriminating between acute and chronic invasion just by determining the IgG reactivity. Additionally, the recombinant MAG1 peptides (107–148 and 422–452 amino acid residues) have the potential to distinguish active from chronic infection (Xiao *et al.*, 2013). Finally, the results of experiments on chronically infected mice non-treated and treated with sulfadiazine to inhibit cerebral tachyzoite proliferation (Hester *et al*., 2012), led authors to speculation that detecting increased levels of IgG antibodies against several *T. gondii* antigens (including MAG1) may offer an alternative method to detect reactivation of latent *T. gondii* invasion.

Acknowledgements

The paper was partially supported by the Polish National Science Centre (grant N N 302 636340) and University of Łódź.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 61–64

SHORT COMMUNICATION

Optimized Protocol for PFGE Analysis of *Anginosus* (*milleri*) Streptococci.

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Submitted 9 December 2014, revised 12 December 2014, accepted 24 December 2014

Abstract

Streptococcus anginosus (*milleri*) is a diverse group of gram positive bacteria. Molecular methods to establish relationship between strains are poorly developed. Therefore, main tool to study genetic variability is restriction fragment length polymorphism combined with pulsed field gel electrophoresis (RFLP-PFGE). In this communication, we present optimized protocol for *S. anginosus* PFGE analysis.

Key words: *Streptococcus anginosus*, PFGE

Streptococci are a group of quite diverse gram positive organisms that includes over 40 species (Kohler, 2007). There are multiple methods of classification within this group, often confusing for the non-specialist. The most common classification is simply based on the hemolysis type: full β- or partial α-hemolysis. However, the classification does not even reflect relationships between species, as the same streptococcal species can sometimes exhibit either α-, β- or no hemolysis. The other common classification that uses capital letter designation (groups A, B, C *etc.*) is based on the presence of specific carbohydrates or lipoteichoic acids on the cell surface, and divides streptococcal species into so called "Lancefield groups" (Lancefield, 1933). This classification, however, can be also confusing as the same Lancefield antigen can be present on the surface of non-related species. In addition species names and evolutionary position also changed over the years (Facklam, 2002). The most recent division into phylogenic groups: pyogenic, anginosus (formerly milleri), mitis/oralis, bovis, salivarius and mutans, reflects evolutionary relationships between species (Kohler, 2007).

The majority of the streptococci that belong to pyogenic group (*Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilis*) have been studied for years and are well or very well characterized. The same is true for other human pathogens such as *Streptococcus pneumoniae*. Methods for molecular characterization of these organisms and detecting evolutionary relationships between strains have been described and are widely introduced (Borek *et al.*, 2012a, 2012b; Borek *et al.*, 2011; Obszanska *et al.*, 2012; Obszanska *et al.*, 2011).

Unfortunately, an increasing number of uncharacterized streptococcal species are being regarded as human pathogens. Often, epidemiology and relationships between strains are not studied due to the lack of precise methods.

Rapid, advanced molecular methods to determine relationships between strains, based on known DNA sequences, are usually not developed for less studied groups such as anginosus. Because the anginosus group is poorly characterized at the genetic level, restriction macro-analysis combined with pulse field gel electrophoresis (PFGE) is currently used as the method of choice. Despite the fact that the method is time and labor consuming, it allows to compare strains of unknown characteristics and is regarded as the golden standard in epidemiological research.

PFGE of *Sma*I (recognized restriction site CCCGGG) digested chromosomal DNA has been for many years the method used to investigate differences between bacterial species/strains with low G + C nucleotide content such as streptococci and staphylococci. However, upon inspection of a newly sequenced in our laboratory *Streptococcus anginosus* strain, we noticed that it contains only few *Sma*I sites, and generates only 4 distinguishable bands (data not shown). Also, published PFGE analyses show that digest with *Sma*I yields sometimes as few as 5–6 bands visible on a gel (Bartie *et al.*,

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2000; Chang and Lo, 2013). Therefore we decided to develop a system of macro-restriction PFGE analysis of bacterial strains that belong to the anginosus (milleri) group yielding more bands visible on gel, allowing better resolution and better discriminating between closely related strains. We propose the use of *Bsp120*I (recognized restriction site GGGCCC), as an less expensive alternative to *Sma*I, and *Eag*I (recognized restriction site CGGCCG) for more precise differentiation between strains of the anginosus group. We also optimized a step by step procedure of plug preparation and digestion, as well as electrophoresis parameters.

To prepare agarose plugs, strains belonging to the anginosus group should be cultured overnight on Columbia blood agar at 37°C in 5% CO_2, then collected using a sterile swab and re-suspended in an ampule of sterile saline. Using densitometer sample density should be adjusted to turbidity 4 on the McFarland scale. One ml of such suspension is then spun in a centrifuge, saline is removed and the pellet re-suspended in 150 µl PIV buffer (1 M NaCl, 10 mM Tris-HCl, pH 8.0). An equal volume of 2% SeaPlaque (Lonza) agarose in ddH_2O pre-warmed to 50°C is then added to the bacteria and gently but thoroughly mixed. Next, 20 µl droplets of bacteria in agarose mix are dispensed on a glass plate covered with Parafilm® M and covered with a microscope slide to form plugs (Chung *et al.*, 2000) and the whole assembly is then placed for 10 min at 4°C. When the agarose with embedded bacteria is cooled, agarose droplets are gently moved with the help of a sterile loop into 1.5 ml tubes filled with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). Plugs can be stored in TE buffer at 4°C. The period of storing plugs containing whole bacteria, before DNA release, can vary in multiple species and is often a crucial factor to achieve high quality restriction patterns after electrophoresis. However, in the case of anginosus streptococci, we did not notice DNA degradation after keeping undigested plugs in TE for over a year.

To release DNA from bacteria embedded in agarose plugs, one to several plugs can be transferred to 1 ml of EC-lysis buffer in 15 ml conical tubes. EC-lysis buffer must be prepared immediately before use (EC buffer – 6 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.1 M EDTA, pH 8.0, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosinate, 0.5% Brij®58; EC-lysis buffer – EC buffer with addition of 1 mg/ml lysozyme, 0.1 mg/ml RNase, 100 U/ml mutanolysin). Plugs in EC-lysis buffer should be incubated for 4 hours at 37°C. After incubation, the buffer is removed and replaced with 1 ml of ES buffer (ES buffer – 0.5 M EDTA, pH 9.0, 1% sodium lauroyl sarcosinate) and 1 mg/ml of proteinase K and the tubes are left overnight at 50°C.

After overnight incubation, ES buffer with proteinase is removed and two 7 ml washes with TE are performed. For each wash plugs must be incubated for 30 min with gentle mixing on an orbital shaker at room temperature. After the second wash, TE is replaced with 1 ml TE buffer with 20 µl 0.1 M PMSF and incubate 30 min at room temperature without shaking. After incubation with TE + PMSF three additional washes with 10 ml of TE are performed.

Washed plugs with released DNA can be digested immediately with restriction enzymes, however, plugs with released DNA can be stored in TE buffer. We recommend that plugs are used for restriction digest and electrophoresis as soon as it is possible, but we did not observe markedly visible loss of quality even after 4 months of storage in TE buffer.

To perform the digest, one plug should be placed in 1.5 ml tube, filled with 100 µl of appropriate 1 × digestion buffer and incubated for 30 min at 37°C. After

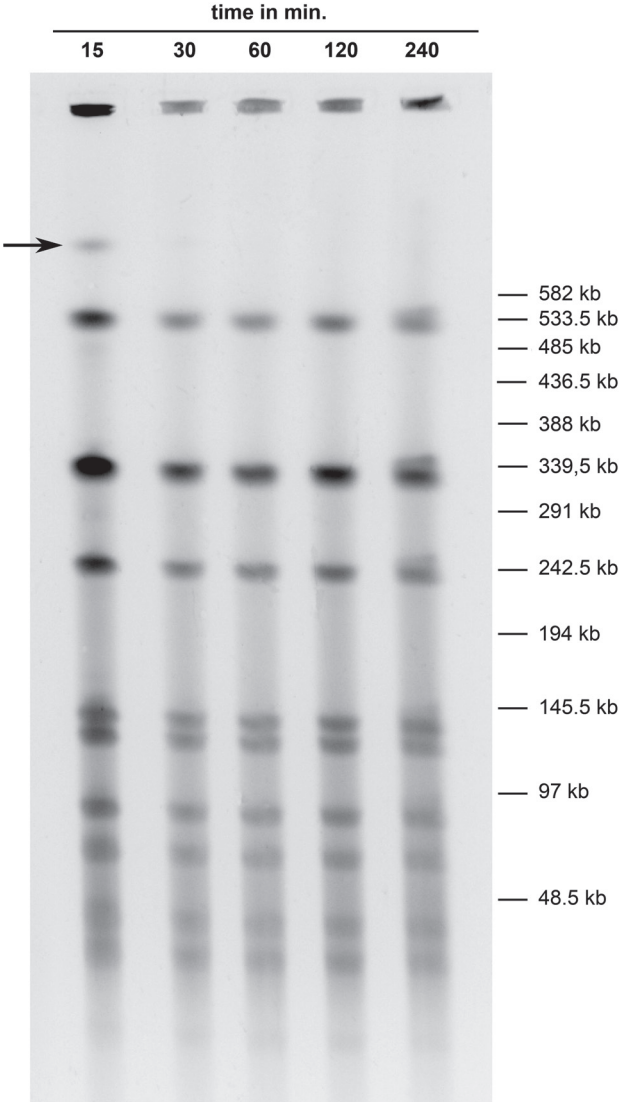

Fig. 1. Majority of chromosomal DNA released from plugs is digested after 15 minutes of incubation with 0.5 µl of FastDigest® *Bsp120*I restriction enzyme. Incompletely cut DNA is marked by a black arrow. Marker – Lambda Ladder PFG Marker (New England Biolabs)

that time the buffer should be replaced with 50 µl of diluted *Bsp120*I or *Eag*I (49.5 µl of the 1 × restriction buffer + 0.5 µl of the enzyme). Digestion time should be optimized, however, we observed that FastDigest® restriction enzymes (available from Thermo Scientific/Fermentas) used in this protocol can noticeably shorten the whole procedure. As can be seen in Figure 1 for *Bsp*120I, chromosomal DNA can be digested almost completely using a single 0.5 µl aliquot of the enzyme within 15 minutes. Only a small amount of uncut DNA (marked by an arrow) is visible on a gel. An hour long incubation with *Bsp*120I always presented completely digested DNA. We usually digested our samples with 0.5 µl of *Eag*I for four hours for complete digest, because of more restriction sites usually present in the genome.

Digestion with both enzymes yields a substantial (10–28) number of bands that can be used to differentiate between strains. *Bsp120*I is a cheap alternative to other restriction enzymes, yet a considerable set of anginosus group strains is not digested by *Bsp120*I. We tested whether this is caused by poorly digested cell wall and unreleased DNA. As a control we used the same lot of plugs for both *Bsp120*I and *Eag*I digestion and we observed that DNA in plugs not digested by *Bsp120*I was digested by *Eag*I (Fig. 2).

Because *Bsp120*I is an enzyme blocked by Dcm methylation, we further tested whether this type of methylation affects digestion of anginosus group strains DNA released from plugs. To assess the influence of Dcm methylation on *Bsp120*I activity, we digested chromosomal DNA isolated from strains whose DNAs were unable to be digested with *Bsp120*I, with *Eco*RII and *Mva*I. *Eco*RII and *Mva*I recognize the same DNA sequence but are sensitive and non-sensitive to Dcm methylation, respectively. We observed that both enzymes digested DNA (data not shown), so the inability of *Bsp120*I to digest DNA released from anginosus group is not related to Dcm methylation. Therefore, we hypothesize that we may observe a similar phenomenon as in the case of *Sma*I digestion of *Staphylococcus aureus* DNA (Bens *et al.*, 2006). *Sma*I, but not its neoschizomers such as *Xma*I or *Cfr9*I, is blocked by the presence of 5-methylcytosine at specific sites in its recognition sequence CCCGGG. It was noticed that a particular line of *S. aureus* MRSA strains cannot be analyzed by PFGE using standard *Sma*I restrictase due to the activity of a specific uncharacterized restriction/methylation system present in this MRSA clone. We suspect that GGGCCC restriction site recognized by *Bsp120*I in anginosus group can be also influenced by an uncharacterized so far methylation system.

The electrophoresis was run in 1% SeaKem® Gold agarose (Lonza) gel in standard electrophoresis 0.5 × TBE buffer. Prior to the run the gel was placed in the electrophoresis chamber of a CHEF Mapper® XA Pulsed Field Electrophoresis System (BioRad), covered with 0.5 × TBE buffer and chilled to 11°C. Digested plugs were washed with TBE buffer, placed in wells, one

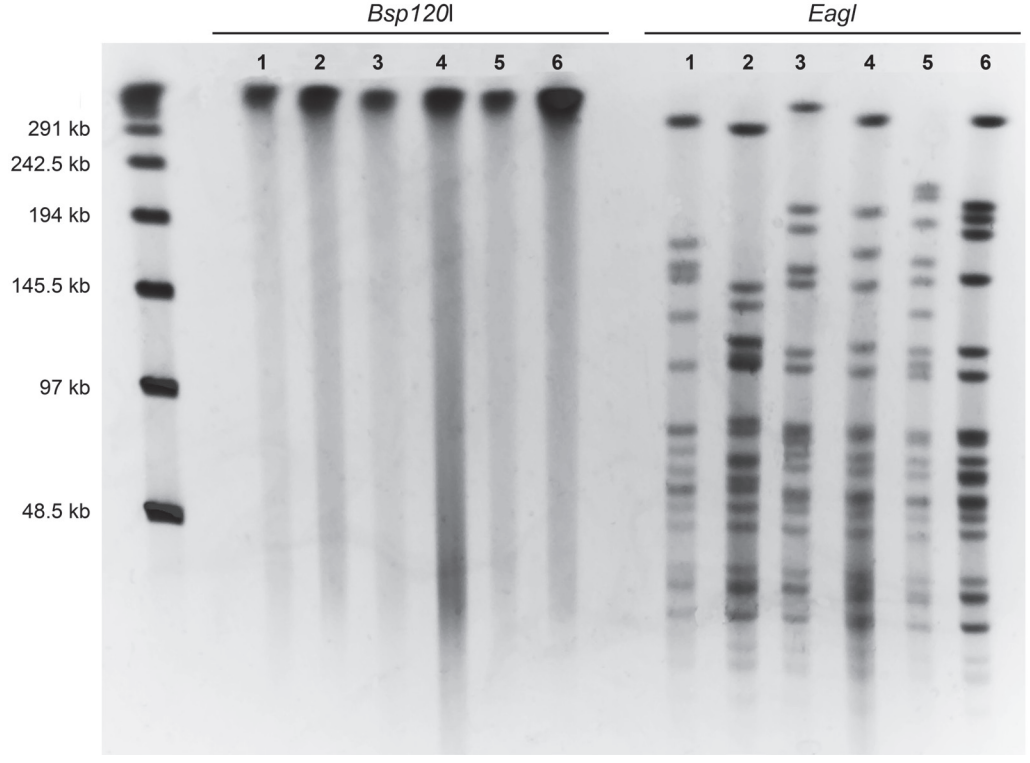

Fig. 2. Chromosomal DNA released from the same lots of plugs (marked 1 through 6) cannot be digested with *Bsp120*I, but is cut by *Eag*I. Marker – Lambda Ladder PFG Marker (New England Biolabs).

plug per well, and covered with 1% SeaKem agarose. Fragments were separated for 23 hours at 6V/cm using a 120° included angle. For *Bsp120*I initial switch time was 2 s and final switch time 50 s, for *Eag*I the initial switch time was 1 s and the final switch time 20 s. After electrophoresis, the gel was stained in 0.5 × TBE buffer with 0.5 μg/ml of ethidium bromide for ~45 minutes with gentle shaking and photographed under UV light. Gels can be kept overnight in staining solution at 4°C. Prolonged staining increases the level of band detection sensitivity and produces images with less background. Also, to decrease the background, stained gels can be briefly de-stained (2 × 5 minutes) with water.

As a final step PFGE patterns on single or multiple gels can were compared using Tenover criteria (Tenover *et al.*, 1995) or various algorithms dedicated to band pattern clustering and dendrogram construction such as BioNumerics software (Applied Maths).

Acknowledgments

The work was financed by the grant from National Center for Science (NCN) number NN401535940.

We would like to thank dr Janusz Fiett and dr Radosław Izdebski for valuable discussions and help with electrophoresis optimization.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 65–68

SHORT COMMUNICATION

PCR Detection of *Scopulariopsis brevicaulis*

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Submitted 9 July 2014, revised 5 November 2014, accepted 13 November 2014

Abstract

Scopulariopsis brevicaulis is known as the most common etiological factor of the mould toenail infections. There are also reports indicating that *S. brevicaulis* could cause organ and disseminated infections. Nowadays microscopic observations from the direct sample and culture are crucial for the appropriate recognition of the infection. In this paper a PCR-based method for *S. brevicaulis* detection is presented. The specificity of the reaction was confirmed, as positive results were obtained only for tested *S. brevicaulis* isolates and no positive results were obtained for other moulds, dermatophytes, yeast-like fungi, and human DNA.

Key words: *Scopulariopsis brevicaulis*, onychomycosis, PCR

Scopulariopsis brevicaulis is a soil saprophytic mould. This non-dermatophytic filamentous fungus colonises plantar, animal and human tissues (Filipello Marchisio *et al.*, 2000). Traditionally, it has not been considered a common human pathogen. However, the number of cases showing its pathogenicity is still increasing. The most common type of infection caused by *S. brevicaulis* is onychomycosis. Its prevalence is estimated to be 3–10% of total number of onychomycosis cases. The clinical manifestation is generally recognised as distal and lateral subungual onychomycosis (DLSO) (Stefanato and Verdolini, 2009; Gupta *et al.*, 2012). There are also cases of smooth skin and subcutaneous tissue infections caused by *S. brevicaulis* (Dhar and Carey, 1993; Bryuynzel and Starink, 1998; Anandan *et al.*, 2008). Moreover, *S. brevicaulis* has been reported as the cause of such infections as endocarditis (Gentry *et al.*, 1995; Migriono *et al.*, 1995; Jain *et al.*, 2011), keratitis (Del Prete *et al.*, 1994; Lotery *et al.*, 1994), endophthalmitis (Gariano and Kalina, 1997), sinusitis (Gluck *et al.*, 2011), fungus ball (Endo *et al.*, 2002; Satyavani *et al.*, 2010), otomycosis (Hennequin *et al.*, 1994; Besbes *et al.*, 2002), pneumonia (Issakainen *et al.*, 2010), cerebral phaeohyphomycosis and brain abscess (Hart *et al.*, 2001), disseminated infection causing skin lesions including a patient with acquired immune deficiency syndrome (AIDS) (Dhar and Carey, 1993), disseminated infections after bone marrow transplant (Neglia *et al.*, 1987; Phillips *et al.*, 1989; Krisher *et al.*, 1995) and after lung transplant (Wuyts *et al.*, 2005). Disseminated *S. brevicaulis* infections are hard to treat and have high mortality rates, especially in immunocompromised patients. *S. brevicaulis* disseminated infections might be clinically and histologically indistinguishable from disseminated aspergillosis, fusariosis or zygomycosis (Salmon *et al.*, 2010; Swick *et al.*, 2010; Vignon *et al.*, 2011). The data considering *S. brevicaulis* susceptibility are scarce and often inconsistent. The reports indicate that *S. brevicaulis* is a multi resistant fungus (Cuenca-Estrella *et al.*, 2003).

In this paper we present a PCR-based method developed for the detection of *S. brevicaulis*. By a two-step extraction procedure followed by PCR and electrophoresis, the method enables fast identification of this pathogen.

Reference strains: *S. brevicaulis* (CBS 112377), *Scopulariopsis fusca* (IHEM 14552; IHEM 25912), *Scopulariopsis asperula* (IHEM 2546), *Scopulariopsis cinerea* (IHEM 25417) and other fungal isolates were obtained from the Microbiology Department of Gdańsk University of Technology (Poland). Three animal (rabbit)-derived *S. brevicaulis* isolates (isolated from superficial infections) were obtained from the Department of Preclinical Sciences of Warsaw University of Life Sciences (Poland). One human-derived *S. brevicaulis* isolate (isolated from superficial infection) was obtained from the Department and Clinic of Dermatology, Venereology and Allergology of Wroclaw Medical University

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(Poland) (Table I). All isolates were identified by observation of macro- and micromorphology. Isolates were cultured on Sabouraud medium (Biomerieux, France) and incubated for up to 14 days at room temperature. DNA from samples (pieces of mycelium of 3–5 mm diameter) was extracted by a 10-min incubation of the sample in 100 µl of extraction buffer (60 mM sodium bicarbonate [$NaHCO_3$], 250 mM potassium chloride [KCl] and 50 mM Tris, pH 9.5) in 95°C and subsequent addition of 100 µl anti-inhibition buffer (2% bovine serum albumin). After vortex mixing, this DNA-containing solution was used for PCR (Brillowska-Dąbrowska *et al.*, 2010). Reagents were, unless otherwise stated, purchased from Sigma (Germany).

On the basis of alignment (VectorNTI; InforMax, Inc., USA) of sequences of β-tubulin gene presented in the NCBI nucleotide database, *S. brevicaulis* – specific primers Sbfor (5'AACAAACCCACTTCCCGTCGTTT3') and Sbrev (5'ACATATTTGTTTCCCGAAGCCTTTAG3') were designed. One *S. brevicau-*

Table I
Organisms used in the study (161 isolates)

Organism		No of isolates
moulds	*Scopulariopsis brevicaulis* (reference strain)	1
	S. brevicaulis (clinical isolates)	4 (1 human-derived) (3 animal-derived)
	S. fusca (reference strain)	2
	S. cinerea (reference strain)	1
	S. asperula (reference strain)	1
	Acremonium charticola	1
	A. kiliense	1
	Alternaria strictum	1
	Alternaria alternata	6
	A. brassicae	3
	A. tenuissima	1
	Alternaria sp.	1
	Aspergillus flavus	2
	A. fumigatus	8
	A. clavatus	3
	Cladosporium cladosporioides	2
	C. macrocarpum	1
	Fusarium solani	1
	F. oxysporum	1
	F. culmorum	1
	Mucor racemosus	2
	M. circinelloides	1
	Ochrocladosporium elatum	1
	Penicillium chrysogenum	1
	P. commune	2
	P. melinii	1
	Penicillium sp.	1
	P. digitatum	1
	P. italicum	1
	P. polonicum	1
	P. crustosum	1
	P. verrucosum	1
	P. paneum	1
	P. hirsutum	1
	P. carneum	1
moulds	*Pleospora papaveracea*	1
	Rhizopus oryzae	1
	R. oligosporus	1
	Trichoderma viridae	1
	Ulocladium tuberculatum	1
	U. chartarum	1
dermato-phytes	*Epidermophyton floccosum*	7
	Microsporum audouinii	5
	M. canis	3
	M. gypseum	5
	M. nanum	1
	M. persicolor	3
	Trichophyton equinum	1
	T. erinacei	2
	T. interdigitale	4
	T. mentagrophytes	5
	T. rubrum	4
	T. schoenleinii	3
	T. soudanense	2
	T. terrestrae	6
	T. tonsurans	6
	T. verrucosum	5
	T. violaceum	3
yeast-like fungi	*Candida albicans*	7
	C. catenulata	1
	C. glabrata	3
	C. guillermondii	1
	C. kefyr	2
	C. krusei	1
	C. magnoliae	1
	C. parapsilosis	5
	C. tropicalis	5
	C. utilis	1
	Geotrichum sp.	1
	Rhodotorula mucilaginosa	1
	Saccharomyces cerevisiae	1
Human		1

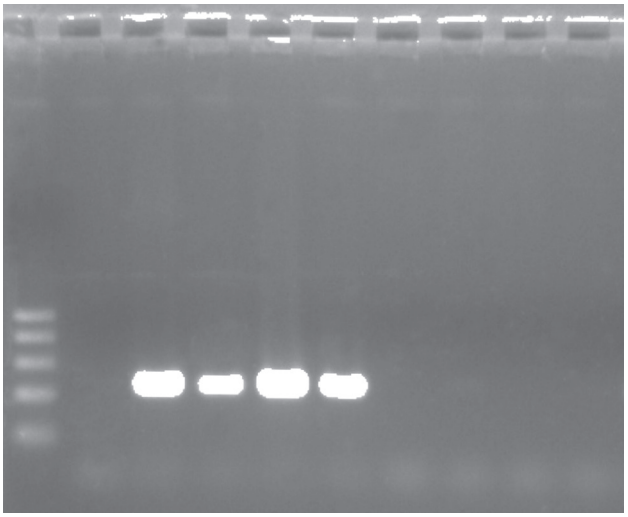

Fig. 1. Example of *S. brevicaulis* specific PCR product analysis. Lane 1 – molecular size marker (fragment sizes 500, 400, 300, 200 and 100 bp); results of *S. brevicaulis*-specific PCR performed for *S. brevicaulis* – reference strain (lane 3), human-derived isolate (lane 4), animal-derived isolates (lanes 5–6); *S. fusca* – reference strains (lanes 7–8); *S. asperula* (lane 9); *S. cinerea* (lane 10); negative control (lane 2).

lis reference strain, four clinical *S. brevicaulis* isolates, two *S. fusca* reference strains, one *S. asperula* reference strain, one *S. cinerea* reference strain and 56 other mould isolates, 65 dermatophyte isolates, 30 yeast-like isolates and one purified human DNA (Table I) were tested. 20 µl PCR mixtures consisted of 10 µl of 2× PCR Master Mix Plus High GC (A&A Biotechnology, Poland), 0,1 µl of each primer (Sbfor, Sbrev – Genomed, Poland) at 100 µM, and 2 µl of DNA. PCR was performed in a Mastercycler ep gradient S-5345 (Eppendorf, Germany). The time-temperature profile for PCR started with initial denaturation for 3 min 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The presence of specific PCR products of 223 bp was examined using electrophoresis on a 2% agarose gel and staining with ethidium bromide.

A 223-bp PCR product corresponding to *S. brevicaulis* was observed for 5/5 *S. brevicaulis* DNA samples. No PCR products were detected for *S. asperula*, *S. cinerea*, *S. fusca* reference strains, 56 other mould isolates, 65 dermatophyte isolates, 30 yeast-like isolates and one purified human DNA (100% sensitivity and 100% specificity for PCR) (Fig. 1).

Nowadays, *S. brevicaulis* identification is based on macro– and microscopic observations. As these methods are laborious, time-consuming and requiring a pure culture for correct identification, they often produce misleading results (Jagielski *et al.*, 2013). Especially diagnosis of disseminated *S. brevicaulis* infections is challenging for several reasons. First, disseminated *S. brevicaulis* infection is clinically difficult to distinguish from disseminated infection caused by *Aspergillus*, *Fusarium* and zygomycosis. Second, in the immunocompromised organ transplant patient the sensitivity of confirmatory blood cultures is poor. There is no rapid blood test to confirm *Scopulariopsis* infection. Third, histopathologic evaluation of a cutaneous biopsy from a suspicious cutaneous lesion is not able to distinguish one disseminated mycelial infection from another. *Aspergillus*, *Fusarium* and other hyalohyphomycoses such as *Scopulariopsis* show identical morphology of biopsy specimens. To enable differentiation between the above listed mycelial fungi the tissue should be submitted for culture studies at the time of biopsy that delays the diagnosis for at least 1 week (Swick *et al.*, 2010). So far, sequencing (Jagielski *et al.*, 2013) and RFLP (Bontems *et al.*, 2009) are the only molecular methods described for *S. brevicaulis* identification. However, these methods are still laborious and time-consuming. All of these factors indicate the need for development of methods that provide simple, rapid and highly specific identification of *S. brevicaulis*. In this study we present a PCR-based method that enables specific detection of *S. brevicaulis* within few hours. Application of primers Sbfor and Sbrev in PCR gives correct results among tested isolates. This potential of the described method as the diagnostic test should be evaluated further by testing patient specimens, because the applied procedure enables DNA extraction directly from specimens thus significantly reducing the time of diagnosis (Brillowska-Dąbrowska *et al.*, 2010).

Acknowledgements

The authors wish to express their gratitude to A&A Biotechnology (Gdynia, Poland) for the free provision of 2× PCR Master Mix Plus High GC. The authors wish to express their thanks to Prof. B. Dworecka-Kaszak, and I. Dąbrowska, MSc from the Department of Preclinical Sciences of Warsaw University of Life Sciences (Poland), Dr Med. A. Hryncewicz-Gwóźdź, and K. Kalinowska, MSc from the Department and Clinic of Dermatology, Venereology and Allergology of Wroclaw Medical University for the identification and delivery of *S. brevicaulis* isolates.

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 69–72

SHORT COMMUNICATION

Effect of Antibiotics on Polymorphonuclear Leukocyte Functions and Myeloperoxidase Activity, Glutathione and Malondialdehyde Levels in Allergic Asthma

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Submitted 31 March 2014, revised 3 November 2014, accepted 13 November 2014

Abstract

We investigated the effect of ciprofloxacin, rifampicine and doxycycline on myeloperoxidase (MPO) activity, glutathione (GSH) and malondialdehyde (MDA) levels in allergic asthma patients and healthy volunteers. Polymorphonuclear leukocytes(PMNs) were isolated with ficoll-hypaque gradient centrifugation method. MPO activity was assayed with modified o-dianisidine, GSH by Ellman's and MDA levels by Beuge's method. PMN functions and MDA levels of patients significantly decreased when compared with healthy volunteers. Ciprofloxacin significantly increased PMN functions, MPO activity and MDA levels of both groups. We have demonstrated that ciprofloxacin has beneficial effects on MPO activity and PMN functions in allergic asthma patients and healthy volunteers.

Key words: allergic asthma, antibiotics, intracellular killing activity, myeloperoxidase, phagocytosis

It is known that neutrophils take place during the allergic process and asthma. Oxidative stress takes place in the pathogenesis of inflammatory diseases such as allergic asthma and rhinitis (Kämpe *et al*., 2011; Öztop *et al*., 2002).

The deterioration of PMN's intracellular killing activity could cause the intracellular microorganisms to become potential pathogens in the phagocytic cells. Işık *et al*. (2010) have stated that PMN's intracellular killing activity of patients with allergic rhinitis was insignificantly low when compared to that before immunotherapy ($p = 0.05$). Also, Gürer *et al.* (2005) have demonstrated that PMN's intracellular killing activity of patients with seasonal allergic rhinitis before allergen-specific short-term immunotherapy significantly decreased ($p = 0.252$).

Today it is known that most important PMN defects are leukocyte adhesion insufficiency and myeloperoxidase deficiency. The relationship between PMN's MPO activity and PMN's intracellular killing activity of patients whose intracellular killing activity was found to be low has not been investigated. Also, the cause of oxidative stress in these patients has not been exactly determined. Glutathione (GSH) is a vital antioxidant in the non-enzymatic antioxidant defence system and scavenges free radicals directly (Shurtz-Swirski *et al.*, 2001; Beier *et al*., 2004).

As an indicator of lipid peroxidation, malondialdehyde (MDA) is made up by free radicals during tissue damage and used in the measurement of oxidative stress (Okur *et al*., 2006).

We aimed to find out the relationship between PMN functions (phagocytosis and intracellular killing activity) of allergic asthma patients and MPO activity, GSH and MDA levels in our study. The antimicrobial drugs used in the treatment of the infection seen in these patients could negatively affect or increase the enzyme levels. That is why the aim of our study is to find out the effect of ciprofloxacin (2.5 μg/ml), rifampicine (7 μg/ml) and doxycycline (2.5 μg/ml) on PMN functions and MPO activity and GSH and MDA levels of patients with allergic asthma and healthy volunteers *in vitro*.

In our study 13 allergic asthma patients (1 male and 12 females, mean age 28.85) and healthy volunteers used as the control group consisted of 13 healthy volunteers (3 male and 10 females, mean age 39.69).

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This study protocol was approved by the Marmara University, Ethics Committee. We used a modified neutrophil function evaluation method of Alexander *et al.* (1968) to detect PMN functions. The PMN's of patients and healthy volunteers was isolated by using Ficoll-Hypaque method. Viability of PMNs was tested by the trypan blue exclusion method (Alexander *et al.*, 1968; Barbior and Cohen, 1981).

A clinical strain of *Candida albicans* was used to determine the PMN's phagocytic and intracellular killing activity. *C. albicans* was opsonizated with sterile human serum (1:4) at 37°C for 30 minutes. Dead yeast cells were assessed by 0.01% methylene blue stain (1:1). The phagocytic activity was determined by the percentage of PMNs that had phagocytosed yeast cells. Intracellular killing activity was assayed by the percentage of PMNs that included dead yeast cells. (Richardson *et al.*, 1992; Gürer *et al.*, 2006).

The PMN suspension stored was in order to measure PMN's MPO activity, GSH and MDA levels (Kurutas *et al.*, 2005). MPO activity was determined by a modification of the o-dianisidine method. The protein content of the homogenate was measured by Spectronic-UV 120 spectrophotometer using Lowry's method (Lowry *et al.*, 1951; Kurutas *et al.*, 2005).

The MDA levels were assayed for the products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as previously described (Beuge and Aust, 1978). GSH levels were measured by modified Ellman procedure (Beutler, 1975).

The *in vitro* effect of ciprofloxacin (2.5 μg/ml), rifampicine (7 μg/ml) and doxycycline (2.5 μg/ml) on PMN functions and MPO activity, GSH and MDA levels at the therapeutic serum concentration was investigated.

The results were expressed by means of ± SD (Standart Deviation). Statistical analyses were performed using Mann Whitney U and Wilcoxon Signed Ranks tests. P values less than or equal to 0.05 were considered to be statistically significant.

PMN's MDA levels of patients with allergic asthma significantly decreased when compared to healthy volunteers ($p < 0.001$, Table I). The PMN's phagocytic ($p < 0.01$) and intracellular killing activity ($p < 0.001$) of patients with allergic asthma significantly decreased when compared to healthy volunteers (Table I).

As it is seen from Table II ciprofloxacin significantly increased PMN's phagocytic ($p < 0.01$) and intracellular killing activity ($p < 0.01$) of healthy volunteers and patients with allergic asthma when compared to drug-free controls. Rifampicine and doxycycline ($p < 0.05$) have significantly decreased PMN's phagocytic ($p < 0.05$) and intracellular killing activity ($p < 0.05$) of healthy volunteers when compared to drug-free controls (Table II).

As it is seen on Table III ciprofloxacin significantly increased PMN's MPO activity ($p < 0.01$) and MDA levels of patients with allergic asthma ($p < 0.01$) and healthy volunteers ($p < 0.01$) when compared to drug-free controls. However, while ciprofloxacin has significantly decreased PMN's GSH levels of patients with allergic asthma ($p < 0.01$), it has significantly increased

Table I
PMN Functions (Phagocytic and Intracellular Killing Activity), PMN's MPO activity, GSH and MDA Levels of Healthy Volunteers (n = 13) and Patients (n = 13) with Allergic Asthma

Group	PA (%)	IKA(%)	MPO (U/mg protein)	GSH (μmol/mg protein)	MDA (nmol/mg protein)
A. asthma	40.38 ± 1.34*	1.54 ± 0.24**	237.03 ± 17	1.93 ± 0.03	1.92 ± 0.29**
Healthy volunteers	48.31 ± 5.27	6.54 ± 1.39	275.96 ± 33	1.94 ± 0.07	2.22 ± 0.50

*$p < 0.01$, **$p < 0.001$. Statistics were done by using Mann Whitney U test and the data shown is by means of ± SD.
PA: Phagocytic Activity, IKA: Intracellular Killing Activity, MPO: Myeloperoxidase GSH: Glutathione and MDA: Malondialdehyde

Table II
Comparison of the Effect of Antibiotics on PMN Functions (Phagocytic and Intracellular Killing Activity of Patients with AA(n = 13) and Healthy Volunteers (n = 13) with Drug-Free Controls

Group	Ciprofloxacin (2.5 μg/ml)		Rifampicine (7 μg/ml)		Doxycycline (2.5 μg/ml)	
	PA (%)	IKA (%)	PA (%)	IKA (%)	PA (%)	IKA (%)
Control	48.31 ± 5.27	6.54 ± 1.39	48.31 ± 5.27	6.54 ± 1.39	48.31 ± 5.27	6.54 ± 1.39
Healthy volunteers	63.54 ± 1.89*	9.92 ± 0.35*	39.08 ± 2.39**	5.62 ± 0.35**	38.15 ± 1.82**	5.69 ± 1.62**
Control	40.38 ± 1.34	1.54 ± 0.24	40.38 ± 1.34	1.54 ± 0.24	40.38 ± 1.34	1.54 ± 0.24
A. asthma	54.85 ± 1.09*	4.39 ± 0.33*	38.69 ± 1.62	1.46 ± 0.33	38.69 ± 1.62	1.46 ± 0.33

* $p < 0.01$, ** $p < 0.05$ .Statistics were done by using Wilcoxon Signed Ranks test and the data shown is by means of ± SD.
PA: Phagocytic Activity, IKA: Intracellular Killing Activity

Table III
Comparison of the Effect of Antibiotics on PMN's MPO activity, GSH and MDA Levels of Patients with AA (n = 13) and Healthy Volunteers (n = 13) with Drug-Free Controls

Group		Control (drug-free)	Ciprofloxacin(2.5 μg/ml)	Rifampicine (7 μg/ml)	Doxycycline(2.5 μg/ml)
MPO	A. asthma	237.03 ± 17	365.17 ± 17*	213.40 ± 17	224.10 ± 16
	Healthy	275.96 ± 33	391.58 ± 30*	272.94 ± 30	284.33 ± 27
GSH	A. asthma	1.93 ± 0.03	1.24 ± 0.12*	1.88 ± 0.02	1.87 ± 0.03
	Healthy	1.94 ± 0.07	2.19 ± 0.13*	1.87 ± 0.11	1.81 ± 0.12
MDA	A. asthma	1.92 ± 0.29	2.70 ± 0.79*	1.85 ± 0.48	1.82 ± 0.31**
	Healthy	2.22 ± 0.50	2.68 ± 0.40*	2.28 ± 0.24*	2.18 ± 0.39

* $p < 0.01$, ** $p < 0.05$. Statistics were done by using Wilcoxon Signed Ranks test and the data shown is by means of ± SD.MPO: Myeloperoxidase, GSH: Gluthathione, MDA: Malondialdehyde

PMN's GSH levels of healthy volunteers ($p < 0.01$) when compared to drug-free controls. While rifampicine has significantly increased PMN's MDA levels of healthy volunteers ($p < 0.01$), doxycycline has significantly decreased PMN's MDA levels of patients with allergic asthma ($p < 0.05$) when compared to drug-free controls.

Patients with allergic asthma are very sensitive against the microorganisms which cause infections. Since the PMN's phagocytic and intracellular killing activity of patients with allergic asthma significantly decreased when compared to healthy volunteers, it must be considered that it is important to identify the antibiotics and the other drugs which are used in the treatment of this diseases.

Beside their successful therapeutic effects, many frequently used drugs could affect the immune system positively or negatively (Badur, 1991; Gemmell, 1993).

Ciprofloxacin significantly increased the PMN's MDA level in healthy volunteers and patients with allergic asthma in our study. Possibly the excessively increased MPO activity by ciprofloxacin has increased the lipid peroxidation in the cell. This might lead to the increase of MDA level. In our opinion there is necessity for much more clinical and *in vitro* investigations.

Szczepaniak *et al*. (2003) showed that there is no significant difference between the MPO release from peripheral blood neutrophils of patients with allergic respiratory tract disease and healthy volunteers. In their study Boner *et al*. (1993) has found that while total IgE levels of 12 asthmatic children increased after exposure to allergen-rich environments, their serum MPO levels did not change.

In our study ciprofloxacin has significantly increased the PMN's MPO activity in patients with allergic asthma ($p < 0.01$). Related with the increase in the MPO activity, there has been an increase in both PMN functions of these patients (Table II, $p < 0.01$).

The PMN's GSH levels of patients with allergic asthma significantly have decreased ($p < 0.01$) after ciprofloxacin, while their MDA have levels significantly increased ($p < 0.01$).

Öztop *et al*. (2002) have stated that the serum GSH-Px levels of patients with mild and moderate asthma were significantly lower than healthy volunteers ($p < 0.05$), but their MDA levels were significantly higher ($p < 0.05$).

There is no investigation showing the interaction between PMN functions, MPO activity, GSH and MDA levels in patients with allergic asthma. Gürer *et al*. (2006) have shown that ciprofloxacin signficantly increased the PMN's phagocytic activity of elderly patients ($p = 0.002$) before zinc supplementation and significantly increased both PMN functions of elderly patients ($p = 0.002$) after zinc supplementation 15 mg/day for 1 month. The same antibiotic significantly increased both PMN functions of healthy young volunteers ($p = 0.005$ and $p < 0.05$, respectively) before and after zinc supplementation when compared to control values (drug-free). However, the same investigators have staded that rifampicine significantly decreased the PMN's phagocytic activity of elderly patients ($p < 0.05$) when compared to drug-free values before zinc supplementation.

Also, Daşdelen *et al*. (1999) have reported that rifampicine (7 μg/ml) and doxycycline (2.5 μg/ml) have significantly decreased PMN's phagocytic ($p < 0.05$) and intracellular killing activity ($p < 0.01$) of healthy volunteers when compared to drug-free values.

The results of our study are in accordance with the results of Gürer *et al*. (2006) and Daşdelen *et al*. (1999). Additionally, the increase of PMN's MDA levels, by rifampicine in healthy volunteers might be related with the increase of PMN's phagocytic and intracellular killing activity.

In conclusion, ciprofloxacin has stimulated cellular immunity by increasing PMN functions and MPO activity in healthy volunteers and patients with allergic asthma. Beside these beneficial effects there might be cellular damage when MDA increases. We believe that the addition of various antioxidants to the treatment of these patients and proving our *in vitro* results by clinical investigations might bring more successful results to the therapy of allergic asthma.

Acknowledgements

The authors are thankful to all volunteers and the Scientific Research Commission at Marmara University, which supported this study by Project SAG-DKR-060907-0184(2010).

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Polish Journal of Microbiology
2015, Vol. 64, No 1, 73–76

SHORT COMMUNICATION

The Prevalence of *Campylobacter* spp. and Occurrence of Virulence Genes Isolated from Dogs

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Submitted 16 July 2014, revised 18 November 2014, accepted 15 December 2014

Abstract

This study was conducted to determine the prevalence of *Campylobacter* spp. isolated from dogs' faecal samples. From June 2012 to June 2013, a total of 210 faecal samples from pet dogs living in different kennels (n = 210) were collected by the owners in Greater Poland Voivodeship, Poznań District, Poland. The study revealed that 105 out of 210 faecal samples (50%) contained *Campylobacter*. The highest prevalence of *Campylobacter* spp. occurred in spring (81%), followed by winter (64%). The *cad*F gene was found in 100% of the isolates tested. The occurrence of the other genes was variable. The isolates from young dogs were characterised by higher occurrence of virulence genes.

Key words: *Campylobacter* spp., dogs, virulence genes

Salmonella spp., *Escherichia coli* and *Campylobacter* spp. are the main causes of diarrhoea both in animals and humans all over the world (Silva *et al*., 2011). According to the EFSA (EFSA, 2014), campylobacteriosis is the most frequently reported zoonotic disease. The most common sources of infection are products of animal origin: meat (primarily poultry), milk and water from natural reservoirs (Acke *et al*., 2011). The most frequently isolated species from dogs in Denmark are: *Campylobacter upsaliensis* 75%, *Campylobacter jejuni* 19.4% and *Campylobacter coli* 0.7% (Hald *et al*., 2004). These *Campylobacter* species are more often isolated from dogs aged between 3 and 12 months than from older dogs (Hald *et al*., 2004). Moreover, some researchers also reported seasonal differences in prevalence (Rahimi and Saljooghian Esfahani, 2010; Andrzejewska *et al*., 2013). The factors which are associated with the pathogenicity of *Campylobacter* include: motility, chemotaxis as well as adhesion, and invasiveness (Bang *et al*., 2001; Krutkiewicz, 2008) and toxicity. These traits are associated with certain virulence genes identified from these bacteria, for example: *fla*A, *cad*F, *iam* and *cdt*B (Biswas, 2011; Selwet and Galbas, 2012a). The investigations which have been carried out so far have revealed varying distribution of these virulence genes among strains. The aim of this study was to determine the frequency of occurrence of *C. upsaliensis*, *C. jejuni* and *C. coli* isolated from two different age groups of healthy dogs and from dogs with the symptoms of diarrhoea in Greater Poland Voivodeship, Poznań District. Apart from that, the frequency of occurrence of selected virulence genes was determined in the collected isolates. From June 2012 to June 2013, a total of 210 samples of faeces from pet dogs living in 5 kennels were collected by their owners in Greater Poland Voivodeship Poznań District, Poland. All the samples were obtained by veterinarian by means of swab kits with a transport substrate (Euro Tubo Collection Swab Rubi, Spain). The animals were aged as follows: 105 dogs were adult (> 12 months), 105 dogs were younger than 1 year. Diarrhoea was reported in 25 dogs. The owners of the other dogs did not report clinical symptoms of diarrhoea. The faecal samples were cultured at 42 ± 1°C in Campy Selective Agar Base (Preston) (Neogen Europe, Scotland UK) for 48 h under microaerophilic conditions**.** DNA was extracted by means of CHELEX-100 chelating resin (Bio-Rad, CA, USA). Bacterial colonies were suspended in 100 µl Tris buffer and 45 µl 20% CHELEX and boiled for 10 min. The samples were immediately placed on ice for 1 min. and centrifuged at 13.000 g for 10 min. at room temperature. A PCR was used for the detection of *C. upsaliensis*, *C. jejuni* and *C. coli*. (Andrzejewska *et al*., 2011). The following positive controls were included in the PCR: *C. upsaliensis*

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Table I
The prevalence of *Campylobacter* spp. isolated from diarrhoeic and healthy dogs

Sources		No. of samples	*Campylobacter* spp. positive	*Campylobacter upsaliensis*	*Campylobacter jejuni*	*Campylobacter coli*
Adult dogs	Healthy	95	37 (38.9%)	18 (48.6%)	15 (40.5%)	4 (10.8%)
	Diarrhoeic	10	5 (50%)	1 (20%)	2 (40%)	2 (40%)
Young dogs	Healthy	90	54 (60%)	29 (53.7%)	18 (33.3%)	7 (13.0%)
	Diarrhoeic	15	9 (60%)	2 (22.2%)	5 (55.5%)	2 (22.2%)

ATCC 43954, *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559. The presence of the *fla*A, *cad*F, *cdt*B and *iam* genes was determined with the primers according to the authors (Nachamkin *et al*., 1993; Konkel *et al*., 1999; Bang *et al*., 2001; Carvalho *et al*., 2001). The statistical analysis was performed according to the GLM procedure of the SAS program (SAS, 1999) and the significance of differences was verified with Tukey's test. The prevalence of *Campylobacter* spp. was determined in the 210 faecal samples. PCR revealed the presence of *Campylobacter* spp. in 105 samples (50%). *Campylobacter* spp. was isolated from 42 samples (40%) from adult individuals and from 63 samples (60%) from young dogs. The numbers of isolates differed significantly between the age groups ($P < 0.05$). The most frequent species in the group of adult dogs were: 45.2% *C. upsaliensis*, 40.5% *C. jejuni* and 14.3% *C. coli*. In the group of young dogs the isolation percentage was similar, *i.e*. 49.2% *C. upsaliensis*, 36.5% *C. jejuni* and 14.3% *C. coli*. There were significant differences observed in the prevalence of *C. upsaliensis* between the age groups of the dogs under study ($P < 0.05$). Out of the 210 samples examined 25 samples derived from individuals with diarrhoea: 10 samples from adult dogs and 15 samples from young individuals (Table I). As far as the adult dogs are concerned, the predominant species were: 45.2% *C. upsaliensis*, 40.5% *C. jejuni* and 14.3% *C. coli* whereas in the young dogs these were: 49.2% *C. upsaliensis* 36.5% *C. jejuni* and 14.3% *C. coli*. In summer and autumn the frequency was similar with: 37.6% and 39.6%, respectively (Table II). The analyses of the percentages of the selected virulence genes (Table III) revealed that the *cadF* gene was found in 100% of all the species. Also, the *flaA* gene was determined in 100% of *C. jejuni* and *C. coli*. As far as the *cdtB* and *iam* genes are concerned, they were detected more frequently in the younger individuals. Although poultry is considered to be the main source of infection with *Campylobacter* spp. in humans, according to Biswas *et al*. (2011), the role of other animals as vectors spreading this bacterium should also be recognised. Parsons *et al*. (2010) in UK and Andrzejewska *et al*. (2013) in Poland reported that the frequency of *Campylobacter* spp. isolation in dogs ranges from 17% in Brazil and Argentina to 76.2% in Denmark. According to the study by Workman *et al*. (2005), the age of animals may also exert significant influence on the degree of occurrence

Table II
The seasonal prevalence of *Campylobacter* spp. isolated from dogs

Seasons	Sources		Total
	Adult dogs	Young dogs	
Spring	14/22 (63.6%)	20/20 (100%)	34/42 (80.9%)
Summer	12/45 (26.7%)	20/40 (50%)	32/85 (37.6%)
Autumn	10/28 (35.7%)	13/30 (43.3%)	23/58 (39.6%)
Winter	6/10 (60%)	10/15 (66.7%)	16/25 (64%)
Total	42/105	63/105	105/210

Table III
The number and percentages of virulence genes in *Campylobacter* spp. isolated from dogs

Isolates/Animals	Genes			
	cadF	*flaA*	*cdtB*	*iam*
C. upsaliensis				
Adult dogs (n = 19)	19 (100%)	18 (94.7%)	17 (89.5%)	18 (94.7%)
Young dogs (n = 31)	31 (100%)	30 (96.8%)	30 (96.8%)	30 (96.8%)
C. jejuni				
Adult dogs (n = 17)	17 (100%)	17 (100%)	16 (94.1%)	15 (88.2%)
Young dogs (n = 23)	23 (100%)	23 (100%)	23 (100%)	21 (91.3%)
C. coli				
Adult dogs (n = 6)	6 (100%)	6 (100%)	5 (83.3%)	5 (83.3%)
Young dogs (n = 9)	9 (100%)	9 (100%)	8 (88.9%)	8 (88.9%)

of *Campylobacter* spp. Andrzejewska *et al*. (2013) reported the highest prevalence of *Campylobacter* spp. in dogs less than one year of age. Westgarth *et al.* (2008) observed that younger dogs were more likely to harbour *C. upsaliensis* and *C. jejuni* than adult animals. It may be related with younger dogs' lower immunity. So far the relationships between the occurrence of *C. upsaliensis* and gastritis as well as diarrhoea in dogs and humans have not been sufficiently explained. However, younger individuals are believed to be the main source of occurrence of gastritis and diarrhoea, as Rahimi *et al*. (2012) noted. Salihu *et al*. (2010), claim that age is not an indicator of the risk of contamination with *C. jejuni*. In our study *C. upsaliensis* was found significantly more often in younger dogs than adult animals whereas there was no such difference observed with *C. jejuni* and *C. coli*. Infections caused by *Campylobacter* spp. are usually correlated with the seasons of the year, although there are reports indicating that the isolations of *Campylobacter* spp. from dogs were more frequent in spring (Sandberg *et al*., 2002). Rahimi *et al.* (2012) recorded an increase of *Campylobacter* spp. isolations from young dogs aged under 1 year as well as from individuals which were over 1 year of age in warm months: in spring – 38.5% and in summer – 38.9%. In our study, the frequency of isolation of *Campylobacter* spp. was higher in spring and in winter. The study by Rizal *et al*. (2010) showed that the following factors are involved in pathogenicity of *Campylobacter* spp.: motility and chemotaxis as well as adhesion and invasiveness. Nowadays it is believed that the following genes are responsible for the potential pathogenicity of *Campylobacter* spp.: the *fla*A gene influencing motility, *cad*F – affecting adhesion, *cdt*B – responsible for toxin production (cytolethal distending toxin) and *iam* – determining invasiveness (Krutkiewicz, 2008). In our investigations the *cad*F gene was determined in 100% in *C. upsaliensis*, *C. jejuni* and *C. coli*. Biswas *et al.* (2011) confirmed the occurrence of this gene in 100% of the examined *C. jejuni* strains derived from human clinical studies and cow faeces. Selwet and Galbas (2012a; 2012b) observed that the *cad*F and *fla*A genes were found in 100% of *C. coli* and *C. jejuni* isolated from broilers, porkers, calves and piglets. All the strains of *C. jejuni*, *C. coli C. upsaliensis* under analysis carried the *cdt*B gene encoding the protein exhibiting toxic properties, *i.e.* cytolethal distending toxin. This exotoxin causes the inhibition of the cell cycle and DNA degradation in the host (Lara-Tejero, 2001) and it may cause the death of sensitive eukaryotic cells (Heywood *et al.*, 2005). CDT is composed of three subunits: CdtA, CdtB and CdtC, which are encoded by three genes: *cdt*A, *cdt*B and *cdt*C. All the three subunits are required for full activity (Rozynek *et al*., 2005). In our study the *iam* gene, which is responsible for invasiveness, was found in all the strains of *Campylobacter* spp. Carvalho *et al*. (2001) observed in their studies that the *iam* gene was detected most frequently in the strains of *C. jejuni* rather than in *C. coli*. The PCR analysis of stool isolates collected from dogs on farms in Wielkopolska (Greater Poland) region revealed the presence of *Campylobacter* spp. with the predominance of *C. upsaliensis*, which occur more frequently in young animals. The presence of *C. jejuni* and *C. coli* was frequently identified in adult dogs with the symptoms of diarrhoea, whereas *C. jejuni* was identified in young dogs. The correlation between diarrhoea and the presence of these bacteria should be taken into consideration in veterinary practice.

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Acknowledgements

Acknowledgements for financial support and for a personal assistance (with the permission of person named) are given in two separate paragraphs below the main text.

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- **Funnel B.E. and G.J. Phillips** (eds). 2004. *Plasmid Biology*. ASM Press, Washington, D.C.
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Pol J Microbiol 2014; 63(3): 267–273 Fournier's gangrene – current concepts.

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On page 267 there is an error in the affiliation.
The affiliation of Dariusz Kawecki is Chair and Department Medical Microbiology, Medical University of Warsaw, Warsaw, Poland.