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ORIGINAL PAPER

Presence of Antibodies Against *Leptospira interrogans* Serovar *hardjo* in Serum Samples from Cattle in Ukraine

ANTON PYSKUN[1]*, VITALII UKHOVSKYI[2], OLENA PYSKUN[1], VITALII NEDOSEKOV[2], VYACHESLAV KOVALENKO[3], SERHIY NYCHYK[2], MYKOLA SYTIUK[2] and WOJCIECH IWANIAK[4]

[1] State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Kyiv, Ukraine
[2] Institute of Veterinary Medicine of the National Academy of Agrarian Sciences, Kyiv, Ukraine
[3] State Scientific Control Institute of Biotechnology and Strains of Microorganisms, Kyiv, Ukraine
[4] Consultation Service, Pulawy, Poland

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Abstract

The article presents data on serological studies of 573 sera samples of cattle that were collected from the farms affected by leptospirosis in different regions of Ukraine in the period of 2014–2015. Samples were investigated by the microscopic agglutination test (MAT), which was conducted within eight serological groups of *Leptospira* and nine serovars: *Sejroe* (serovars *polonica* and *hardjo*), *Hebdomadis* (serovar *kabura*), *Tarassovi* (serovar *tarassovi*), *Pomona* (serovar *pomona*), *Grippotyphosa* (serovar *grippotyphosa*), *Canicola* (serovar *canicola*), *Icterohaemorrhagiae* (serovar *copenhageni*), and *Australis* (serovar *bratislava*). The circulation of *L. interrogans* serovar *hardjo* among cattle has been observed in all 11 regions of Ukraine investigated within 25.8–60.0% of the leptospirosis-positive serum samples in these regions. Antibodies in the cattle sera against serovar *hardjo* (serogroup *Sejroe*) were detected in 139 of the 370 cows reacting positively in MAT. Overall, they were detected in 24.3% animals out of the total of 573 cows investigated. These are the preliminary results, however, in our opinion, they should allow to include the serovar *hardjo* in a standard panel of strains for MAT in Ukraine.

Key words: antibody, cattle, leptospirosis, microscopic agglutination test, serovar *hardjo*

Introduction

Leptospirosis is a dangerous zoonotic infection with a worldwide distribution that is recognized as an emerging disease (Levett 2001; Sykes et al. 2011). There are no available data about leptospirosis in animals and humans in a number of countries, and thus its global burden remains mostly unknown (Hartskeerl et al. 2011). In general, leptospirosis has been reported in over 150 mammalian species (Ko et al. 2009), but the infectious agent can also be detected in other classes of animals (reptiles, amphibians, etc.) (Levett 2001; Adler and Moctezuma 2010).

To date, there are about 20 species of pathogenic *Leptospira*: *L. kirschneri*, *L. borgpetersenii*, *L. mayottensis*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, *L. alstonii* etc. that include serogroup and serovars (Ko et al. 2009). Over 250 pathogenic serovars of *Leptospira* have been recognized (Adler and Moctezuma 2010). Susceptibility to them in species of animals is different. According to Ukrainian and foreign scientific literature, rodents are considered maintenance hosts for leptospires in serogroups *Grippotyphosa* and *Icterohaemorrhagiae*, and dogs are hosts for serogroup *Canicola*. Pigs in most cases are infected by *Pomona*, *Icterohaemorrhagiae*, and *Australis* (serovar *bratislava*). Cattle are the maintenance hosts of serovar *hardjo* (serogroup *Sejroe*) and are often infected by *polonica* (serogroup *Sejroe*) and *kabura* (serogroup *Hebdomadis*) (Levett 2001; Sykes et al. 2011; Ukhovskyi et al. 2014).

In Ukraine, the standard diagnostic panel included all the mentioned above serovars for MAT except serovar *hardjo*.

The wide spectrum of symptoms confuses the clinical diagnosis and makes it unreliable (Sharma et al. 2007). The laboratory diagnosis of leptospirosis in animals, a prerequisite for their treatment, is usually achieved either by isolation of the causative agent with

* Corresponding author: A. Pyskun, State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Kyiv, Ukraine; **e-mail: anton.pyskun1989@gmail.com**

further identification (PCR) or by serological analysis revealing the infection (Werts et al. 2001; OIE 2018). Although the serological diagnosis is easier than isolation *Leptospira* by culture from biological specimens it is quite difficult since a wide range of antigens is used to detect infections in different countries where uncommon or previously undetected serovars may occur (Katz et al. 1991). Furthermore, the number of serovars and serogroups constantly increase due to the discovery of new strains, cultured from macroorganisms and the environment (Levett 2001).

Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA), but serological diagnosis of leptospirosis generally is based on detecting antibodies by MAT which is a referent method for this disease (Sykes et al. 2011; OIE 2018). MAT involves the serial dilutions of patient` sera that react with live *Leptospira* followed by an assessment of organism's agglutination with a dark field microscopy. According to World Organization for Animal Health (OIE) recommendations, a titer 1/100 can be considered as positive, but on practice, it differs from country to country. For example, in Ukraine, it is 1/50. Because of a large amount of *Leptospira* serovars, standard panels for MAT vary in different countries and include generally from five to seven serovars (Sykes et al. 2011). In Ukraine, in accordance with the current instruction the standard panel of *Leptospira* strains for MAT includes eight serovars (*polonica*, *kabura*, *tarassovi*, *pomona*, *grippotyphosa*, *canicola*, *copenhageni*, and *bratislava*).

In addition, leptospirosis may be registered as the incidence of the disease in animals caused by strains that are not included in the standard panel of strains for MAT in certain countries (exotic strains). So, the sensitivity of the assay can be improved by the use of local isolates rather than reference strains, but reference strains assist in the interpretation of results between laboratories (Pinto et al. 2015).

Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is linked mainly to serovar *hardjo* (Levett 2001). Nowadays, cattle are the maintenance hosts for this serovar (Ellis et al. 1981; Ryan et al. 2012), and shed *leptospires* both in urine and the discharges from the genital tract (Ryan et al. 2012). The infection with this pathogen occurs worldwide: in Malaysia, Argentina, Chile, India, and the European countries (Myers and Jelambi 1975; Bahaman et al. 1988; Sehgal 2000; Salgado et al. 2015). Ellis et al. reported in 1981 that a combined random survey of both beef and dairy cattle in Northern Ireland resulted in positive antibody titers in 34.7% of the population sampled toward serovar *hardjo* using the MAT assay (Ellis et al. 1981). The serological herd prevalence of serovar *hardjo* in beef herds in England was 72% in 1987 (Pritchard et al. 1987); herd prevalence was 11% among beef herds in 2001 in Spain (Alonso-Andicoberry et al. 2001); and in the USA 42% of suckler herds were infected with *Leptospira hardjo* in 2007 (Wikse et al. 2007).

This strain was isolated in 1938 from a patient in Sumatra, Indonesia, by J.W. Wolff, who called it serovar *hardjo* (Wolff 1953). This serovar was first mentioned in the Wolff and Broom list in 1954 (Wolff and Broom 1954). The strain was subjected to analysis by Kmety in 1977, who based on the results placed it in the subgroup *Wolffi* (Kmety 1977). Since 1999, when the DNA relatedness was determined among *Leptospira* strains, a strain *Hardjoprajitno* serovar *hardjo* belongs to *L. interrogans* spp. (Brenner et al. 1999).

There is no information in the literature about the prevalence of serovar *hardjo* (serogroup *Sejroe*) in animals in Ukraine, and this serovar was not included in the routine panel of strains for MAT in our country. Therefore, the aim of the study was to monitor the circulation of this pathogen among cattle in the farms affected by leptospirosis (previously confirmed cases in cattle by other serovars of *Leptospira*) of 11 regions of Ukraine.

Experimental

Materials and Methods

Cattle sera. Serum samples from 573 cattle were analyzed at the Laboratory of Leptospirosis of the Institute of Veterinary Medicine of National Academy of Agrarian Sciences in Kyiv during 2014–2015. Samples have been selected from the leptospirosis-affected farms (confirmed cases in cattle by other serovars of *Leptospira*) in 11 regions (oblasts) of Ukraine: Khmelnytskyi, Chernihiv, Kyiv, Volyn, Donetsk, Poltava, Kharkiv, Vinnytsia, Odesa, Dnipropetrovsk, and Cherkasy. Samples were collected randomly from cattle in herds where the cases caused by other serovars of *Leptospira* had been confirmed. Serum samples were tested immediately or stored frozen at –20°C. Subsequently, sera were thawed and analyzed for the presence of *Leptospira hardjo* antibodies.

Antigens. Research was conducted with eight reference strains of *Leptospira's* serological groups and eight serovars included in the diagnostic panel for the MAT analysis as the most common causative agents of leptospirosis among animals in Ukraine: *Sejroe* (serovar *polonica*), *Hebdomadis* (serovar *kabura*), *Tarassovi* (serovar *tarassovi*), *Pomona* (serovar *pomona*), *Grippotyphosa* (serovar *grippotyphosa*), *Canicola* (serovar *canicola*), *Icterohaemorrhagiae* (serovar *copenhageni*) and *Australis* (serovar *bratislava*). In addition,

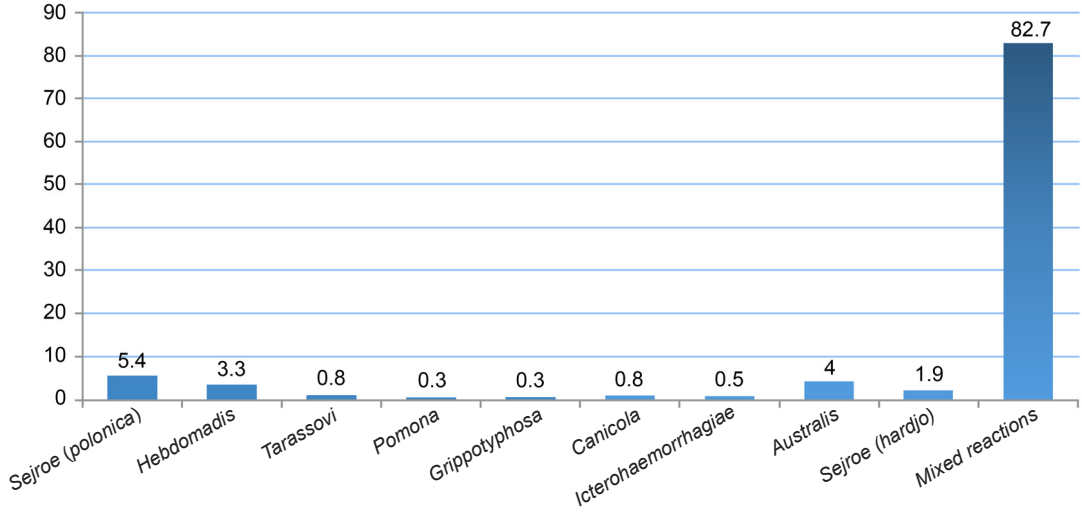

Fig. 1. The etiological structure (%) of *Leptospira* serological groups in the cattle positive sera from the affected farms in different regions of Ukraine (N = 370).

MAT was conducted with serovar *hardjo* (serogroup *Sejroe*). All reference strains were provided by the OIE and National Collaborating Centre for Reference and Research on Leptospirosis (Amsterdam, Netherlands) and cultivated in the Laboratory of Leptospirosis at the Institute of Veterinary Medicine of National Academy of Agrarian Sciences in Kyiv. Each serovar was grown in 10 ml volumes in liquid Korthof medium, incubated at 28–30°C for 6–10 days (depending on the serovar) in aerobic conditions. The concentration of bacteria was approximately $1–2 \times 10^8$ organisms/ml.

Microscopic Agglutination Test. The MAT procedure was carried out according to the Manual of Standards for Diagnostic Tests and Vaccines of the World Organization for Animal Health (OIE). The results were recorded in accordance with the current Ukrainian regulations. The samples of sera diluted 1/25 were mixed with an equal volume of each of the *Leptospira* serovars. Final serum dilution (including the antigen added) 1/50 was used during the preliminary examination. For the positive samples in the preliminary examination that reacted with one or more serovars, the series of two-fold dilutions were prepared to titer endpoint – 50% agglutination. The samples showing titers equal to or higher than 1/50 were recognized as positive.

MAT was conducted using four titers: 1/50, 1/100, 1/500 and 1/2500. The results have been evaluated using a dark field microscope (with magnification × 300).

Results

The etiological structure of leptospirosis in cattle from the affected farms (previously confirmed cases in cattle by other serovars of *Leptospira*) in different regions of Ukraine was studied during 2014–2015. Over the whole period, 573 samples of cattle sera were investigated, and 370 positive reactions have been detected, which constitutes 64.6%.

The etiological structure of MAT-positive *Leptospira* serological groups in cattle is shown in Fig. 1.

Analysis of the data showed that the dominant *Leptospira* serological groups, which were circulating among cattle and recorded as monoreactions, were the follows: *Sejroe* (serovar *polonica*) (5.4%), *Australis* (4.0%), and *Hebdomadis* (3.3%). Other serological groups and serovars were detected in a smaller quantity: *Sejroe* (serovar *hardjo*) in 1.9% of cows, *Tarassovi* and *Canicola* – in 0.8% each, *Icterohaemorrhagiae* – in 0.5%, and *Pomona* and *Grippotyphosa* – in 0.3% each.

Since the antibodies against serovars *hardjo*, *tarassovi*, *canicola*, *copenhageni*, *pomona* and *grippotyphosa* in the serum samples were diagnosed to a lesser extent, one could conclude about their minor role in the etiology of leptospirosis in the cattle studied. However, at the same time, a significant number of mixed reactions (antibodies to more than one serovar) were noticed in 305 cows (82.7% from the total number of the positive-reacting cows). This phenomenon, as shown in Table I, is probably associated with the addition of serovar *hardjo* (serogroup *Sejroe*) to the panel of reference strains used in MAT, because antibodies to this pathogen were recorded in a large percentage of positive mixed reactions (23.2%).

As shown in Table I, 570 positive reactions to different serovars in mixed reactions were observed. The dominant, as previously, were serological groups *Sejroe* (serovar *polonica*) and *Australis* (serovar *bratislava*). They consisted of 24.9% and 18.4%, respectively, of positive mixed reactions for several serogroups of *Leptospira*. Simultaneously, antibodies to *L. interrogans* serovar *hardjo* were diagnosed by MAT almost at the same

Table I
The prevalence of antibodies to different *Leptospira* serogroups in mixed reactions diagnosed with MAT.

Total number of positive reactions to serovars in mixed reactions (%)			570 (100%)
The average number of serovars per mixed reaction			2
Specific combinations of multiple serovars that were commonly seen in samples			Serovars *polonica* and *kabura*, *polonica* and *hardjo*, *hardjo* and *bratislava*
Number of positive reactions in the samples with antibodies against *Leptospira* serogroups	*Sejroe* (serovar *polonica*)	number	142
		%	24.9
	Hebdomadis	number	84
		%	14.7
	Tarassovi	number	31
		%	5.4
	Pomona	number	37
		%	6.5
	Grippotyphosa	number	14
		%	2.5
	Canicola	number	10
		%	1.8
	Icterohaemorrhagiae	number	15
		%	2.6
	Australis (serovar *bratislava*)	number	105
		%	18.4
	Sejroe (serovar *hardjo*)	number	132
		%	23.2

level as *Sejroe* (serovar *polonica*) and accounted for 23.2%. Antibodies to serogroups *Hebdomadis* (14.7%), *Pomona* (6.5%) and *Tarassovi* (5.4%) were noted less frequently. Positive reactions to serological groups *Icterohaemorrhagiae* (2.6%), *Grippotyphosa* (2.5%) and *Canicola* (1.8%) were recorded at lower levels.

For 132 sera a positive reaction with serovar *hardjo* and for 142 with serovar *polonica* was shown out of 305 animal's sera with mixed positive reactions. Of those, 97 samples were obtained from the same animals, therefore it may be assumed that there are cross-reactions between both serovars, which is probably due to their belonging to the same serological group *Sejroe*.

Analyzing the data by regions, it has to be noted that antibodies to these pathogens were registered in a different number of animals. The least difference between rates of infection by these serovars was detected in Cherkasy (positive reactions to serovars *hardjo* and *polonica* were observed, respectively, for four and five cows sera), Poltava (three and five) and Donetsk (one and four) Regions. In Dnipropetrovsk Region, there were no cases of mono- or mixed-positive reaction in which the serovar *hardjo* was diagnosed separately from *polonica*. Antibodies to serovar *polonica* were also noted in four cows and together both pathogens with other serogroups, in three cows. In other regions of Ukraine, from which the serum samples were collected, the rates of infection with these serovars differed significantly from seven (in Volyn Region) to 22 (in Kharkiv Region) cows (Table II).

As shown in Table II, antibodies to serovars *hardjo* and *polonica* (serogroup *Sejroe*), were detected in the cattle from all 11 regions of Ukraine from where the serum samples were collected. Of 370 cows, which sera that reacted positively, 100 (27.0%) cows were infected by both serovars. Positive reactions to *Sejroe* (serovar *hardjo*) and other serogroups (excluding serovar *polonica*) were detected in 39 cows' sera, which accounted for 10.5%. In total, antibodies to *L. interrogans* serovar *hardjo* (serogroup *Serjoe*) were diagnosed in 139 (37.5%) of 370 animals, which sera reacted positively. That shows that a significant portion of leptospirosis observed in this study were caused by this serovar. This pathogen was found in 24.3% of the total number of investigated cattle (139 out of 573 animals).

Figure 2 illustrates the positive reaction to serovar *hardjo* in the serum samples from the MAT-positive cows with leptospirosis in different regions of Ukraine during 2014–2015.

As shown in Fig. 2, antibodies to serovar *hardjo* (serogroup *Sejroe*) were diagnosed by MAT in 25.8% of the infected cattle in Poltava Region. In the cattle

Table II
The indicators of cattle infection with serovars *hardjo* and *polonica* from leptospirosis-affected farms in different regions of Ukraine.

Regions	Number of the cattle investigated	Number of the positively reacting cattle sera (% from total sera investigated)	Number of the positively reacting cattle sera to serovars *hardjo* and *polonica* (%)			Number of the sera positively reacting with leptospirosis antigens that did not react to *hardjo* and *polonica* (% from the positive reacting sera)
			Sejroe (*hardjo*) and other serogroups without *Sejroe* (*polonica*) (% from the positive reacting sera)	*Sejroe* (*polonica*) and other serogroups without *Sejroe* (*hardjo*) (% from total positive reacting)	Two serovars together and other serogroups (% from the positive reacting sera)	
Khmelnytskyi	38	31 (81.6)	3 (9.7)	13 (41.9)	14 (45.2)	1 (3.2)
Chernihiv	86	41 (47.7)	6 (14.6)	14 (34.1)	9 (22.0)	12 (29.3)
Kyiv	73	45 (61.6)	4 (8.9)	23 (51.1)	13 (28.9)	5 (11.1)
Volyn	33	29 (87.9)	1 (3.4)	8 (27.6)	7 (24.1)	13 (44.9)
Donetsk	12	10 (83.3)	1 (10.0)	4 (40.0)	5 (50.0)	–
Poltava	53	31 (58.5)	3 (9.7)	5 (16.1)	5 (16.1)	18 (58.1)
Kharkiv	106	71 (67)	11 (15.5)	33 (46.5)	26 (36.6)	1 (1.4)
Vinnytsia	65	57 (87.7)	8 (14.0)	18 (31.6)	9 (15.8)	22 (38.6)
Odesa	40	25 (62.5)	2 (8.0)	16 (64.0)	5 (20.0)	2 (8.0)
Dnipro	32	10 (31.3)	–	4 (40.0)	3 (30.0)	3 (30.0)
Cherkasy	35	20 (57.1)	4 (20.0)	5 (25.0)	4 (20.0)	7 (35.0)
Total	**573**	**370 (64.6)**	**39 (10.5)**	**143 (38.7)**	**100 (27.0)**	**88 (23.8)**

from Volyn, Odesa, Vinnytsia, and Dnipro Regions, antibodies to this serovar were found in 27.6–30.0% of cattle. In Chernihiv, Kyiv and Cherkasy Regions they were detected in 36.6–40.0% of animals. The high rate of infection caused by *hardjo* was detected in Kharkiv and Khmelnytskyi Regions. In these areas, the infection

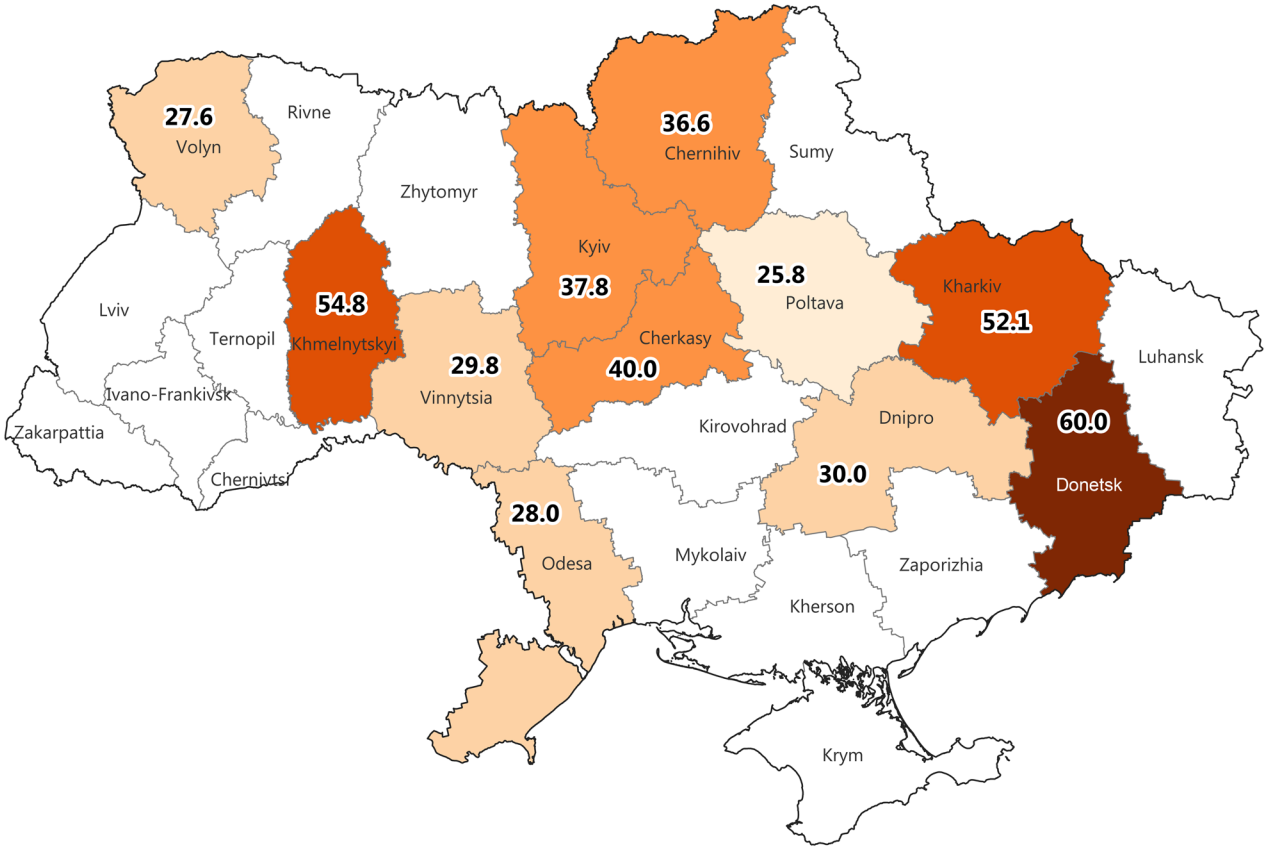

Fig. 2. The percentage of antibodies to serovar *hardjo* among the serum samples from cows with leptospirosis in the regions investigated during 2014–2015 (N = 370).

by *L. interrogans* serovar *hardjo* (serogroup *Sejroe*) was recorded in 52.1–54.8% of the MAT-positive cows. The highest levels of antibodies to this serovar were diagnosed in Donetsk Region and reached 60.0%.

In general, the highest level of antibodies against serovar *hardjo* was in western regions of the country (52.1–60.0%), the lower – in the east (27.6–54.8%) and the north (36.6–40.0%) regions. The lower incidence of positive samples was in the central part of the country (25.8–30.0%) when compared to the east and west regions. In all cases, antibodies to serovar *hardjo* were detected by MAT in titers of 1/50–1/100.

Discussion

Leptospirosis is the most widespread zoonosis worldwide, which is present on all continents except Antarctica and evidence for the carriage of *Leptospira* has been found in virtually all examined mammalian species (Adler and Moctezuma 2010). It is an important zoonotic bacterial infection of livestock that may cause reproductive failure, loss of milk production, economic losses and can result in human infection (Carole and Bolin 2001).

Different species of animals are hosts for various serovars of *Leptospira*. Thus, cattle are maintenance host for serovar *hardjo* (Ellis et al. 1981; Ryan et al. 2012) but the scientific literature from different countries describes cases of infection by this pathogen in other species too (red deer in Italy, brown bears in Croatia, wild boars in Poland, etc.) (Slavica et al. 2010; Andreoli et al. 2014; Żmudzki et al. 2015). The infections with this pathogen are registered at the present time in many countries including Andaman and Nicobar Islands (Sehgal 2000; Sharma et al. 2003; Salgado et al. 2015; Chideroli et al. 2016; Balamurugan et al. 2018; Miyama et al. 2018; Sunder et al. 2018).

Leptospira serovar *hardjo* mainly causes reproductive diseases and failures, such as abortion, mummification, stillbirth, premature and term birth of weak calves, as well as the loss of milk production in dairy herds (Carole and Bolin 2001, Ryan et al. 2012). Moreover, serovar *hardjo* has been isolated from physiologically normal fetuses, the genital tracts of pregnant cattle (Ellis et al. 1982), and vaginal discharge after calving (Levett 2001).

Before our study, there was no information in Ukrainian and foreign literature regarding the circulation of serovar *hardjo* (serogroup *Sejroe*) in animals in Ukraine and this serovar was not included to the standard panel of strains for MAT in our country. In the neighboring countries of eastern Europe, the infections caused by the serovars from the *Sejroe* serogroup were reported in Hungary (Fuzi et al. 1957), Romania (Combiesco et al. 1958.), Moldavia (Matveeva et al. 1977) and Russia (Bondarenko et al. 2002) but these infections were mainly caused by serovar *polonica*. In the south-western region of Poland, during 2010–2011 the seroprevalence of antibodies against serovar *hardjo* was 4.6 and 4.1%, respectively, in herds of 51–100 and 101–500 animals (Rypuła et al. 2014). At the same time, the infections with this serovar occurred among humans in Georgia (Mamuchishvili et al. 2015).

Therefore, we investigated the circulation of this pathogen among cattle from farms affected by leptospirosis and compared the percentage of MAT-positive samples to serovar *hardjo* with a total number of cattle blood sera seropositive to leptospirosis.

The results of our serological studies indicated that infection among cattle caused by *L. interrogans* serovar *hardjo* was detected in 37.5% of the total number of cattle that reacted positively in MAT in the farms affected with leptospirosis in different regions of Ukraine. It is a high number but similar to the numbers reported in other countries such as Northern Ireland in 1981 – 34.7% (Ellis et al. 1981), England in 1987 – 72% (Pritchard et al. 1987), Spain in 2001 – 11% (Alonso-Andicoberry et al. 2001), and the USA in 2007 – 42% (Wikse et al. 2007).

In the majority of the samples investigated, antibodies against serovar *hardjo* were detected together with antibodies to other serogroups of *Leptospira* (mixed reactions) and accounted for 23.2% of these.

Regarding recent cases of leptospirosis caused by serovar *hardjo*, in 2018 the scientists from India published the results that are similar to our results. They investigated 373 cattle serum samples by MAT from 45 farms in 11 states in India. Samples were collected from animals with a history of reproductive disorders like abortion, repeated breeding, anoestrus, and endometritis, and also from apparently healthy animals. The *Leptospira* antibodies against the serovar *hardjo* were shown in 27.76% of cattle (Balamurugan et al. 2018).

Analyzing the results in different regions of Ukraine, least frequently leptospirosis caused by serovar *hardjo* (serogroup *Sejroe*) was detected in Poltava Region, where it was in 25.8% of MAT-positive cattle. The highest number of *hardjo* positive-sera (60%) were registered in the Donetsk Region. In general, infection by *L. interrogans* serovar *hardjo* was detected in all regions of Ukraine, from which samples of sera were received.

Antibodies in titers of 1/50–1/100 can be interpreted as the early stage of infection or chronic leptospirosis. The results obtained in this study that titer of 1/100 or greater should be taken as significant in the cattle infected with serovar *hardjo* were supported by others (Carole and Bolin 2001; OIE 2018).

In our opinion, further research is needed to perform a more meaningful analysis of the epizootic situation of this pathogen in different regions. However,

the data obtained here show that cattle in 11 regions of Ukraine were affected by serovar *hardjo*. Perhaps this serovar has already been circulating in the territory of Ukraine constantly or it entered the country due to the import of animals from other countries. This hypothesis is supported by the fact that serovar *hardjo* is not a component of the typical panel of strains for MAT used to control the sera of imported animals to Ukraine.

For the first time, we investigated the possible circulation of serovar *hardjo* in Ukraine. At the same time, in the world, the highest risk of leptospirosis infection is associated with dairy farming and linked to serovar *hardjo*. In addition, cattle are the maintenance hosts of this pathogen. In our opinion, the results of our research indicate the circulation of this pathogen among cattle in Ukraine. The differences in the detection of positive samples between the regions can be related to the fact that samples were randomly collected. For further improvement of this monitoring and the definition of infection indicators, we need to investigate more samples from each region and cover all territory of Ukraine.

In conclusion, there is a need of conducting more meaningful analysis (to investigate more samples from each region and cover all territory of Ukraine) of the epizootic situation regarding the serovar *hardjo* in different regions of Ukraine and incorporating of this serovar as obligatory into a routine diagnostic panel of *Leptospira* strains used for MAT in the country.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Prevalence and Antifungal Susceptibility of the Emerging Fungal Species, *Candida nivariensis*, Isolated in a Teaching Hospital in Poland

MAGDALENA SIKORA[1, 2], ROBERT KUTHAN[2, 3]*, KATARZYNA PISKORSKA-MALOLEPSZA[2, 3], MARLENA GOLAS-PRADZYNSKA[3], DARIUSZ DOMAŃSKI[4], EWA AUGUSTYNOWICZ-KOPEĆ[5] and EWA SWOBODA-KOPEC[2, 3]

[1] Department of Dental Microbiology, Medical University of Warsaw, Warsaw, Poland
[2] Department of Microbiology, Infant Jesus Teaching Hospital, Warsaw, Poland
[3] Chair and Department of Medical Microbiology, Medical University of Warsaw, Warsaw, Poland
[4] Salus Medycyna Medical Center, Siedlce, Poland
[5] Department of Microbiology, National Tuberculosis and Lung Diseases Research Institute, Warsaw, Poland

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Abstract

The data on susceptibility to antifungals of new species within *Candida glabrata* complex are limited. Our study was to enrich a global knowledge of yeast epidemiology and drug resistance. The study was focused on the identification of species within clinical isolates of the *C. glabrata* complex and on the determination of their resistance to antifungals. Four hundred forty-five clinical *C. glabrata sensu lato* strains were isolated from different clinical samples at routine mycological exams at the Infant Jesus Teaching Hospital in Warsaw. The identification of the most of tested isolates to species complex level was performed using the ID 32 C system. The identification of *C. nivariensis* and *C. bracarensis* species within the *C. glabrata* complex was performed by DNA sequencing. The MICs of amphotericin B, fluconazole, itraconazole, posaconazole, voriconazole, caspofungin, anidulafungin, and micafungin were determined by E-test. Twenty-four isolates did not have an ITS-1 region, characteristic of *C. glabrata sensu stricto* and their D1/D2 regions of the 26S rRNA were 99% homologous to *C. nivariensis* 26S rRNA. No strains of *C. bracarensis* were recovered. *C. nivariensis* strains were very susceptible to amphotericin B, anidulafungin, micafungin, and caspofungin. Ninety-two percent of *C. nivariensis* were resistant to itraconazole. The halves of the strains was resistant to posaconazole. Eighty-three percent of *C. nivariensis* were susceptible to voriconazole. None of the tested strains were susceptible to fluconazole. In the present study, none of the *C. nivariensis* strains were simultaneously resistant to azoles and echinocandins. *C. nivariensis* should be recognized as an emerging pathogen, resistant to azoles.

Key words: *Candida glabrata* complex, *Candida nivariensis*, emerging pathogen, resistance to azoles

Introduction

Non-*albicans Candida* (NAC) yeast-like fungi play a more active role in fungal infections. *Candida glabrata*, one of the most important yeast-like fungi of this group, is the second most common cause of candidiasis.

Distribution of *C. glabrata* varies depending on the geographical area. Relatively high incidence of *C. glabrata* was observed in the northern part of Europe and in the USA in contrary to southern countries of Europe and Latin America where *C. parapsilosis* infections are more often found.

The global prevalence of *C. albicans* is decreasing, in contrary to *C. glabrata* and *C. krusei*, which remain stable. The incidence of *C. parapsilosis* and *C. tropicalis* is increasing. It was also demonstrated that *C. glabrata* is more often isolated from elderly patients (Falagas et al. 2010; Alexander et al. 2013; Guinea 2014).

The reason for the change in the profile of *Candida* infection remains unknown, however, an increase in NAC infections, especially *C. glabrata*, seems to be attributable to unreasonable antifungal prevention (Basetti et al. 2009; Gołaś et al. 2014). *C. glabrata* candidemias are on the increase (Quindós 2014). In ten years

* Corresponding author: R. Kuthan, Chair and Department of Medical Microbiology, Medical University of Warsaw, Warsaw, Poland; Department of Microbiology, Infant Jesus Teaching Hospital, Warsaw, Poland; **e-mail: rkuthan@yahoo.com**

(since 1989) *C. glabrata* candidemias have increased from the fourth to the second most common cause of ICU infections (Lockhart et al. 2012).

Nowadays, the *C. glabrata* species complex has been identified including three closely related new species: *C. glabrata sensu stricto*, *C. nivariensis*, and *C. bracarensis* (Alcoba-Flórez et al. 2005; Miranda-Zapico et al. 2011).

C. nivariensis and *C. bracarensis* represented less than 5% within the *C. glabrata* complex (Bishop et al. 2008; Lockhart et al. 2009; Sharma et al. 2013). *C. nivariensis* was more frequently isolated than *C. bracarensis*. These species could become the new sources of opportunistic infections. However, the mycological routine diagnostics cannot differentiate between *C. glabrata sensu stricto*, *C. bracarensis*, and *C. nivariensis*. It can only classify these species as a part of the *C. glabrata* species complex. A complete species differentiation within the complex may be achieved with molecular biology techniques, which can identify closely related yeast-like fungi (Angoulvant et al. 2016). In order to assess the clinical impact of the new species within the *C. glabrata* species complex, it is important to determine their virulence factors. Virulence factors enable yeast-like fungi to begin and carry out the subsequent stages of an infection. Amongst the most important pathogenicity determinants of *C. glabrata sensu lato* is their ability to adhere to abiotic and biotic surfaces, to form biofilm on both surfaces, and to secrete phospho- and lipases, hemolysins, and other cytotoxic enzymes. (Tamura et al. 2007; Silva et al. 2012; Rodrugues et al. 2014).

Little is known about the drug resistance of the *C. glabrata* species complex. Some studies suggest that the new species within the complex may be more resistant to antifungals than *C. glabrata sensu stricto* (Tamura et al. 2007; Silva et al. 2012; Li et al. 2014, Rodrugues et al. 2014; Angoulvant et al. 2016). *C. nivariensis* and *C. bracarensis*, two new species recently discovered, exhibit many traits common to *C. glabrata sensu stricto*, but are isolated less frequently. Despite being detected more commonly, these two species are still unknown and require further investigation.

This study was to identify the clinical species within the *C. glabrata* complex and to determine their resistance to antifungals.

Experimental

Materials and Methods

Four hundred forty-five clinical *C. glabrata sensu lato* strains were isolated from different clinical samples (urine – 154, tracheal aspirate – 63, throat swab – 44, sputum – 34, feces – 32, peritoneal fluid – 27, skin ulcer – 26, vagina – 18, post-surgical wound drainage – 12, bile – 11, blood – 7, bronchoalveolar lavage – 7, other – 10) at routine mycological exams at the Infant Jesus Teaching Hospital in Warsaw in the years from 2014 to 2016. All samples were collected from adult patients. The samples were cultured following routine microbiological diagnostic guidelines on Sabouraud agar and incubated at 30°C for 24–72 h until representative single colonies were formed. The ID 32 C yeast identification system (bioMérieux, France) was used for species identification within the complexes.

Species identification within *C. glabrata* complex. The isolation of genomic DNA of the *C. glabrata* complex isolates was performed using the Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit (EurX, Poland) following the manufacturer's guidelines.

***C. nivariensis* and *C. bracarensis* identification within the *C. glabrata* complex.** There were two stages of species identification within the *Candida glabrata* complex. In the first stage, the internal transcripted spacer 1 (ITS-1), characteristics of *C. glabrata sensu stricto* was identified using PCR. The NL-1 (5'-GCAT ATCAATAAGCGGAGGAAAAG') and NL-4 (5'-GGT CCGTGTTTCAAGACGG') primers were used for the amplification. The amplification were performed at following conditions: initial denaturation 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 1 min, elongation at 72°C, 30 sec., and then final elongation at 72°C for 10 min. In the second stage the DNA was sequenced. Strains without the ITS-1 amplicon specific for *C. glabrata* had the D1/D2 region of the 26S rRNA sequenced. The NL-2A (5'-CTTGTTCGCTATCGGTCTC') and NL-3A (5'-GAGACCGATAGCGAACAAG') primers were used for sequencing (Kurtzman et al. 2003). The sequencing results were analyzed with the BLAST (the Basic Local Alignment Search Tool) software to compare the nucleotide sequences obtained with the reference sequence databases and calculate the statistical significance.

The resistance of *C. nivariensis* to antifungals. E-test (bioMérieux, France) was used on RPMI agar; the minimal inhibitory concentrations (MIC [μg/ml]) for amphotericin B (AMB), fluconazole (FLU), itraconazole (ITC), posaconazole (POS), voriconazole (VOR), caspofungin (CAS), anidulafungin (ANF), and micafungin (MCF) were measured. MIC results (S – susceptible and R – resistant) of FLU, AMB, CAS, MCF, and ANF were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST 2018). In details, the interpretation for antifungals MICs was as follows: AMB ≤ 1 susceptible, > 1 resistant, FLU ≤ 0,002 susceptible, MCF ≤ 0,032 susceptible, > 0,032 resistant, ANF ≤ 0,064 susceptible, > 0,064 resistant, CAS – isolates that are susceptible to anidulafungin as well as

micafungin should be considered susceptible to caspofungin. MIC results (S – susceptible and R – resistant) of ITC, POS, and VOR the CLSI guidelines were used (CLSI 2008). In details, the interpretation for antifungals MICs was as follows: ITC ≤ 0,125 susceptible, > 1 resistant, POS ≤ 1 susceptible, > 4 resistant, VOR ≤ 1 susceptible, > 1 resistant.

Results

Species identification within the new *C. glabrata* complex. Four hundred forty-five strains were identified as *C. glabrata* and analyzed using PCR. For twenty-four isolates (5.4% of all strains) the ITS-1 amplicon characteristic of *C. glabrata sensu stricto* was not observed and their D1/D2 regions of the 26S rRNA were sequenced. They contained sequences 98% homologous to *C. nivariensis* 26S rRNA. A representative BLAST analysis of the sequenced D1/D2 regions of the 26S rRNA is shown in Fig. 1. Based on the sequencing results, no strains of *C. bracarensis* were identified. Table I presents the source of isolation of *C. nivariensis* and the drug susceptibility results.

Drug susceptibility of *C. nivariensis*. The strains of the new species were isolated from various clinical samples, including primarily sterile specimens. Their sensitivity to antifungals varied.

C. nivariensis strains were all susceptible to amphotericin B, anidulafungin, micafungin, and caspofungin. The MIC50 and MIC90 for amphotericin B were 0.19 mg/l and 0.5 mg/l respectively, and the MIC50 and MIC90 for caspofungin were 0.19 mg/l and 0.19 mg/l respectively. The MIC_{50} = 0.012 mg/l, MIC_{90} = 0.016 mg/l, and micafungin MIC_{50} = 0.008 mg/l, MIC_{90} = 0.023 mg/l strains also had low anidulafungin.

Forty-one percent of *C. nivariensis* were resistant to itraconazole with the MIC in the range of 1.5–32 mg/l. The half of the strains (50%) was resistant to posaconazole with the MIC of 1.5–32 mg/l. Eighty-three percent of *C. nivariensis* were susceptible to voriconazole (the MIC in the range of 0.008–2.0 mg/l). All of the strains tested were intermediate-susceptible or resistant to fluconazole with the MIC in the range from 0.25 to 256 mg/l.

Discussion

C. glabrata candidemias are on the increase, as it has been suggested in the literature due to the overuse of fluconazole treatments (Tapia et al. 2012; Colombo et al. 2013; Quindós 2014). There is little information on the isolation of *C. nivariensis* and *C. bracarensis*. These are estimated to constitute 0.2–4.0% of the *C. glabrata* complex (Bishop et al. 2008; Lockhart et al. 2009, Sharma et al. 2013). In the present study, *C. nivariensis* was more frequently isolated and made up for 5.4% (24 strains). Sharma et al. (2013)assessed 100 *C. glabrata* isolates and found five *C. nivariensis* strains. Chowdhary et al. (2010) analyzed 366 *C. glabrata* complex strains and established that two of them were *C. nivariensis*. Li et al. (2014) conducted a study on 301 isolates of *C. glabrata* complex cultured from vulvovaginal candidiasis cases. They found seven isolates of *C. nivariensis*. Similarly, the drug resistance of the new species within the *C. glabrata* complex is not well known. As there are very few species in the world identified as *C. nivariensis* or *C. bracarensis,* the information about their drug resistance profile is very scarce. Li et al. (2014) showed that all *C. nivariensis* isolates were susceptible to nystatin and susceptible or susceptible dose-dependent to fluconazole, itraconazole, miconazole, and clotrimazole. The authors did not provide the MICs values of the antifungals tested.

Both species were susceptible to amphotericin B; their MIC was not higher than 1.0 μg/ml. The international data on *C. nivariensis* and *C. bracarensis* resistance to azoles is different. The MIC values for azole (especially fluconazole) was high against some strains what may suggest that they could have the same resistance mechanisms as *C. glabrata sensu stricto.* Previous studies suggested that the MIC values for echinocandin against *C. nivariensis* and *C. bracarensis* were low. None of these strains was proved resistant to this medication (Angoulvant et al. 2016).

C. glabrata sensu lato may nowadays be resistant to amphotericin B. In 1993 Pfaller et al. discovered 25% of *C. glabrata sensu stricto* with MIC > 1 μg/ml (Pfaller et al. 2004). More and more often the MIC value for amphotericin B against *C. nivariensis* was ≥ 0.5 μg/ml (Angoulvant et al. 2016). In the present study, both *C. glabrata sensu stricto* and *C. nivariensis* were susceptible to amphotericin B.

The *C. nivariensis* strains evaluated in the present study were resistant to fluconazole (100%), itraconazole (41.7%), posaconazole (50%), and voriconazole (17%). One of the few studies of the British Reference Laboratory conducted by Borman et al. (2008) performed on 16 clinical *C. nivariensis* isolates has reported similar results regarding *C. nivariensis in vitro* sensitivity to azoles. However, in contrary to their findings on the resistance to voriconazole and posaconazole (MIC_{50} at 4 mg/l and 1 mg/l, respectively), in the present study only 17% of the strains were resistant to voriconazole and 50% to posaconazole (MIC_{50} at 0.125 mg/l and 0.75 mg/l, respectively).

Other studies also suggested high value of the MIC for fluconazole (between 16–128 mg/l) against *C. nivariensis* (Fujita et al. 2007; Borman et al. 2008;

Sharma et al. 2013). In the present study, the MIC for fluconazole ranged from 0.25 to 256 mg/l.

In our opinion, all strains that had the MIC above 0.002 mg/l should be considered as the intermediate susceptible to fluconazole since there are no established interpretive breakpoint criteria to designate the *C. nivariensis* strain as either susceptible or resistant to fluconazole. Our opinion is supported by the EUCAST guidelines on the susceptibility to fluconazole of *C. glabrata*, which classify all stains above this value as the intermediate susceptible *in-vitro*.

In contrary to the findings of the present study, Li et al. (2014) proved that *C. nivariensis* was susceptible to fluconazole and itraconazole. Also Tay et al. (2014) established that *C. nivariensis* was susceptible to fluconazole and voriconazole.

Our study results also differ from the results presented by Huo et al. (2017) who studied 12 *C. nivariensis* strains and one *C. bracarensis strain* isolated from ten Chinese hospitals, and found that all strains were susceptible to azoles but not to fluconazole. Based on these findings we believe that susceptibility to azoles may vary geographically and can be attributed to the divergent use of azole in various locations. It is worth to mention that five of our isolates with the MIC value of 256 mg/l against fluconazole showed also the highest MICs values for itraconazole, posaconazole, and voriconazole. This finding may suggest cross-resistance among azoles, similar to the one described for *C. glabrata* (Panackal et al. 2006). This has to be elucidated for *C. nivariensis*.

Echinocandins, i.e. caspofungin, anidulafungin, and micafungin, are the newest available antifungal medication. However, the number of *C. glabrata sensu lato* strains with a lowered sensitivity to echinocandins have been described in several publications (Katiyar et al. 2006; Cleary et al. 2008; Thompson et al. 2008; Garcia-Effron et al. 2009; Arendrup et al. 2013).

Pfaller et al. (2011) evaluated 215 *C. glabrata sensu lato* isolates and established that 16.7% of them were resistant to echinocandins. Low values of the MIC for echinocandin against *C. nivariensis* have already been reported. In the present study, all strains *C. nivariensis* were susceptible to echinocandins.

Our data are in concordance with the data published by Morales-López et al. (2017) regarding the strains isolated during 30 years in Argentina. They tested resistance to echinocandins of five *C. nivariensis* strains isolated from a collection of 122 *C. glabrata* complex strains. All *C. nivariensis* strains tested were resistant to echinocandins with the MICs ranging from 0.015 to 0.03 mg/l and from 0.06 to 0.13 mg/l for anidulafungin and caspofungin, respectively. Since the relatively low number of *C. nivariensis* has already been examined worldwide it is difficult to estimate the real resistance rate to echinocandins. *C. glabrata sensu stricto* simultaneous resistance to azoles and echinocandins was observed in the past years and it is an alarming phenomenon (Pfaller et al. 2011; Alexander et al. 2013; Morales-López et al. 2017). Cleveland et al. (2015) observed a simultaneously increased resistance to azoles and echinocandins, from 1.8% to 2.6% within five years of observation. In the present study, any *C. nivariensis* strain was simultaneously resistant to azoles and echinocandins.

Since data on the epidemiology and susceptibility to antifungals of *C. nivariensis* are limited, our study may enrich the global knowledge about its epidemiology and drug resistance. Moreover, it indicates the need for proper microbiological analysis with the use of molecular methods or with the updated spectra of the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *C. nivariensis* should be recognized as an emerging, azoles-resistant pathogen.

ORCID
Robert Kuthan 0000-0002-9680-1632

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Literature

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ORIGINAL PAPER

Chemical Composition of *Aspergillus creber* Extract and Evaluation of its Antimicrobial and Antioxidant Activities

AFAF SAKHRI[1, 2]*, NOREDDINE KACEM CHAOUCHE[1], MARIA ROSARIA CATANIA[3], ALBERTO RITIENI[4] and ANTONELLO SANTINI[4]

[1] Laboratoire de Mycologie, de Biotechnologie et de l'Activité Microbienne (LaMyBAM), Département de Biologie Appliquée, Université des Frères Mentouri Constantine-1, Constantine, Algeria
[2] Department of Medicine, University of Batna 2, Batna, Algeria
[3] Department of Molecular Medicine and Medical Biotechnologies, University of Napoli Federico II, Naples, Italy
[4] Department of Pharmacy, University of Napoli Federico II, Naples, Italy

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Abstract

Among the species belonging to the *Aspergillus* section *Versicolores*, *Aspergillus creber* has been poorly studied and still unexplored for its biological activities. The present study was undertaken to analyze *A. creber* extract and to evaluate its *in vitro* antimicrobial and antioxidant activities. UHPLC-MS/MS analysis of *A. creber* extract allowed the characterization of five known fungal metabolites including: asperlactone, emodin, sterigmatocystin, deoxybrevianamide E, and norsolorinic acid. The highest antimicrobial activity was displayed against *Candida albicans*, with a mean strongest inhibition zone of 20.6 ± 0.8 mm, followed by Gram-positive drug-resistant bacteria. The MIC values of *A. creber* extract varied from 0.325 mg/ml to 5 mg/ml. *A. creber* extract was shown a potent antioxidant activity and a high level of phenolic compounds by recording 89.28% scavenging effect for DPPH free radical, 92.93% in ABTS assay, and 85.76 mg gallic acid equivalents/g extract in Folin-Ciocalteu assay. To our knowledge, this is the first study concerning biological and chemical activities of *A. creber* species. Based on the obtained results, *A. creber* could be a promising source of natural antimicrobial and antioxidant compounds.

Key words: Antimicrobial activity, antioxidant activity, *Aspergillus creber*, UHPLC-MS/MS, *Versicolores*

Introduction

Fungal secondary metabolites represent a diverse group of bioactive natural products often produced at a restricted part of the life cycle (Keller et al. 2005). *Aspergillus* species are among the major contributors to the secondary metabolites of fungal origin (Dewi et al. 2012; Bai et al. 2014). Although the functions of these compounds for the producing fungi are obscure or unknown, they confer important benefits to humankind as many of them have antibiotic and pharmaceutical activities (Yu and Keller 2005; Siddiquee et al. 2015). Isolation of a new fungal strain is often conducted to the identification of new natural products (Brakhage and Schroeckh 2011).

Aspergillus species assigned to section *Versicolores* are among the most ubiquitous fungi, frequently isolated from environmental samples (Gautier et al. 2016). The important characteristics that render these species of interest to the scientific community are their prevalent in indoor environments, their capacity to produce sterigmatocystin (STC), a carcinogenic mycotoxin, and their diverse biotechnological applications (Siqueira et al. 2016). Before using molecular methods for species identification, isolates belonging to section *Versicolores* were commonly reported as *A. versicolor* (Despot et al. 2016). In 2012, Jurjevic et al. revised the section *Versicolores* and accepted 13 species; among them, *Aspergillus creber* was described for the first time as a new species. Currently, the section *Versicolores* comprises 17 distinct species (Despot et al. 2017); among them, *A. versicolor* and *A. sydowii* are still the most commonly reported and studied species (Siqueira et al. 2016). However, very few reports are available concerning the species *A. creber* and those reports were only concerned with contamination by *A. creber* and its ability to

* Corresponding author: A. Sakhri, LaMyBAM, Département de Biologie Appliquée, Université des Frères Mentouri Constantine-1, Constantine, Algeria; Department of Medicine, University of Batna2, Batna, Algeria. **e-mail: a.sakhri@univ-batna2.dz, afsakhri@hotmail.fr**

produce STC. *A. creber* was reported as the most prevalent species in indoor air environments in USA (Jurjevic et al. 2012) and in Italian libraries (Micheluz et al. 2015). *A. creber* was also reported as among the lower producers of STC (Jurjevic et al. 2013).

Therefore, the lack of knowledge of this species recently assigned to section *Versicolores* has attracted our attention to more explore and study this species. In our research *A. creber* was assayed for its secondary metabolites diversity using UHPLC-MS/MS technique. Then, the antimicrobial activity of *A. creber* against some human pathogens and its ability to scavenge different free radicals, and to produce antioxidant compounds were also evaluated to improve the knowledge of this species, which has never been tested before for its biological activities.

Experimental

Materials and Methods

Fungal strain. The strain of *A. creber* employed in this study was isolated from maize grains collected from Batna region, Northeast Algeria. Morphological identification of the selected strain was made following the recommended method and media (Samson et al. 2014). Then, the identity was confirmed by the German Collection of Microorganisms and Cell Cultures (DSMZ) using the analysis of the Internal Transcribed Spacer rDNA region (ITS) and the calmodulin gene (CaM) sequences. The strain was kept on Potato Dextrose Agar (PDA) slants at 4°C in the fungal collection of the Laboratory de Mycologie, de Biotechnologie et de l'Activité Microbienne (LaMyBAM), University des Frères Mentouri Constantine-1.

Production and extraction of *A. creber* secondary metabolites. Under sterile conditions, a small amount of *A. creber* was transferred onto new PDA plates and incubated at 25°C until sporulation. The fungal spores were harvested by pouring 9 ml of sterile distilled water, containing 0.1% Tween 80, on the agar plate surfaces and transferred into sterile Falcon tubes. The prepared fungal suspension was inoculated aseptically in five 1000 ml Erlenmeyer flasks [5% (v/v)], each containing 250 ml of Czapek-Dox broth supplemented with yeast extract (Slack et al. 2009). The Erlenmeyer flasks were covered with the aluminum foil and incubated at 28 ± 2°C with shaking at 125 rpm for two weeks.

The culture broth was filtered, using Whatman filter paper number 1, to remove the mycelia. The filtered broth was extracted three times with equal volumes of ethyl acetate (EtOAc) and evaporated until dryness using a rotary evaporator at 45°C. The resulted ethyl acetate extract of the filtrate (EAF) was reconstituted in methanol to get a concentrated stock solution of 200 mg/ml, which was used for further analysis.

Ultrahigh-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) analysis of *A. creber* extract. A Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, USA) coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, USA) via a HESI-II electrospray ionization (ESI) source was used for the analysis of EAF. A volume of 1 µl of EAF was injected on a Luna Omega C18 column (50 × 2.1 mm, 1.6 µm particle size). The column thermostat was maintained at 25°C. A mobile phase consisting of eluent A (0.1% formic acid in water, 5 mM ammonium formate) and eluent B (0.1% formic acid in methanol, 5 mM ammonium formate) was used at a flow rate of 0.4 ml/min. The gradient elution was performed as follows: 0 min 20% eluent B; 0.5 min 40% eluent B; 6 min 100% eluent B; 8 min 20% eluent B; 10 min 20 % eluent B.

The Orbitrap mass spectrometer was operated in both positive and negative mode with the use of the following parameter settings: spray voltage, 4 kV; sheath gas (N2 > 95%), 35 arbitrary units; auxiliary gas (N2 > 95%), 10 arbitrary units; capillary temperature, 290°C; S lens RF level, 50; heater temperature, 305°C. Two scan events were carried out, the LC-MS was used in full scan mode at a resolution of 70 000 fwhm (full width at half maximum) in the range m/z 90–1000 without the use of any lock masses. The maximum injection time (MIT) was 100 ms with one micro scan, and the automatic gain control (AGC) target was set to 1e6. The MS/MS was performed in parallel reaction monitoring (PRM) mode, in order to obtain two product ions for each target compound, at a resolution of 35 000 fwhm with collision energy (CE) of 30, the AGC and MIT were set at 2e5 and 200 ms, respectively. The instrument control and data analysis were performed by Thermo Fisher Xcalibur v. 3.0.63 software.

Antimicrobial activity assay

Test microorganisms. Eight clinically isolated microorganisms, obtained from the laboratory of Clinical Microbiology, University of Federico II Napoli-Italy, were used to evaluate the antimicrobial activity of EAF. Four Gram-negative pathogenic bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Salmonella* Typhi) and two Gram-positive drug-resistant bacteria (DRB, *Staphylococcus aureus* producing beta-lactamase (SABL) and methicillin-resistant *Staphylococcus epidermidis* (MRSE)) were used for the antibacterial tests. Two pathogenic yeasts (*Candida albicans* and *Candida glabrata*) were used for anticandidal tests. Pathogenic bacteria were inoculated into the nutrient broth and incubated at 37°C for 24 h. *Candida* species were inoculated into Sabouraud Dextrose broth at 28°C for 48 h.

Antimicrobial activity determination. Antimicrobial activity of EAF against the test organisms was examined using disc diffusion method according to Turkoglu et al. (2007) with slight modification. Suspension of the organisms tested (0.5 McFarland standards) was spread on the sterile growth media plates containing Nutrient agar and Sabouraud Dextrose agar for bacteria and yeasts, respectively. Sterilized paper disks (6 mm) were impregnated with 20 µl of EAF and dried aseptically. The impregnated discs were placed on the surface of plates seeded with the test organisms and incubated at 37°C for 24 h for bacteria and at 28°C for 48 h for yeasts. Both chloramphenicol (10 µg/disk) and ketoconazole (30 µg/disk) were employed as positive controls for pathogenic bacteria and *Candida* species, respectively. Similarly, 20 µl of methanol and clean disks (without solvent) were used as negative controls. All experiments were carried out in triplicate. After incubation, antimicrobial activity was determined by measuring the diameter of inhibition zones around the discs.

Determination of minimum inhibitory concentration (MIC). The minimum inhibitory concentration (MIC) of EAF was carried out against the sensitive test microorganisms by using broth microdilution method following the recommendations of Clinical and Laboratory Standards Institute (CLSI) protocols for bacteria (CLSI 2012) and for yeasts (NCCLS 2002). Chloramphenicol and ketoconazole were used as a positive control. The MIC was taken as the lowest concentration at which no visible growth was observed.

Antioxidant analysis

2,2-diphenyl-1-picryl hydrazyl (DPPH) assay. The DPPH radical scavenging activity was determined according to Jakovljević et al. (2014). Different concentrations of EAF were prepared: 12.5, 25, 50, 100, 200, 400 µg/ml. To 1 ml of DPPH solution, 1 ml of each concentration was added. After incubation for 30 min at 37°C, the absorbance was measured at 517 nm against the blank. Ascorbic acid was used as positive control. The percentage of the DPPH scavenging activity was calculated using the formula:

$$\% \text{ inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 represents the absorbance of the blank sample, and A_1 represents the absorbance of the mixture.

2,2'-azino-bis-3- ethylbenzthiazoline-6-sulphonic acid (ABTS) assay. Using the same concentrations, ABTS assay was used as reported by Dudonné et al. (2009). The ABTS radical cation ($ABTS^{\bullet+}$) stock solution was prepared by mixing 7 mM of ABTS with 2, 45 mM of potassium persulfate. The mixture was left in the dark at room temperature for 12–16 h. For the study of EAF, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. To 3 ml of $ABTS^{\bullet+}$ diluted solution, 100 µl of each concentration was added, and the absorbance reading at 734 nm was taken at the end of the tenth minute. Percent inhibition of $ABTS^{\bullet+}$ was calculated using the previous formula.

Total phenolic content (TPC) assay. TPC of *A. creber* was determined using the Folin-Ciocalteu method. To 0.5 ml of EAF, 2.5 ml of Folin-Ciocalteu reagent was added. After 4 min, 2 ml of sodium carbonate (7.5%, w/v) were added, and the absorbance was measured at 760 nm after incubation for 2 h at room temperature (Gan et al. 2010). The TPC of the extract was expressed as mg Gallic acid equivalent per g of extract (mg GAE/g extract).

Results

Fungal strain. *Aspergillus* isolate showed the typical macro- and micro-morphological features described for section *Versicolores*. For species designation, sequencing of the ITS rDNA and CaM gene followed by a BLAST search revealed that the isolated strain had a high identity (99%) with *A. creber*. Thus, according to these results, the strain was identified as *A. creber* and its sequences data were submitted at GenBank with accession numbers: MH796366 and MH796367.

UHPLC-MS/MS analysis. After two weeks of growth in the fermentation broth, the secondary metabolites of *A. creber* were extracted, through EtOAc, filtered and concentrated *in vacuo* to give EAF, which was subjected to UHPLC-MS/MS analysis. On the basis of the fungal metabolites list established by Lehner et al. (2011), molecular mass data, MS/MS analysis and references, five known fungal metabolites were tentatively identified as asperlactone **(1)**, emodin **(5)**, sterigmatocystin **(7),** deoxybrevianamide E **(9)** and norsolorinic acid **(10)** (Table I) whereas five metabolites remained unidentifiable. The Total Ion Current (TIC) chromatograms of the ten peaks of detected compounds are shown in Fig. 1.

Antimicrobial activity. Results obtained for the antimicrobial activity of EAF are shown in Table II. In the case of bacteria, the highest zone of inhibition was displayed against Gram-positive DRB, including MRSE and SABL, followed by Gram-negative bacteria *K. pneumoniae* and *S. typhi* with an inhibition zone ranged from 8.5 ± 0.6 to 14.0 ± 0.2 mm. No activity was observed against *E. coli* and *P. aeruginosa* at the concentration used. In the case of yeasts, the EAF inhibited the growth of all tested yeasts (*C. albicans* and *C. glabrata*) with maximum inhibitory activity against *C. albicans*, which displayed an important zone of inhibition (20.6 ± 0.8 mm).

Table II showed also the MIC values of *A. creber* extract against the tested organisms, which showed an inhibition zone in the disc diffusion assay. The EAF of *A. creber* showed MIC values ranging from

Table I
Metabolites of *Aspergillus creber* as determined by UHPLC-MS/MS. The metabolites were tentatively identified using molecular mass data, MS/MS analysis and references.

Peak No.	Putative compound name	Adduction	Measured mass (m/z)	Productions (m/z)	t_R[a] (min)	Ref[b]
1	Asperlactone	[M+H]+	185.08081	141.05444, 113.05948	1.73	Vishwanath et al. 2009
2	NI	[M+Na]+	211.06121	195.03455, 133.02816	1.78	–
3	NI	[M+H]+	308.10992	280.95587, 145.10104	3.38	–
4	NI	[M+H]+	327.04734	309.18594, 191.15396	4.28	–
5	Emodin	[M+H]–	269.04590	ND	5.42	Sulyok et al. 2007; Lehner et al. 2011; Micheluz et al. 2016
6	NI	[M+H]+	251.09145	233.09526, 204.09319	5.49	–
7	Sterigmatocystin	[M+H]+	325.07053	ND	6.62	Lehner et al. 2011; Micheluz et al. 2016
8	NI	[M+Na]+	423.25041	405.27911, 239.14821	7.27	–
9	Deoxybrevianamide E	[M+H]+	352.20352	ND	8.04	Lehner et al. 2011; Micheluz et al. 2016
10	Norsolorinic acid	[M+H]–	369.09892	ND	8.24	Micheluz et al. 2016

[a] – Retention time; [b] – Reference; ND – not detected; NI – not identified

Table II
Antimicrobial activity and minimum inhibitory concentration (MIC) of ethyl acetate extract of *Aspergillus creber* against human pathogens.

	Zone of inhibition (mm)[a]		MIC of ethyl acetate extract (mg/ml)
	Ethyl acetate extract	Positive control[b]	
Bacteria			
Escherichia coli	0	23.4 ± 0.3	–
Klebsiella pneumoniae	10.0 ± 0.3	25.0 ± 0.4	2.5
Pseudomonas aeruginosa	0	20.8 ± 0.4	–
Salmonella Typhi	8.5 ± 0.6	21.5 ± 0.3	5
SABL[c]	12.8 ± 0.3	19.8 ± 0.2	0.625
MRSE[d]	14.0 ± 0.2	19.2 ± 0.2	0.625
Yeasts			
Candida albicans	20.6 ± 0.8	22.8 ± 0.4	0.325
Candida glabrata	13.0 ± 0.3	25.7 ± 0.5	1.25

[a] – Mean of three replicates (±) SD
[b] – Chloramphenicol and ketoconazole were used as the positive control for bacteria and yeasts respectively
[c] – *Staphylococcus aureus* producing beta lactamase
[d] – Methicillin resistant *Staphylococcus epidermidis*
0 – no zone of inhibition

0.325 to 5 mg/ml. The higher MIC values were recorded against *C. albicans* (0.325 mg/ml) and Gram-positive DRB (0.625 mg/ml) whereas the lower MIC values were recorded against *C. glabrata* (1.25 mg/ml) and Gram-negative bacteria *K. pneumoniae* (2.5 mg/ml) and *S. typhi* (5 mg/ml).

Antioxidant analysis. In the DPPH assay, the EAF of *A. creber* extract demonstrated a dose-dependent scavenging activity and the highest decolorization was recorded at 400 µg/ml (Fig. 2a). In the ABTS assay, like the DPPH assay, the EAF exhibited a rich scavenging effect and the highest decolorization was also recorded at a concentration of 400 µg/ml (Fig. 2b). In both assays, the scavenging activity of EAF was slightly lower than that of ascorbic acid. In the Folin-Ciocalateu assay, the EAF showed a high level of total phenols (Table III).

Table III
Antioxidant activities of *Aspergillus creber* ethyl acetate extract by DPPH and ABTS assays and its total phenolic content.

Activity	Extract	Ascorbic acid[a]
DPPH[a]	89.28 ± 0.32	91.39 ± 0.39
ABTS[a]	92.93 ± 0.30	93.03 ± 0.45
Total phenolic content (mg GAE/g)	85.76 ± 0.96	–

[a] – Percentage of inhibition at a concentration of 400 µg/ml. Values are mean of three replicates (±) SD

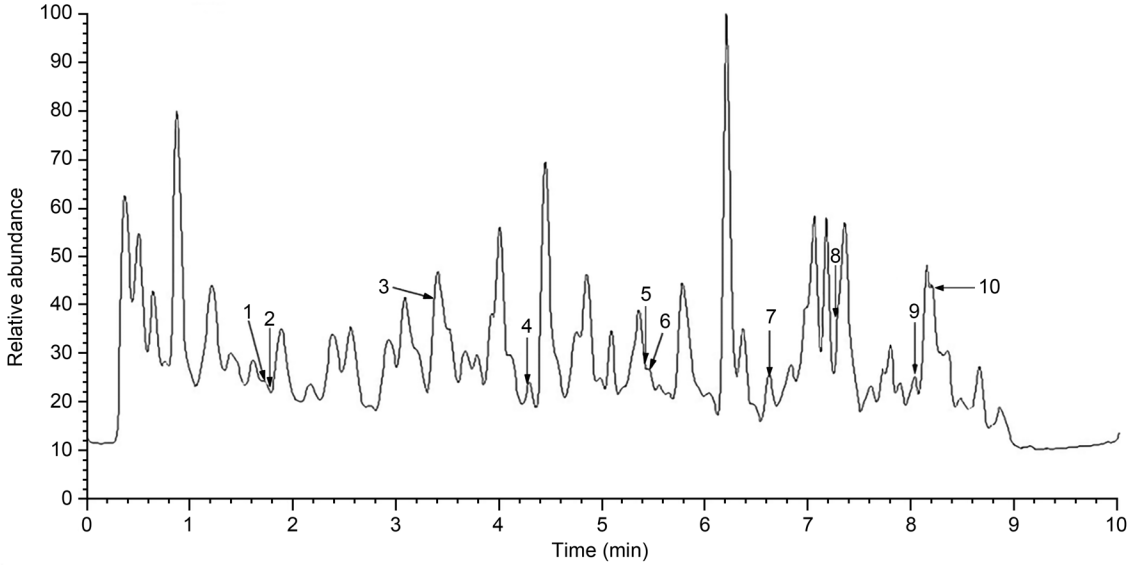

Fig. 1. Total Ion Current chromatogram of *Aspergillus creber* extract obtained with UHPLC-MS/MS. The retention times of the peaks (1–10) and their corresponding molecules names are presented in Table I.

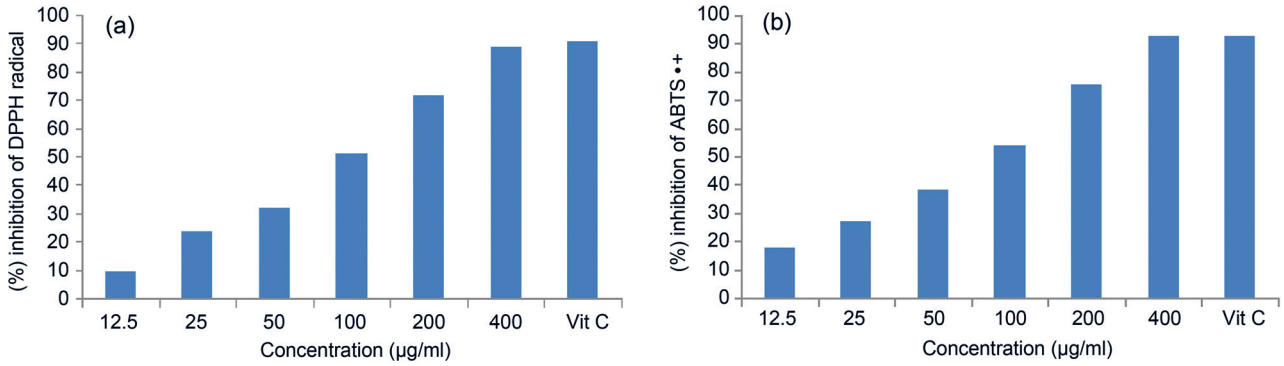

Fig. 2. Free radical-scavenging activities of *Aspergillus creber* extract and ascorbic acid (400 μg/ml) measured (a), in DPPH assay and (b), in ABTS assay.

Discussion

Fungi are major producers of secondary metabolites with different biological activities and various chemical structures (Abo-Elmagd 2014). Until today, except for *A. versicolor* and *A. sydowii*, there is no thorough study on metabolic profiles for the *Aspergillus* species belonging to section *Versicolores* (Despot et al. 2017). Moreover, to our knowledge, there are no published reports recorded the antimicrobial and the antioxidant activities of *A. creber*.

Our study demonstrated that *A. creber* is able to produce a variety of secondary metabolites previously reported as potent biologically active compounds. The biological activity of asperlactone, a polyketide metabolite, had been established for the first time by Balcells et al. (1995). It was the first fungal compound that exhibited insect growth regulating activity in *in vivo* tests. Asperlactone has also been reported as a potent antibacterial and antifungal metabolite (Chen et al. 2014). Emodin is the most studied anthraquinone derivative for its diverse biological activities, including antibacterial (Hatano et al. 1999), antifungal (Kim et al. 2004), antioxidant (Izhaki 2002), and anticancer properties (Cheshmi et al. 2017; Zhao et al. 2017). STC is a polyketide mycotoxin and a precursor of Aflatoxin B1 (AFB1), however, its toxicity is lower than that of AFB1 (Piontek et al. 2016). Deoxybrevianamide E is a prenylated indole alkaloid that belongs to the family of brevianamides. Although a variety of biological activities were exhibited by many brevianamides including antibacterial, anti-insect pests and antitubercular properties (Xu et al. 2017) there are no reports in the literature concerning the bioactivities of deoxybrevianamide E. Norsolorinic acid is an anthraquinone derivative fungal metabolite. Wang et al. (2008) reported that norsolorinic acid has antiproliferative activity on T24 human bladder cancer cells.

The ability of *A. creber* to produce STC is in accordance with previous studies demonstrated that most of *Aspergillus* section *Versicolores* are STC producers (Jurjević et al. 2013, Despot et al. 2016). Moreover, our findings are in agreement with the study of Micheluz et al. (2016) reported that *A. creber* is able to produce emodin, STC, deoxybrevianamide E and norsolorinic acid. However, to our knowledge, this is the first report concerning the production of asperlactone from the species *A. creber*.

In the antimicrobial assays, our study indicated that *A. creber* exhibited more potent antibacterial activity against Gram-positive than Gram-negative bacteria. This variation of susceptibility could be attributed to the morphologic difference in the composition of their cellular membranes, which influences their reaction to antibacterial compounds (Valle Jr et al. 2015). In addition to its antibacterial activity, the EAF of *A. creber* exhibited higher anticandidal activity against *Candida* species. In the case of *Candida* species, ergosterol is the main target of antifungal drugs (Martins et al. 2015). These results are in contrast to the previous studies reported that the metabolites produced by *A. versicolor,* the most studied species of *Aspergillus* section *Versicolores*, exhibited lower or no antibacterial and anticandidal activities (Zhuang et al. 2011; Song et al. 2012; Ebada et al. 2018). Another attractive finding on *A. creber* was the higher MIC values of its ethyl acetate extract against *C. albicans* (0.312 mg/ml) and Gram-positive DRB (0.625 mg/ml) since these species are the most common opportunistic pathogens (Martins et al. 2015; Knafl et al. 2017). Moreover, our findings can be considered very promising since the extract was crude and obtained by using non-optimized fermentation, which usually produces a poor yield of active compounds (Noor Ifatul et al. 2016).

Our study also brings additional data on *A. creber*, since the antioxidant activity and the TPC of this species were evaluated for the first time. The antioxidant ability of *A. creber* was tested using two different procedures, DPPH and ABTS assays. The DPPH assay is a widely used method that based on the reduction of the purple DPPH free radical to a yellow colored compound 1,1-diphenyl-2-picryl hydrazine (Floegel et al. 2011) whereas the ABTS assay is based on the reduction of the blue-green pre-formed $ABTS^{\bullet+}$ to the colorless ABTS form (Re et al. 1999). Thus, the higher scavenging activities of the EAF indicated its bioactive potential to neutralize the DPPH free radicals and $ABTS^{\bullet+}$. These results are in agreement with many other studies reported the antioxidant potency of filamentous fungi from different sources (Abo-Elmagd 2014; Kumaresan et al. 2015; Sharma 2015; Smith et al. 2015; Sugiharto et al. 2016). The slight difference between the antioxidant capacities of EAF and ascorbic acid may be explained either by the purity of ascorbic acid, whereas the extract of *A. creber* is a mixture of multiple compounds or by the capacity of some compounds in the fungal extract to neutralize or to inhibit the effect of active compounds (Dhankhar et al. 2012). Moreover, our data revealed that *A. creber* is able to produce much higher content of phenols than the other filamentous fungi in the literature (Jakovljević et al. 2014; Smith et al. 2015; Sugiharto et al. 2016). Many previous studies attributed the antioxidant activity of filamentous fungi to their TPC (Abdel-Monem et al. 2013; Smith et al. 2015, Nwobodo et al. 2017). In accordance with this, the higher percentages of inhibition of DPPH radical and $ABTS^{\bullet+}$ in our fungal extract might contribute to the high level of phenolic compounds.

In conclusion, UHPLC-MS/MS analysis revealed that *A. creber* could produce a variety of secondary metabolites including polyketides, anthraquinones, and alkaloids. Our findings suggest that *A. creber* might represent a novel source of natural bioactive products since we have demonstrated its ability to inhibit the growth of many pathogen microorganisms, mainly *C. albicans* and Gram-positive DRB, and its high antioxidant activity. Our work will increase the knowledge about the species A. creber newly described and serve as a prelude to a better understanding of the biology and the chemistry of its metabolites.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Evaluation of the *pol*/S Gene Overlapping Mutations in Chronic Hepatitis B Patients in Northern Cyprus

AYSE ARIKAN[1, 3]*, MURAT SAYAN[2, 3], TAMER SANLIDAG[3, 4], KAYA SUER[5], SINEM AKCALI[4] and MERYEM GUVENIR[6]

[1] Near East University, Faculty of Medicine, Medical Microbiology Department, Nicosia, Northern Cyprus
[2] Kocaeli University, Research and Education Hospital, Clinical Laboratory, PCR Unit, Kocaeli
[3] Near East University, Research Center of Experimental Health Sciences, Nicosia, Northern Cyprus
[4] Manisa Celal Bayar University, Faculty of Medicine, Medical Microbiology Department, Manisa, Turkey
[5] Near East University, Faculty of Medicine, Infectious Disease and Clinical Microbiology Department, Nicosia, Northern Cyprus
[6] Near East University, Vocational School of Health Services, Microbiology, Nicosia, Northern Cyprus

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Abstract

Mutations associated with the *pol* and the S gene can emerge as a consequence of the high replication capacity and proofreading deficiencies of hepatitis B virus during replication. The current study was constructed to evaluate primary, partial, compensatory and the escape mutations in chronic hepatitis B patients in Northern Cyprus. The samples of HBsAg positive treatment naïve 100 patients were involved in this study. HBV *pol* gene region was sequenced, amplified and HBV *pol*/S gene mutations were determined. The samples of thirty-two patients were excluded because of their low viral load (HBV < 1000 ıu/ml). Among the sequenced 68 samples, there was a partial mutation (1.5%) and 36.7% displayed a resistance profile to lamivudine, adevofir, and telbivudine. Immune response escape, vaccine escape, HBIg and diagnosis escape mutations were determined in 24%, 10%, 6%, and 4% samples of the patients, respectively. Additionally, there were six different combined mutations. These data underscored that there is no concern for primary mutations in Northern Cyprus, however, we have identified a compensatory mutation (rtV173M) that may have primary mutation characteristics by combining with other mutation patterns. Additionally, HBsAg escape mutants demonstrated that detection of the S gene together with the *pol* gene mutations might be beneficial and important to monitor the surveillance of S variants.

K e y w o r d s: Drug resistance, mutations, hepatitis B, Northern Cyprus

Introduction

Hepatitis B virus (HBV) affects more than 2 billion people worldwide and approximately 247 million chronic individuals are known to be chronic carriers (Caligiuri et al. 2016; WHO 2018). HBV can cause severe liver infections and cirrhosis may develop in 15–40% of individuals if they are not treated (Tang et al. 2018). There are several novel treatment strategies that have been used for the treatment of chronic hepatitis B infection in order to prevent the risk of developing liver failure, cirrhosis, and cancer (Lapiński et al. 2013). Interferon alpha (IFN-α) 2a- 2b, PEGylated interferon-α-2a (*Pegasys*), PEGylated interferon-α-2b (*Pegintron*), and nucleos(t)ide analogues (NAs) are worldwide used for fighting chronic hepatitis B (CHB) infection by suppressing HBV replication (Ward et al. 2016). Lamivudine (LAM), telbivudine (LdT), entecavir (ETV), adefovir (ADV), and tenofovir (TDF) are antiviral agents that have been approved in Europe, America and many Asia-Latin America countries for the treatment of chronic HBV, however, there is a big concern of long term use of these agents as they are associated with the development of antiviral resistance mutations (Sun et al. 2016; Ozguler and Sayan 2016; Zhao et al. 2016; Ozguler and Sayan 2018). These mutations are associated with the amino acid substitutions in the reverse transcriptase (RT) domain and are classified as primary or secondary compensatory resistance mutations (Rugieri Pacheco et al. 2017). Mutations at the amino acid positions rt169, rt181, rt184, rt194, rt202, rt204, rt236, rt250 are classified as the primary resistance mutations which

* Corresponding author: A. Arikan, Near East University, Faculty of Medicine, Medical Microbiology Department, Nicosia, Northern Cyprus; **e-mail: aysearikancy@yahoo.com**

are associated with reducing the sensitivity to antivirals, whereas, the secondary compensatory resistance mutations occur at the amino acid positions rt80, rt173, rt180 and generally restore the replication capability of viral polymerase (Choi et al. 2018).

HBV is characterized by its high replication capacity ($>10^{12}$ virion/day) and a lack of proofreading activity during replication (10^{-5} substitution/base/cycle). This means that each nucleotide on the HBV genome can be changed within a day and cause antiviral resistance-related mutations before treatment (Tezcan et al. 2015). Also, the S gene is completely overlapped by the *pol* gene. Because of this overlap between the S and the *pol* gene, changes that are related to antiviral resistance in the *pol* gene may also occur in the S gene as well (Sayan et al. 2012). This overlap leads to amino acids structure, found in surface antigen (HBsAg) of HBV to change and thus form naturally occurring antiviral resistance mutations. The vaccine escape, diagnosis escape, hepatitis B immunoglobulin (HBIg) escape, and immune escape variants against oral antivirals that are used in chronic HBV treatment may emerge. Another reason for the emerging of these mutations is the occurrence of the mutant viruses deriving from antiviral-treated patients (Zhao et al. 2016). These changes cause reactivation of HBV in vaccinated people and concerns during diagnosis and/or HBIg vaccination failure (Wang et al. 2017; Asan et al. 2018).

Cyprus is an island located in the Mediterranean Sea and has been divided into two communities (Northern and Southern Cyprus). Northern Cyprus, officially the Turkish Republic of Northern Cyprus, is a multinational society due to universities, casinos, luxury hotels, beaches, and other entertainment centers; thus, there are many people coming from foreign countries for education, business and/or tourism purposes. For HBV infection, the prevalence rate ranges from 1.2% (160/13 892) (Arikan et al. 2016) to 1.35% (339/25 442) (Guler et al. 2018). For Southern Cyprus, this rate was given as 0.77% and 1.01% (Altindis et al. 2016). Although there are few studies regarding the prevalence, up to now, there has been no publication on drug resistance in patients infected with HBV in Northern Cyprus (Altindis et al. 2006; Suer et al. 2014). Therefore, we aimed to show the presence of resistance mutations and their clinical significance in untreated chronic hepatitis B patients in Northern Cyprus.

Experimental

Materials and Methods

In our study, the samples of 100 patients with HBsAg positive who had never been treated with NAs or IFN, were involved retrospectively. The study group consisted of 13 892 people who applied to the Near East University Microbiology Laboratory for assessment of hepatitis markers during three years. Of these individuals, 100 samples with positive HBsAg were included in the study for drug resistance analysis. The levels of viral markers [HBsAg, anti-hepatitis B core antigen (anti-HBc), anti-hepatitis B e antigen (anti-HBe), hepatitis B e antigen (HBeAg), alanine aminotransferase (ALT), and aspartate aminotransferase (AST)] were determined by using the chemiluminescent enzyme immunoassay kits according to the manufacturer instructions [(Architect i200, Abbott, USA), (Roche, Cobas E411),

Table I
Demographic and clinical characteristics of the study group.

Variable	Study Group
Patient, n	100
Gender, F/M, n (%)	13 (13%)/87 (87%)
Age, median year (range)	35 (18–65)
Nationality, region/country, n (%)	
Asia	**68 (68)**
Turkey	43 (63)
Northern Cyprus	14 (21)
Pakistan	3 (4)
China	3 (4)
Turkmenistan	3 (4)
Syria	1 (2)
Georgia	1 (2)
Africa	**29 (29)**
Nigeria	28 (97)
Benguela	1 (3)
North America	**1 (1)**
Mexican	1 (100)
Europe	**2 (3)**
Azerbaijan	1 (50)
Bulgaria	1 (50)
Anti HBc IgG positivity, n (%)	96 (96)
HBeAg positivity, n (%)	5 (5)
ALT (average ± SD) (U/L)	23 ± 19
AST (average ± SD) (U/L)	28 ± 19
HBV DNA median IU/ml (range)	1.0 + E7 (1.9 + E1 – 2.8 + E8)
Genotype/subgenotype of HBV, n (%)	
Genotype D	**53 (78.0)**
D1	48 (70.6)
D2	4 (5.9)
D3	1 (1.5)
Genotype A	**7 (10.2)**
A1	5 (7.3)
A2	2 (2.9)
Genotype E	**8 (11.8)**
Treatment status, n (%)	
Naïve	100 (100%)
Under treatment	–

F – female, M – male, ALT – alanine aminotransferase, AST – aspartate aminotransferase, SD – standard deviation

(Olympus AU680, Beckmann Coulter IFCC)]. HBsAg positive sera were stored at –80°C until use. The ethical approval of the study was taken from Near East University Scientific Researches Evaluation Ethics Committee (YDUBADEK, 20/06/2013 date and NEU/2013/16-88 decision number).

The viral loads of positive samples were determined by using real-time polymerase chain reaction (PCR) technique according to the manufacturer instructions (artus HBV QS RGQ Qiagen, Hilden, Germany). The HBV *pol* gene (RT region between 80.–250. aa) was sequenced (between 254.–995. nucleotides), amplified and analyzed for *pol*/S gene mutations (Sayan et al. 2010a). Hepatitis B virus DNA (HBV DNA) was extracted from positive serum samples (Anatolia Geneworks, Bosphore® Viral DNA Extraction Spin Kit ve Magnesia® 16 Magnetic Bead Extraction System, Istanbul, Turkey). HBV polymerase region was amplified by using a pair of primers (forward: 5'-TCGTGGTGGACTTCTCTCAATT-3'and reverse: 5'-CGTTGACAGACTTTCCAATCAAT-3') (Sayan et al. 2010a; 2010b). PCR conditions were 95°C for 10 min, followed by 35 cycles consisting of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s (Sayan et al. 2010a). High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to purify all PCR products. The sequencing was performed on ABI PRISM 3130 (Applied Biosystems Inc. Foster City, USA) platform by using Phire Hot Start DNA polymerase (FinnzymesOy) enzyme and BigDye Terminator v3.1 Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc.), 36 cm capillary and POP-7 TM polymer (Applied Biosystems Inc.). The obtained data were analyzed by using Geno2pheno Drug Resistance program (the Center of Advanced European Studies and Research, Bonn, Germany) which searches for HBV drug resistance mutations at amino acid positions 80., 84., 85., 91., 169., 173., 180., 181., 184., 191., 194., 202., 204., 214., 215., 233., 236.–238., and 250. in the RT domain of the polymerase (Shaw et al. 2006). At the same time, the overlapping S gene segments at amino acid positions 121., 135., 137., 139.–149., 151.–153., 155.–157., 161., 164., 172., 173., 175., 176., 182., 193.–196. were also analyzed and monitored for HBsAg vaccine escape, diagnosis escape, HBIg escape, and immune escape mutations (Avellon and Echevarria 2006).

Results

The current study consisted of HBsAg positive-treatment naïve 100 CHB patients, of whom 13 were female and 87 were male, whose ages varied between 18–65 ages (with a median of 35 years). The origins of the patients were from Asia (68%), Africa (29%), North America (1%) and Europe (2%), and none of them have been taken antiviral therapy when their serum samples were collected. Ninety-six percent and 5% of the study group were positive for anti-HBc IgG and HBeAg, respectively. The serum HBV DNA level was calculated as a median of 1.0 + E7 (range: 1.9 + E1 – 2.8 + E8). ALT and AST levels were reported as 23 ± 19 and 28 ± 19, respectively. We sequenced only the samples of 68 patients in this study because HBV DNA was determined below 1000 IU/ml in 32 (32%) patients' samples. The distribution of genotypes of the sequenced patients (68/100) was determined as D1 (n:48, 70.6%), D2 (n:4, 5.9%), D3 (n:1, 1.5%), A1(n:5, 7.3%), A2 (n:2, 2.9%), and E (n:8, 11.8%) (Arikan et al. 2016). Demographic and laboratory findings of the study are displayed in Table II.

In the *pol* region, there was no primary drug resistance mutation detected in this study. There was only one treatment-naïve patient (1.5%) who had the partial resistance mutation at rt173M amino acid substitution that may be related with LAM and LdT resistance. Twenty-five patients (36.7%) had secondary/compensatory mutations and the mutations patterns (rtL91I, rtQ149K, rtQ215H/P/S and rtN238D) were depicted in Table II.

In the *S* region, 30 (44%) patients had typical HBsAg escape mutations. Vaccine escape, diagnosis escape,

Table II
Mutation characteristics of the HBV *pol*/S gene mutation patterns to the nucleos(t)ide analogues in treatment naïve HBV carriers.

HBV virion	Mutation characteristic	Mutation pattern	Nucleos(t)ide analogues	n (%)
Pol gene	Primary resistance mutation	ND	ND	–
	Partial resistance mutation	rtV173M	LAM-LdT related	1 (1.5)
	Compensatory mutation	rtL91I	LdT related	25 (36.8)
		rTQ149K	ADV related	
		rtQ215H/P/S	LAM-ADV related	
		rtN238D	LAM-ADV related	

ND – not determined, LAM – lamivudine, ADV – adevofir, LdT – telbivudine

Table III
Typical HBsAg escape and combined mutations of the study patients (n = 68).

Typical HBsAg escape mutation	Mutation patterns	n (%)	Combined mutations	Mutation patterns	n (%)
Immune escape	sY100C, sI110L, sP120L/R, sT123N, sT127L, sP127T, sA128V, sT131N, sS132P, sY134F/H, sT140I/S, sS143T, sD144E, sS144T, sP210S	16 (24)	Immune response -vaccine	sI110L + sS193L	1 (1.5)
Vaccine escape	sT126I, sD144A/E, sG145A/R, S193L, sP210T	7 (10)	Vaccine-HBIg	sP120L + sT123N + sT126I + sA128V + sY134H + sD144E + sG145A	1 (1.5)
HBIg* escape	sT118A, sP120T, sD144A/E, sG145A/E/R	4 (6)	Immune response -HBIg	sT118A + sP127T	1 (1.5)
Diagnostic escape	sT118A, sT131I, sP120T, sC121Y, sD144A, sG145R	3 (4)	Immune response-vaccine-HBIg Immune response-diagnosis	sI110L + sP120T + sD144A + sG145R sP120R + sC121Y, sT131I + sS132P	1 (1.5) 2 (3)
Total		30 (44)			6 (9)

* HBIg – Hepatitis B immunoglobulin
** Patients may have more than one mutation pattern

HBIg, immune escape mutations were determined in 7/68 (10%), 3/68 (4%), 4/68 (6%), 16/68 (24%) samples of the patients, respectively and these mutations are presented in Table III.

There were also six different combined mutations. The mutation patterns were sI110L + sS193L, sP120L + + sT123N + sT126I + sA128V + sY134H + sD144E + + sG145A, sT118A + sP127T, sI110L + sP120T + sD144A+ + sG145R and sP120R + sC121Y + sT131I + sS132P. The resistance mutation patterns are given in Table III.

Discussion

Even in treatment naïve patients because of the high replication capacity and the overlapping reading frames, antiviral resistance mutations may occur throughout the HBV genome (Yano et al. 2015). To our knowledge, this is the first study that focused on the resistance mutations in the HBV *pol* and S gene regions in treatment naïve CHB patients in Northern Cyprus. This information has confirmed that there was no primary drug resistance mutation in untreated patients. So, even if patients undergo long-term therapy with NA, they may not require periodic monitoring for the primary drug resistance. The previous studies indicated that primary resistance mutations could occur not only in patients undergoing an NA therapy but also might be detectable in patients receiving any treatment (Bottecchia et al. 2016; Zhao et al. 2016; Asan et al. 2018). The mutations that occur mainly at the amino acid positions 181, 204, 233 and 236 have been shown to be related with the primary resistance mutations in the treatment naïve patients (Asan et al. 2018). However, the mutations at the amino acid positions rt184, rt194, rt202, rt250 are mainly classified as partial resistance mutations and often act as compensatory mutations. The main functions of primary and partial resistance mutations are to reduce drug susceptibility and restore the viral replication defects, respectively (Asan et al. 2018). Thereof, the drug resistance should be analyzed during and/or before initiating the treatment to reduce the risk of liver damage and consequently progression of hepatocellular carcinoma. Sayan and his colleagues from Turkey have shown in many studies that acyclic phosphonate related mutations could occur in CHB patients (Sayan 2010b; Asan et al. 2018; Ozguler and Sayan 2018). Similarly, resistance mutations have been identified from other studies conducted in China and Pakistan (Mahmood and Anwar 2017; Zhao et al. 2016) which supports that primary resistance mutations can occur all over the world.

In the current study, we detected rtV173M partial resistance mutation in one patient according to Geno2pheno Drug Resistance Program, however, the rtV173L substitution has been reported more commonly in the literature (Lin et al. 2012; Gürsoy et al. 2019). Both amino acid substitutions are regarded as compensatory mutations and enhance viral fitness (Asan et al. 2018). The single compensatory mutation profiles are generally associated with low-level resistance; however, they may cause high-level resistance when they combine with other patterns (Lazarevic 2014). Thus, screening to detect resistance may be

regarded to be a benefit in the control of CHB. Sayan has shown in one of his studies that rtV173L mutation combined with sD144E produced HBV vaccine escape + HBIG escape (Sayan and Bugdaci 2013). LAM-associated resistance triple mutation pattern (rtV173L + rtL180M + rtM204V) has also been shown to enhance viral replication compared with rtL180M + rtM204V (Sheldon and Soriano 2008).

In the current study, among 68 patients, there were 25 (37%) secondary/compensatory resistance mutations (rtL91I, rtQ149K, rtQ215H/P/S, rtN238D) which are related to LdT, LAM, and ADV resistance (Saran et al. 2017; Asan et al. 2018). It may be a result of naturally emerging mutations due to the biology of HBV. The most common compensatory mutations have been reported to be rtQ149K, rtQ215H/P/S and rtL91I (Asan et al. 2018). These compensatory mutations are important as they assist viral replication and fitness, and hence are associated with drug resistance (Ahn 2015). In our study, we determined one of the most commonly detectable compensatory mutation rtQ215/H/P/S, which is associated with LAM and ADV resistance (Shaw et al. 2006; Altindis 2016; Asan et al. 2018).

Mutations in the *pol* gene, due to the structure of HBV, also occur in the overlapping HBsAg (Zaaijer et al. 2007; Simon et al. 2013) and some typical HBsAg escape mutations emerge. The immune response escape, HBV vaccine escape, HBIg escape, and HBsAg diagnosis escape were detected in 44 patients in this study. sP120T, Sm133I, Ss143L, sD144A/E, sD145R and Se164D are the most frequently detected typical HBsAg escape mutations in CHB patients (Sayan et al. 2010b). Misdiagnosis in the HBsAg, failure to prevent with vaccination and HBIg, reactivation of hepatitis B, and re-infection in HBV infected recipients and/or orthotropic liver transplantations may be a consequence of the typical escape mutations (Asan et al. 2018). All patients that receive or not receive antiviral treatment should be monitored for these mutations.

In conclusion, these findings on drug resistance mutations show that treatment-naïve CHB patients have low HBV polymerase resistance mutation (1.5%) rate. Hence, primary drug resistance analysis may not be necessary to be performed before the initiation of antintiviral therapy; however, we have discovered a novel compensatory mutation, rt173M that may act as a primary mutation together with other patterns. To draw attention, we preferred to define this mutation as a partial resistance mutation rather than compensatory resistance mutation in this study. Additionally, HBsAg escape mutants in patients living in Northern Cyprus demonstrated us that early detection of possible S gene mutations may be beneficial and important to monitor the surveillance of S variants. Even though national compulsory HBV vaccination program, these mutants may develop in hepatitis B vaccinated and/or HBIG vaccinated individuals with CHB, in chronic carriers and/or patients undergoing antiviral treatment and may pose danger for public health. In addition, due to the arrival of people from many countries to North Cyprus for the reasons we have stated, drug resistance mutations should be analyzed as *pol* and S gene together for monitoring CHB.

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Ethical Approval

Near East University Scientific Researches Evaluation Ethics Committee (YDUBADEK, 20/06/2013 date and YDÜ/2013/16-88 decision number.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Evidence for Infections by the Same Strain of Beta 2-toxigenic *Clostridium perfringens* Type A Acquired in One Hospital Ward

DOMINIKA SALAMON[1], DOROTA OCHOŃSKA[1], ILONA WOJAK[2], EWA MIKOŁAJCZYK[2], MAŁGORZATA BULANDA[3] and MONIKA BRZYCHCZY-WŁOCH[1*]

[1] Department of Molecular Medical Microbiology, Chair of Microbiology, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland
[2] Department of Microbiological Diagnostics, Blessed Father Jerzy Popieluszko Provincial Specialist Hospital, Wloclawek, Poland
[3] Department of Epidemiology of Infections, Chair of Microbiology, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland

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Abstract

This study conducts a comparative phenotypic and genetic analysis of *C. perfringens* strains isolated from two patients hospitalized at the same time in 2017 in the surgical ward of the Provincial Specialist Hospital in Włocławek (Kujawsko-Pomorskie Province) who developed necrotizing soft tissue infections (NSTI). To explain the recurring cases of this infection, a comparative analysis was performed for these strains and the ones originating from infections recorded at the same hospital in three patients with gas gangrene in 2015. The two *C. perfringens* isolates studied in 2017 (8554/M/17 from patient No. 1 and 8567/M/17 from patient No. 2) had identical biochemical profiles. A comparison of research results using multiplex PCR from 2017 with a genetic analysis of strains from 2015 enabled us to demonstrate that the strains currently studied have the genes encoding the same toxins (α and β2) as the two strains analyzed in 2015: no. 7143 (patient No. 3) and no. 7149 (patient No. 2). A comparative analysis of the strain profiles obtained with pulsed-field gel electrophoresis (PFGE) in 2017 with the results from 2015 has found one identical and genetically unique restriction profile, corresponding to one clone of *C. perfringens* comprising of two strains: no. 8567/M/17 (patient No. 2 in 2017) and no. 7143 (patient No. 3 in 2015). The epidemiological data and detailed analysis of the course of both events suggest that this clone of *C. perfringens* possibly survived in adverse conditions of the external environment in the operating block of this hospital for many months.

Key words: *Clostridium perfringens,* beta 2 (β2)-toxin, NSTI, molecular diagnostics

Introduction

Clostridium perfringens, a Gram-positive, anaerobic bacillus, is a bacterium commonly found in nature. Its presence can be confirmed in both the external environment (water, soil, and sewage) as well as in the digestive tract of humans and animals, where it is part of the microbiome. The important carriers of this microbe are the elderly and people engaged in the processing and distribution of food. Nonetheless, its ability to produce numerous toxins and enzymes as well as to form spores makes this bacterium a dangerous pathogen of humans and animals (Kędzielska et al. 2012).

The scientific literature indicates several toxins produced by *C. perfringens* that play a vital role in the pathogenicity of this bacterium, they are: alpha (α, *C. perfringens* alpha toxin – CPA) and a synergistic theta, i.e. perfringolysin (θ, perfingolysin O – PFO), as well as beta (β, *C. perfringens* beta toxin – CPB), epsilon (ε, epsilon toxin – ETX), iota (ι, iota toxin – ITX), enterotoxin (*C. perfringens* enterotoxin – CPE), necrotic enteritis B-like toxin (NetB), and β2 toxin, discovered in 1997, the role of which is not fully explained and still requires further research (van Asten et al. 2010; Brzychczy-Włoch and Bulanda 2014; Navarro et al. 2018). It is known that β2-toxin is produced by animal strains of

* Corresponding author: M. Brzychczy-Włoch, Department of Molecular Medical Microbiology, Chair of Microbiology, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland; **e-mail: m.brzychczy-wloch@uj.edu.pl**

C. perfringens, especially in the course of necrotic enteritis in pigs. Studies on human strains of this bacterial species, isolated from patients with food poisoning, an antibiotic-associated diarrhea and sporadic diarrhea, but also from healthy carriers, have shown that, in some cases, they can have *cpb2* gene for β2-toxin (Johansson et al. 2006, van Asten et al. 2010; Allaart et al. 2014).

Owing to their ability to produce various toxins, strains belonging to the species *C. perfringens* were divided into 6 toxinotypes, A – G, which are responsible for gastrointestinal tract infections in humans (types A, C, F) and animals (types B, C, D, E, G) as well as severe soft tissue infections in humans and animals (type A) (Stevens et al. 2012; Navarro et al. 2018; Rood et al. 2018).

The term *necrotizing soft tissue infections* (NSTI) comprises a group of diseases (especially necrotizing fasciitis – NF) causing rapid and extensive soft tissue necrosis, which often leads to systemic infection, shock, and multiple organ failure, and ultimately, death (Stevens et al. 2017). These infections have different causes, risk factors, location, and pathomechanisms. NSTIs are often divided into two types. Type 1 is a multi-bacterial infection, the significant parts of which are anaerobic bacteria (among others, from the genus *Bacteroides* or *Clostridium*) and facultative anaerobes (among others of the family Enterobacteriaceae). It is often diagnosed in the elderly and the risk factors are diabetes, bedsores, hemorrhoids or anal surgery, and urological surgery or gynecological procedures. A peculiar infection of this type is a Fournier gangrene, which may develop as secondary to damaged mucous membranes of the gastrointestinal tract or urinary tract (Żaba et al. 2009; Stevens et al. 2017; Kuzaka et al. 2018). *C. perfringens* is listed as one of the numerous bacteria identified in the course of this infection. Although in recent years, its share in the infection seems to be getting smaller (Kuzaka et al. 2018), the literature still describes cases of Fournier gangrene in which *C. perfringens* was cultured among the other infectious agents (Wróblewska et al. 2014; Stevens et al. 2017). Type 2 of NSTI is usually associated with infection with a single bacterial species (e.g. MRSA) and often affects limbs. Some people distinguish Type 3, i.e. infection caused by a particular bacterial species: *Aeromonas hydrophila*, *Vibrio vulnificus* or a species from the genus *Clostridium*, which is most often isolated from gas gangrene cases (Stevens et al. 2017).

From the point of view of epidemiology and future management in cases of recurring NSTI in the same hospital, it is important to identify the differences and similarities among bacterial strains from every patient. The use of molecular methods allows for fast and precise identification of the bacteria isolated. The objective of this study is a comparative phenotypic and genetic analysis of *C. perfringens* strains isolated from two patients hospitalized at the same time in 2017 in the Provincial Specialist Hospital in Włocławek (Kujawsko-Pomorskie Province) who developed soft tissue infections. Trying to explain recurring cases of this infection, we also carried out an additional comparative analysis of *C. perfringens* strains isolated in 2017 with the strains isolated in 2015 from three patients with gas gangrene in the same hospital that have been already described by our team (Brzychczy-Włoch et al. 2016).

Experimental

Materials and Methods

The study involved the microbiological analysis of *C. perfringens* strains isolated from biological specimens originating from two patients with NSTI who were hospitalized between 17th May 2017 and 4th July 2017 in a particular Department of General Surgery in the hospital in Włocławek. The analysis of microbiological and epidemiological data concerning three patients hospitalized in the same hospital between 15th April 2015 and 20th April 2015 (Brzychczy-Włoch et al. 2016) was also performed.

Source of the isolates and epidemiological data from 2017. Patient 1. A man, aged 60, admitted on 17.05.2017 to the General Surgery Department due to critical vascular insufficiency of the lower limbs. Due to the ischemia of the lower skin flap covering the stump and inflammatory infiltration in the postoperative wound on 31.05.2017, the stump wound was swabbed. The growth of *C. perfringens* on microbiological media under anaerobic conditions and of coagulase-negative staphylococci (CNS) under aerobic conditions was demonstrated. Control swabs from the healing wound were microbiologically negative. The microbiological examination of the blood taken from the patient did not show bacterial growth.

Patient 2. A man, aged 60, admitted urgently on 31.05.2017 to the General Surgery Department due to anorectal abscess. In the course of the diagnostics undertaken, disseminated sigmoid colon cancer was eventually diagnosed. Past medical history revealed a stay in the same unit in May 2014, when the patient underwent surgery due to right-sided incarcerated inguinal hernia.

During the relevant hospitalization, on 31.05.2017, abscess contents were collected from the patient for microbiological diagnostics. Microbiological testing of the purulent content detected the growth of *Bacteroides fragilis* and *C. perfringens* on microbiological media under anaerobic conditions, and *Escherichia coli* and *Pseudomonas aeruginosa* under aerobic conditions. Microbiological testing of the patient's blood did not show bacterial growth. During the microbiological

examination of a rectal swab carried out on 6.06.2017, growth of *C. perfringens* was detected.

The prevention of epidemic outbreak and epidemiological investigations were initiated**.** Contact isolation was employed with both patients. Lavasepsis was used for wound dressing with 0.9% NaCl solution and superoxidized solution in the form of Microdacyn (Oculus). A sporicidal agent, Incidin Active (Ecolab), was applied for surface disinfection in the ward. When microbiological results were obtained, the entire General Surgery Department and the Main Operating Block were covered by the control. Growth of *C. perfringens* was not demonstrated in cultures collected from the wounds of other patients, the skin of the staff's hands, or on the surfaces in all rooms and on the tools.

Microbiological diagnostics of *C. perfringens* strains. Samples of two patients were collected during a routine check-up by medical staffs and were diagnosed in the Department of Microbiological Diagnostics of the Provincial Specialist Hospital in Włocławek. Specimens from the patients (wound swab containing activated carbon (COPAN) from patient No. 1 and abscess contents from patient No. 2) were put into sterile test tubes with a transport medium. The culture was carried out on Columbia Agar (BioMerieux) as well as in the fluid thioglycollate medium with resazurin (BioMaxima). Solid media with the inoculated materials were incubated under aerobic conditions at 37°C for 48 h and under anaerobic conditions at 37°C for 24 h, and the liquid medium under aerobic conditions (with a closed cap) at 37°C for 48 h. Identification of the cultured bacteria was carried out using the Vitek 2 compact system (BioMérieux). The biochemical patterns for two bacterial isolates were received with the use of the ANC ID card of the Vitek 2 compact system (BioMérieux).

In view of the fact that the same bacterial species, *C. perfringens*, was isolated from both patients and because of its pathogenic potential and epidemiological consequences, the following strains of *C. perfringens*: from patient No. 1 – isolate no. 8554/M/17; from patient No. 2 – isolate no. 8567/M/17, were preserved and transferred to the Chair of Microbiology, Jagiellonian University Medical College, Krakow, Poland for further studies. The *C. perfringens* strain cultured from the specimen obtained from a rectal swab from patient No. 2 was not preserved, which made it impossible to conduct further analyses for this strain.

The strains 8554/M/17 and 8567/M/17 were stored with the use of Cryobank (BioMaxima) at –70°C. *C. perfringens* 3624 ATCC (The American Type Culture Collection) standard was used as a reference strain.

Antibiotic susceptibility testing. To determine the drug-resistance profiles, the E-test method was used, enabling determination of MIC (Minimal Inhibitory Concentration) for: amoxicillin – AML, penicillin – P,

Table I

Antibiotic susceptibility for *C. perfringens* isolates (The E-test method, according to EUCAST 2017).

Isolate no.		Penicillins				Cephalosporins		Carbapenems				Fluoroquinolones	Glycopeptide and lipoglycopeptides		Macrolides, incosamides		Tetracyclines	Miscellaneous agents		
		AML	P	PRL	XL	CRO	CTX	DOR	ETP	IMP	MEM	MXF	TEC	VA	E	DA	TE	C	M	RD
8554/M/17 (Patient 1)	MIC mg/l	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	1.5	0.047	0.50	0.50	0.50	4	0.19	0.016	0.032
	S/R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	R	S	S	S
8567/M/17 (Patient 2)	MIC mg/l	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	2	0.047	0.50	0.50	0.50	4	0.125	0.016	0.032
	S/R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	R	S	S	S

AML – amoxicillin, P – penicillin, PRL – piperacillin, XL – amoxicillin/clavulanic acid, CRO – ceftriaxone, CTX – cefotaxime, DOR – doripenem, ETP – ertapenem, IMP – imipenem, MEM – meropenem, MXF – moxifloxacin, TEC – teicoplanin, VA – vancomycin, E – erythromycin, DA – clindamycin, TE – tetracycline, C – chloramphenicol, M – metronidazole, RD – rifampicin, MIC – Minimal Inhibitory Concentration, S/R – susceptibility, R – resistant, S – susceptible

piperacillin – PRL, amoxicillin/clavulanic acid – XL, ceftriaxone – CRO, cefotaxime – CTX, doripenem – DOR, ertapenem – ETP, imipenem – IMP, meropenem – MEM, moxifloxacin – MXF, erythromycin – E, clindamycin – DA, tetracycline – TE, chlor+amphenicol – C, metronidazole – M, rifampicin – RD and with Glycopeptide Resistance Detection (GRD) for: teicoplanin – TEC and vancomycin – VA. The results were interpreted according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST 2017) – Table I.

PCR multiplex. To isolate DNA, the Genomic Mini Set (A&A Biotechnology) was used according to the manufacturer's protocol. The presence of the genes encoding toxins of *C. perfringens* was confirmed using multiplex PCR amplification according to van Asten et al. (2009) with specific primers (Genomed). The following fragments of the genes were detected (the gene product, length of the fragment): *cpa* (α-toxin, 324 bp); *cpb* (β-toxin, 195 bp); *cpb*2 (β2-toxin, 548 bp); *etx* (ε-toxin, 376 bp); *iap* (ζ-toxin, 272 bp); *cpe* (enterotoxin, 485 bp). The final images from electrophoresis were processed using QuantityOne software, as well as GelDoc2000 device (Bio-Rad, USA).

Molecular typing with PFGE. The chosen *C. perfringens* isolates underwent molecular typing using the PFGE method according to the methodology described by Maslanka et al. (1999). Chromosomal DNA of the bacterial strains was isolated in agarose blocks and then digested with the use of restriction enzyme *Sma*I (MBI Fermentas). Electrophoretic separation was performed with the CHEF-DR II (Bio-Rad) instrument and restriction analysis was carried out using the GelCompar II (Applied Maths) software with the application of UPGMA clustering method and Jaccard index. The obtained genetic profiles were interpreted according to the guidelines given by van Belkum et al. (2007).

The profiles of the strains under investigation that were obtained using PCR multiplex and PFGE were compared with restriction patterns of the strains that came from the event in 2015, when in the Orthopedics and Traumatology Department of the same hospital, three cases of gas gangrene caused by *C. perfringens* were detected simultaneously (Brzychczy-Włoch et al. 2016).

Results

The two *C. perfringens* isolates studied (8554/M/17 from patient No. 1 and 8567/M/17 from patient No. 2) had identical biochemical profiles. Based on the results obtained using E-test, the same pattern of antibiotic susceptibility of the strains from the two examined patients was demonstrated (Table I).

Multiplex PCR confirmed the presence of the *cpa* gene encoding α-toxin for both *C. perfringens* isolates studied. Moreover, both strains demonstrated the presence of the *cpb*2 gene encoding β2-toxin (Fig. 1). A comparison of the results from 2017 and the genetic analysis of the strains from the event in 2015 enabled us to demonstrate that the currently examined strains have the genes encoding the same toxins (α and β2) as the two strains analyzed in 2015: no. 7143 (patient No. 3) and no. 7149 (patient No. 2) (Brzychczy-Włoch et al. 2016).

As a result of the molecular analysis conducted using PFGE, two genetically different, unique restriction profiles were found corresponding to two different clones of the *C. perfringens* isolates studied in 2017: isolate no. 8554/M/17 – clone A; isolate no. 8567/M/17 – clone B (Fig. 2A).

Profiles of the strains under investigation that were obtained using PFGE were then compared with restriction patterns of the strains from the event in 2015 (Brzychczy-Włoch et al. 2016). A comparative analysis of PFGE profiles from 2017 and the results from the event from 2015 found one identical and genetically unique restriction profile, corresponding to one clone of *C. perfringens* for two strains: no. 8567/M/17 (patient No. 2 in 2017) and no. 7143 (patient No. 3 in 2015) (Fig. 2B).

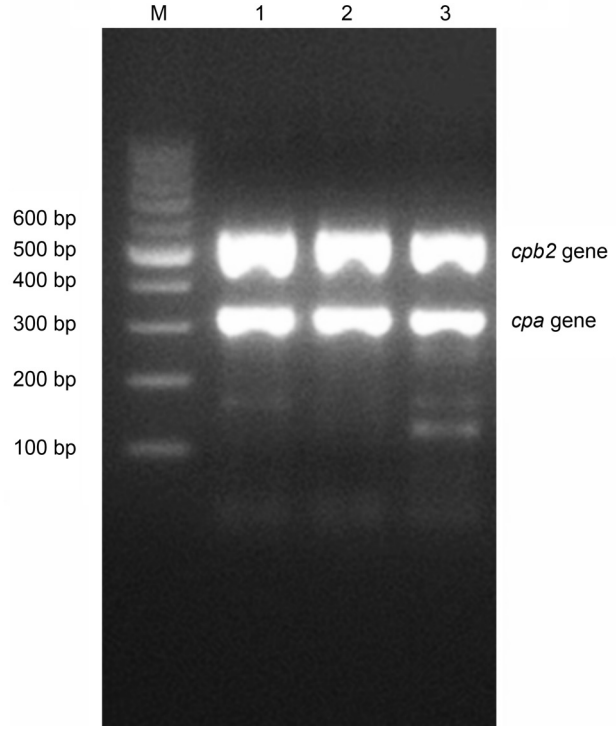

Fig. 1. Detection of genes encoding virulence factors of *C. perfringens* isolates in 2017 tested by multiplex PCR.

Legend: M – size marker,
1 – *C. perfringens* isolate no. 8567/M/17 from patient no. 2
2 – *C. perfringens* isolate no. 8554/M/17 from patient no. 1
3 – reference strain of *C. perfringens* ATCC 3624
*cpb*2 – gene of β2-toxin; *cpa* – gene of α-toxin

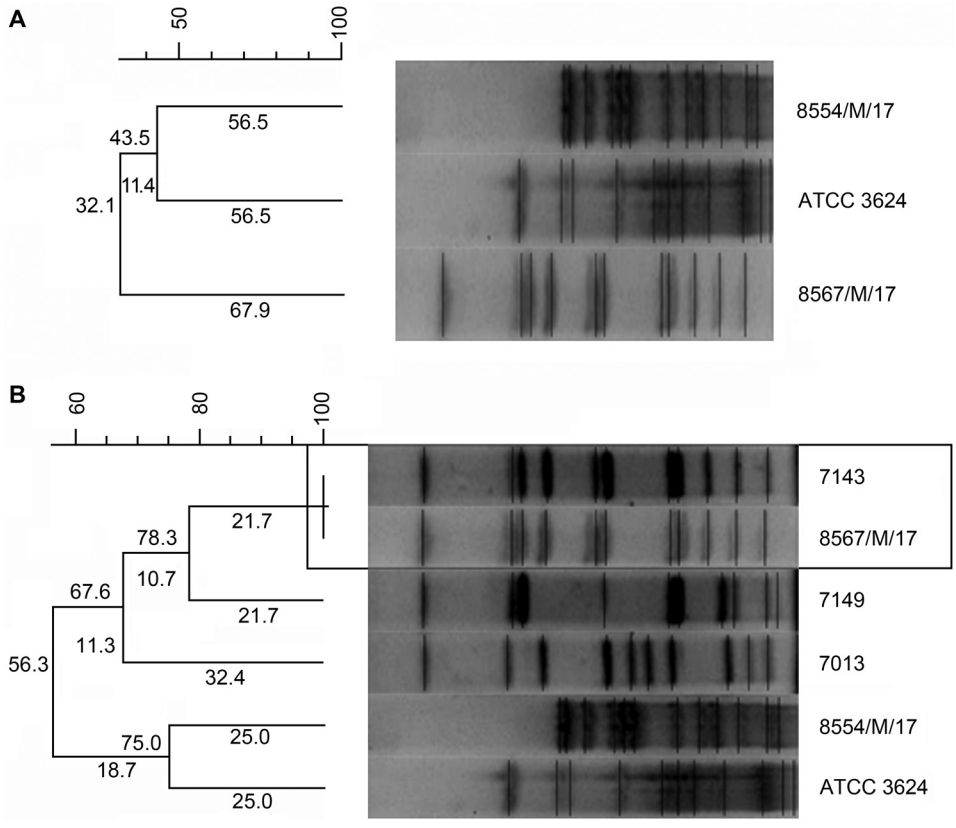

Fig. 2. Genetic profiles of *C. perfringens* isolates, subjected to DNA digestion by restriction enzyme *Sma*I with the use of pulsed-field gel electrophoresis (PFGE) and GelCompar II software.

Legend: A – Comparison of two isolates from 2017; B – comparison of five isolates: three from 2015 and two from 2017
8554/M/17 – *C. perfringens* isolate isolated from patient no. 1 in 2017; ATCC 3624 – reference strain of *C. perfringens*
8567/M/17 – *C. perfringens* isolate isolated from patient no. 2 in 2017
7143 – *C. perfringens* isolate isolated from patient no. 3 in 2015; 7149 – *C. perfringens* isolate isolated from patient no. 2 in 2015
7013 – *C. perfringens* isolate isolated from patient no. 1 in 2015

Due to this fact, an attempt was undertaken to find a connection between patient No. 2 from 2017 and patient No. 3 from 2015. In the course of an epidemiological investigation, it was only found that both patients were hospitalized in the same hospital, but they were in different wards at different times. The patient from whom the strain no. 7143 was isolated (patient No. 3) was hospitalized in April 2015 at the Department of Orthopedics and Traumatology. The currently described patient No. 2 from whom strain no. 8567/M/17 was isolated in 2017 was in the General Surgery Department in May 2014. Both patients underwent surgery in the main operating block of the hospital. Kinship, close contact, possession of the same animals and residence in each other's neighborhood were excluded.

Discussion

The cases presented above illustrate the clinical picture of necrotizing soft tissue infection, caused by *C. perfringen*s toxinotype A. The infection leading to amputation of the left lower limb in the patient No. 1 makes us to assume that he developed type 3 NSTI. Atherosclerosis of the lower extremities, alcoholism, and neuropathy with subsequent vascular insufficiency contributed to the rapid progression of the disease. The course of the infection in patient No. 2, which was classified as Fournier gangrene and isolation of the etiologic agent from the wound, i.e. *C. perfringens*, as well as medical history pointing to decreased immunity and advanced neoplastic disease of the large intestine, allowed to confirm this infection as type 1 NSTI.

The results of microbiological testing with phenotypic methods (biochemical patterns and antibiotic susceptibility testing) did not demonstrate differences between the strains isolated from biological specimens from the patients. On the other hand, the application of molecular methods allowed to characterize each of them accurately and compare them with strains isolated during the epidemiological investigation, which took place in the same hospital during the event in 2015 (Brzychczy-Włoch et al. 2016).

Determination of the toxinotype of *C. perfringens* isolates was possible owing to the use of multiplex PCR. The strains identified for both patients described

in this study were type A and had the genes encoding α-toxin (*cpa* gene) and β2-toxin (*cpb2* gene). The role of α-toxin, which is the main virulence factor in of *C. perfringens* in gas gangrene, seems to be well known and is chiefly associated with hemolysis and dermonecrosis. To the best of our knowledge, there is no evidence pointing to β2-toxin's contribution to necrotizing soft tissue infections in humans. Despite this, in the work reporting three simultaneous cases of gas gangrene associated with *C. perfringens* type A strains in 2015, two patients carried the strains that had the *cpb2* gene, indicated an extremely severe course of these infections (Brzychczy-Włoch et al. 2016). The presence of the gene for β2-toxin in *C. perfringens* strains causing soft tissue infections in humans requires further observation, which could be assisted by the application of genetic analysis of this pathogen in every clinical case involving *C. perfringens*.

Molecular diagnostics of the strains isolated also allowed their final differentiation and an attempt to determine their origin. Owing to macrorestriction analysis of chromosomal DNA combined with PFGE, it was possible to determine that the strain of the described patient No. 1 turned out to be different from the strains isolated from the patient No. 2. However, the identification of an identical restriction profile for the *C. perfringens* isolate from patient No. 2 and the profile of the strain isolated from one of the patients during the event in 2015 in the same hospital (isolate no. 7143 from patient No. 3) deserves more attention. According to the literature, the species *C. perfringens* is characterized by remarkable diversity and numerous mutations due to the presence of genes encoding toxins not only in the chromosome but also in plasmids (Myers et al. 2006; Park et al. 2016; Kiu et al. 2017). Hence, a random detection of the presence of an identical strain in two distant, independent events is unlikely. However, a high genetic similarity between *C. perfringens* strains can be demonstrated when they cause epidemiologically related infections (Johansson et al. 2006; Xiao et al. 2012). Our epidemiological analysis points to the fact that patient No. 2 (from the event in 2017) had already been subjected to a surgical procedure within the abdominal cavity in this hospital in 2014. There is a possibility that he had already been a carrier of *C. perfringens* in the gastrointestinal tract at that time and that he became the source of the infection and as result of the surgical procedure the bacterium appeared in the operating block of the hospital. It is known that *C. perfringens* may be present everywhere, even in dust, and is capable of producing spores, which can survive in unfavorable conditions for many months (van Asten et al. 2010; Kędzielska et al. 2012; Brzychczy-Włoch and Bulanda 2014). In 2014, there was no reason for routine use of sporicidal substances in the operating block (no symptomatic cases of infection with the bacillus *C. perfringens* at the hospital). One should, then, take into consideration the possibility of survival of *C. perfringens* spores in the hospital after the hospitalization of patient No. 2 in 2014 and the possible infection of one of the three patients undergoing surgery in the same operating block in 2015. Even more, other connections between both patients were excluded (kinship or being neighbors). During the event in 2015, the appropriate epidemiological and protective procedures against an outbreak were implemented (including the application of sporicidal agents). Therefore, it is unlikely that the *C. perfringens* strain survived two more years in the operating block (Brzychczy-Włoch et al. 2016). Additionally, the result of rectal swab testing in patient No. 2 in 2017 indicated that the isolate from that sample is an endogenous (own) strain of the patient. It was patient No. 2 who probably had been a carrier of the described *C. perfringens* strain in the gastrointestinal tract for years and his decreased immunity together with cancer created the favorable conditions for the growth of bacteria and development of infection in 2017 (Brzychczy-Włoch and Bulanda 2014; Kuzaka et al. 2018).

Our research has some limitation as there are no environmental or hospital staff studies that could have precisely demonstrated the source where *C. perfringens* clone that was able to survive. However, owing to the application of molecular methods, it was possible to determine that the patients simultaneously hospitalized in 2017, in a single Department of General Surgery developed two different types of NSTI caused by two different *C. perfringens* clones. Archival data from 2015 allowed the identification of an identical clone from the same hospital. The employment of genetic analyses also enabled us to document, in the strains studied in 2015 and in 2017, the presence of the *cpb2* gene encoding β2-toxin. Moreover, the epidemiological data and detailed analysis of the course of both events in the hospital made it possible to attempt to understand the reasons of the survival of the *C. perfringens* clone in the operating block and suggest that this bacterium may have survived in adverse conditions of the external environment for many months, posing a potential threat to patients. Hence, compliance with the procedures concerning the operating block hygiene must always be strictly observed. Maybe it is also worthwhile to consider modifying them and to introduce periodic (e.g. every three months) mandatory application of sporicidal agents regardless of whether there were clinical cases of *C. perfringens* infection or not.

ORCID
Dominika Salamon 0000-0002-5974-5520
Małgorzata Bulanda 0000-0002-4679-9263
Monika Brzychczy-Włoch 0000-0002-7415-0154

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Purification, Characterization and Inhibition of Alanine Racemase from a Pathogenic Strain of *Streptococcus iniae*

MURTALA MUHAMMAD, YANGYANG LI, SIYU GONG, YANMIN SHI, JIANSONG JU, BAOHUA ZHAO* and DONG LIU*

College of Life Science, Hebei Normal University, Shijiazhuang, China

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Abstract

Streptococcus iniae is a pathogenic and zoonotic bacteria that impacted high mortality to many fish species as well as capable of causing serious disease to humans. Alanine racemase (Alr, EC 5.1.1.1) is a pyridoxal-5'-phosphate (PLP)-containing homodimeric enzyme that catalyzes the racemization of L-alanine and D-alanine. In this study, we purified alanine racemase from *S. iniae* that was isolated from an infected Chinese sturgeon (*Acipenser sinensis*), as well as determined its biochemical characteristics and inhibitors. The *alr* gene has an open reading frame (ORF) of 1107 bp, encoding a protein of 369 amino acids, which has a molecular mass of 40 kDa. The enzyme has optimal activity at a temperature of 35°C and a pH of 9.5. It belongs to the PLP-dependent enzymes family and is highly specific to L-alanine. *S. iniae* Alr (SiAlr) could be inhibited by some metal ions, hydroxylamine and dithiothreitol (DTT). The kinetic parameters K_m and V_{max} of the enzyme were 33.11 mM, 2426 units/mg for L-alanine, and 14.36 mM, 963.6 units/mg for D-alanine. Finally, the 50% inhibitory concentrations (IC_{50}) values and antibiotic activity of two alanine racemase inhibitors (homogentisic acid and hydroquinone), were determined and found to be effective against both Gram-positive and Gram-negative bacteria employed in this study.

Key words: *Streptococcus iniae*, alanine racemase, inhibitors, homogentisic acid, hydroquinone and peptidoglycan

Introduction

Streptococcus iniae is a Gram-positive and most commonly reported Streptococcal pathogen of fish responsible for high economic loses of aquaculture industries around the world. The zoonotic bacteria was also reported to cause bacteremia, cellulitis, meningitis, and osteomyelitis in human (Guo et al. 2018; Tavares et al. 2018). Vaccines and antibiotics were currently employed for minimizing the impact of the disease. However, recent studies revealed that the bacteria has so far developed resistance against many potential antibiotics (Tavares et al. 2018). As such, additional efforts for developing more effective vaccines and antibiotics are necessary steps for circumventing the threat of its infection (Saavedra et al. 2004).

Alanine racemase (Alr; E.C. 5.1.1.1) is an enzyme that catalyzes the interconversion of L-alanine and D-alanine using a pyridoxal 5-phosphate (PLP) as a cofactor (Tassoni et al. 2017). It provides D-alanine for the synthesis of peptidoglycan of the bacterial cell wall, D-alanine is directly involved in cross-linking of adjacent peptidoglycan strands and also present in lipoteichoic acids of Gram-positive bacteria (Liu et al. 2018; Ray et al. 2018). There are two isoforms (non-homologous) of the alanine racemase genes (*alr* and *dadX*). The *alr* gene, which is constitutively expressed encodes for an essential enzyme for cell wall synthesis. While the expression of *dadX* is induced in the presence of high concentrations of L- or D-alanine. *DadX* is required for L-alanine catabolism, forming a substrate for D-alanine dehydrogenase (*dadA*) (Duque et al. 2017). The bacterial cell wall is indispensable for the survival and viability of bacteria (Liu et al. 2019) and has always been an attractive target for many antibiotics and antimicrobial agents (Anthony et al. 2011). Alanine racemase is ubiquitous among bacteria and rare in eukaryotes but absent in humans (Kawakami et al. 2018); hence, it emerges as an attractive and potential therapeutic target for the antimicrobial drugs development (Wang et al. 2017).

Numerous inhibitors were identified as able to affect the activity of alanine racemase (Kim et al. 2003a; Kim et al. 2003b). Many of these inhibitors were structural

* Corresponding author: B. Zhao and D. Liu, College of Life Science, Hebei Normal University, Shijiazhuang, China;
e-mail: Baohua519@126.com, pqw1234@163.com

analogs of alanine; they interact with the enzyme-bound PLP, covalently bound to some eukaryotic PLP-dependent enzymes and lead to cellular toxicity (Toney 2005). PLP-related off-target effects could be overcome by using enzyme inhibitors that are not substrate analogs. Structure-based approach and molecular modeling have been employed to discover novel alanine racemase inhibitors, which are devoid of affinity for the PLP and hence off-target effects (Lee et al. 2013; Azam and Jayaram 2018).

In this study, we identified and purified the alanine racemase from *S. iniae* HNM-1 strain that was previously isolated from an infected Chinese sturgeon (*A. sinensis*). We have characterized its enzymatic properties, substrate specificity, and kinetic parameters. We have also explored the potentiality of the enzyme as an attractive antimicrobial target against *S. iniae*. We determined the 50% inhibition concentrations (IC_{50}) of two alanine racemase inhibitors (homogentisic acid and hydroquinone) and their antimicrobial susceptibility against six opportunistic pathogens including *S. iniae*, in quest of providing the possible solutions against antibiotics resistance and bacterial infections.

Experimental

Materials and Methods

Strains, plasmids, and growth conditions. The characteristics of bacterial strains and plasmids used in this context were summarized in Table I. *S. iniae* HNM-1 was cultured at 35°C in the Tryptone soy yeast extract (TSYE) medium. *Escherichia coli* DH5α, *E. coli* BL21 strains, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were cultured in Luria Bertani (LB) medium at 37°C or 35°C. 100 g/ml final concentration of ampicillin (Amp) was used in this study.

Cloning of alanine racemase gene. Primers were designed based on the *alr* gene sequence of *S. iniae* 89353 strain (NCBI accession number CP017952.1). The genomic DNA of *S. iniae* HNM-1was extracted and amplified using the following primers, Alr-F-(5'-GCACCATGGATGATTTCAAGTTTG-3') and Alr-R-(5'-TCACTCGAGATCCCGATAAAGC-3'), with *Nco*I and *Xho*I restriction sites, underlined respectively. PCR product was cloned in pMD19-T cloning vector to construct pMD-alr and transformed to *E. coli* DH5α. The *alr* gene was digested with restriction endonucleases and cloned into expression vector pET-22b (+), forming recombinant plasmid pET22b-*alr*. The deduced amino acid sequence of the ORF was analyzed by the Blast software. Multiple amino acid sequence alignment and phylogenetic relationships among alanine racemase of *S. iniae* and other bacteria were constructed with Clustal Omega.

The evolutionary distances of the phylogenic tree were computed using the p-distance method and are reported in the units of the number of amino acid differences per site. The bootstrap consensus tree inferred from 500 replicates was used to represent the evolutionary history (Felsenstein 1985). The analysis involved 10 amino acid sequences from *S. iniae* (NCBI Genbank accession number: APD32491.1), *P. aeruginosa* PAO1 (NCBI Genbank accession number: AF165882), *Streptococcus agalactiae (*NCBI Genbank accession number: WP_037624882), *Enterococcus feacalis* (NCBI Genbank accession number: AFO43552.1), *Streptococcus pyogenes* (NCBI Genbank accession number: MGAS10750), *Staphylococcus aureus* (NCBI Genbank accession number: CAG41139.1), *Corynebacterium glutamicum* (NCBI Genbank accession number: AAL77207.1), *Aeromonas hydrophyla* (NCBI Genbank accession number: ABK36160.1), *Streptococcus pneumoniae* (NCBI Genbank accession number: AAL00344.1), and *E. coli* (NCBI Genbank accession number: YP_002407858).

Table I
Strains and plasmids used in this study.

Strains/Plasmids	Description	Source
Strains		
Streptococcus iniae HNM-1	Isolated from infected *A. sinensis*	This study
Escherichia coli DH5α	Used for cloning and propagation of plasmids	Novagen
Escherichia coli BL21(DE3)	Used for protein expression	Invitrogen
Salmonella typhimurium		This study
Staphylococcus aureus		This study
Acinetobacter baumannii		This study
Pseudomonas aeruginosa		This study
Plasmids		
pMD19-T	Carries ampR gene; used for cloning PCR product with A at 3' ends	Takara
pET 22b (+)	Carries ampR gene; used for expressing *S. iniae* Alanine racemase	Novagen

Expression and purification of alanine racemase. *E. coli* BL21 (DE3) was transformed with expression vector pET22b-Alr for protein expression, a single colony of the transformed *E. coli* was inoculated in 100 ml LB medium at 35°C. Protein expression was induced when the OD_{600} reaches 0.6 by addition of IPTG at a final concentration of 1 mM, and re-incubated overnight at 16°C or at 35°C for 5 hours. Cells were collected and resuspended in 20 ml binding buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole), lysed on ice by sonication for 40 minutes, and centrifuged at 8000 g, 4°C for 10 minutes. The supernatant was collected and purified using Nickel ion affinity chromatography (Qiagen), according to the manufacturer's protocol. The protein solution was dialyzed against phosphate buffered saline (PBS, pH 7.4). Protein purity and concentration were determined by SDS-PAGE and BCA protein assay kit (Takara), respectively. Western blotting was conducted using a monoclonal antibody against the poly-Histidine tag attached to the Alr protein as described previously (Liu et al. 2015).

Enzyme assay. Alanine racemase racemization assay was conducted in two coupled enzyme reactions, using a standard racemization mixture (Wang et al. 2017). The reaction was initiated by addition of the suitable concentration of SiAlr in the final reaction volume of 200 µl, incubated at 35°C for 10 minutes, terminated by addition of 25 µl of 2 M HCl and neutralized with 25 µl of 2 M sodium hydroxide. Then, the reaction mixture was centrifuged at 14 000 rpm, 4°C for 10 minutes. The concentration of D-alanine was analyzed in the second reaction containing 200 mM Tris- HCl pH: 8.0, 0.2 mg/ml 4-aminoantipyrine, 0.2 mg of N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methyl aniline, 1 unit of HRP, and 0.1 unit of D-Amino acid oxidase. The absorbance was measured using a microplate reader at 550 nm after 20 min incubation at 37°C.

Effect of temperature and pH on enzyme activity and stability. The influence of temperature was determined according to a standard enzyme assay by measuring the initial rate of reaction at various temperatures (10°C to 50°C), while the effect of pH was determined by measuring the initial rate of reaction in Britton-Robinson buffer (pH 2.0 to 12.0) at the optimum temperature. The relative residual activity was calculated with the highest activity as 100%. The thermal stability of the enzyme was examined by preincubation in the reaction buffer without substrate at temperatures of 30°C, 35°C, and 40°C for 2 h followed by addition of L-alanine as a substrate for determination of the relative residual enzyme activity. The pH stability of the enzyme was examined by incubation of the enzyme in the reaction buffers without substrate at pH ranging from 8.5 to 11.0 on ice for 2 hours, and subsequently, L-alanine was added and the relative residual enzyme activity was measured.

Substrate specificity of alanine racemase. The substrate specificity of SiAlr was determined according to the standard racemization reaction mixture using 18 kinds of L-amino acids as the substrates and incubated at the optimum temperature for 10 minutes.

Effect of metal ions, reducing agents and PLP on the enzyme activity. The influence of some metal ions and chemical compounds on the activity of SiAlr were determined by incubating the enzyme with the chemical compounds in the reaction mixture for 30 min, afterward, the substrate was added and the relative residual activity was determined according to the standard protocol (Liu et al. 2015).

SiAlr and different concentrations of hydroxylamine (0.1, 1, and 10 mM) were added to the reaction mixture without the substrate, dialyzed in phosphate buffered saline for 40 min, and its activity was determined without the addition of PLP. The effect of Dithiothreitol (DTT) on the activity of SiAlr was also determined by incubating the enzyme in different concentrations of DTT (1 and 3 mM) for 30 minutes and the relative activity was measured. To confirm SiAlr is a PLP-dependent enzyme, the purified Alr was treated with 10 mM hydroxylamine and dialyzed to obtain the apoenzyme. The apoenzyme was incubated in different concentrations of PLP (0.01, 0.04, and 0.06 mM) and its relative activity was measured.

Kinetic parameters. The alanine racemase activity was determined by measuring the amount of both enantiomers of alanine by high-performance liquid chromatography (HPLC) using a fluorescence detector according to the method described earlier (Hashimoto et al. 1992). The reaction mixture comprised of 10 µm PLP, 200 mM carbonate buffer pH 9.5, and various concentrations of either L or D forms of alanine. The enzymatic reaction was initiated by adding the purified SiAlr, followed by 10 min of incubation at 35°C. The reaction was terminated by the addition of 40 µl of 2 M HCl on ice for 2 min, neutralized with 40 µl of 2 M NaOH, and centrifuged at 10 000 g, 4°C for 5 min. A 40 µl aliquot of the reaction was derivatized by addition of 280 µl of 0.4 M boric acid pH 9.0, 0.1% N-tert-butyloxycarbonyl-L-cysteine (Sigma), and 0.1% O-phthaldialdehyde. One unit of the enzyme was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of L- or D-alanine from either enantiomer per minute. Graph Pad Prism 6.0 was used for results analysis.

Enzyme IC_{50} determination. Inhibitory effects of homogentisic acid and hydroquinone on the activity of alanine racemase were determined as described previously (Wang et al. 2017). Fivefold dilution series (in DMSO) was prepared for the compounds, and the

solutions were added to the wells of a 96-well plate to yield the final inhibitory concentrations. Each concentration was tested in triplicate. The substrate was added after 30 min of incubation, and the fluorescence intensity was measured after the reaction. The negative control was prepared without adding chemicals to the control wells and the D-cycloserine (DCS) was used as a positive control. Percentage inhibition at each inhibitor concentration was calculated with respect to the negative control. Graph Pad Prism 6.0 was used for the calculation of the concentration that causes 50% inhibition (IC_{50}).

Antimicrobial susceptibility tests. Minimum inhibition concentrations (MIC) of hydroquinone and homogentisic acid against both Gram-positive and Gram-negative bacteria were determined by microdilution assay according to the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI 2008), as described previously (Dal Pozzo et al. 2011). An overnight culture was subcultured to OD_{600} of 0.3, diluted tenfold, five times. Aliquots were spread on agar plates in triplicate to determine the number of colony-forming units (CFU)/ml. Compounds were diluted in DMSO at concentrations of 200, 100, 80, 40, 20, or 10 µg/ml. DMSO solvent was used as a negative control of growth inhibition and DMSO alone was used as the blank control. All tests were performed in triplicate. The inoculums were prepared in LB medium (1×10^8 CFU/ml) and incubated at 30°C for 20 h. Subsequently, 100 µl (1×10^5 CFU) of the inoculums and the inhibitors were added to each microplates wells and incubated at 30°C for 48 h. The MIC values were determined as the lowest concentration of the inhibitors at which no growth of the bacteria was observed after incubation.

Results

Identification of *S. iniae* alanine racemase gene. According to the genomic sequence of *S. iniae*, the bacteria have a single putative alanine racemase (*alr*) gene. The *alr* gene has an open reading frame of 1107 bp that encodes a 369 amino acids protein (SiAlr) with a molecular mass of 39.82 kDa. The nucleotide sequence of *alr* has been submitted to Genbank under accession number MK620909.

The deduced amino acid sequence has 76%, 67%, 63%, and 47% similarities with alanine racemase of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*, respectively. Phylogenetic analysis of alanine racemase from different bacteria revealed an evolutionary relationship among them. The phylogenetic tree consists of two distinct clades. The enzyme is clustered with other *Streptococci* species, such as *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, and *E. faecalis*. These sequences, from Gram-positive bacteria, were classified into one group. The sequences from Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Corynebacterium glutamicum* were classified into another (Fig. 1). The evidence indicated that these alanine racemases evolved independently from a common ancestor and formed two isolated genes.

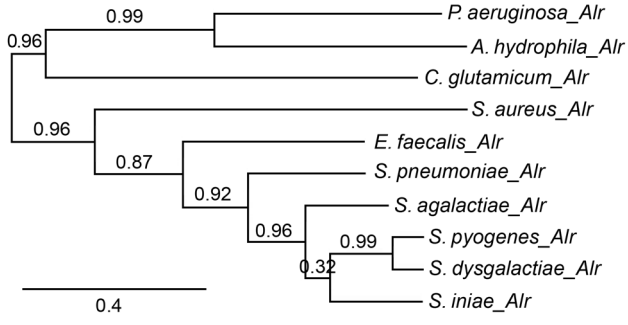

Fig. 1. Phylogenetic relationships of SiAlr and homologs alanine racemases from *Pseudomonas aeruginosa* PAO1, *Corynebacterium glutamicum* ATCC 13032, *Aeromonas hydrophyla* ATCC 7966, *Staphylococcus aureus* ABFQT, *Enterococcus feacalis* D32, *Streptococcus pneumoniae* MDRSPN001, *Streptococcus agalactiae* 2603V/R, *Streptococcus pyogenes* MGAS10750 and *Streptococcus dysgalactiae* Kdys0611. The tree was constructed using Neighbor end-joining. Maximum likelihood tree based on complete coding sequences deposited in Genbank. The evolutionary distances were computed using the p-distance method and are reported in the units of the number of amino acid differences per site.

Multiple sequence alignment of SiAlr with sequences of other 10 species Alr suggested that some regions are conserved in SiAlr, which includes PLP binding motif near the N-terminus (AVVKANAYGHG) and the two catalytic amino acid residues of the active center (Lys 40 and Tyr 274). The eight residues making up the entryway to the active site (inner layer: Ala 174, Tyr 273, Tyr 282 and Tyr 366; middle layer: Asp 166, Arg 298, Arg 318 and Ile 364) (Fig. 2).

Expression and Purification of SiAlr. The SiAlr protein was expressed in *E. coli* (DE3) incubated overnight at 16°C, and purified to homogeneity using Ni-agarose affinity chromatography. The protein has a relative molecular mass of 39.82 kDa as estimated by SDS-PAGE, which was similar to the calculated relative molecular mass. Western blotting analysis using the anti-poly-His antibody confirmed that 39.82 kDa protein is SiAlr (Fig. 3).

Characterization of the enzyme. The optimal temperature of SiAlr was approximately 35°C. The enzyme was found to be very stable at the temperature of 30°C and 35°C, with more than 50% residual activity. The optimal pH of SiAlr was approximately 9.5 at 35°C. The enzyme was found to be very stable, with more than 50% residual activity after incubation for 2 hours at a pH range of 8.5 to 9.5 (Fig. 4). Thus demonstrating that SiAlr is a basophilic enzyme.

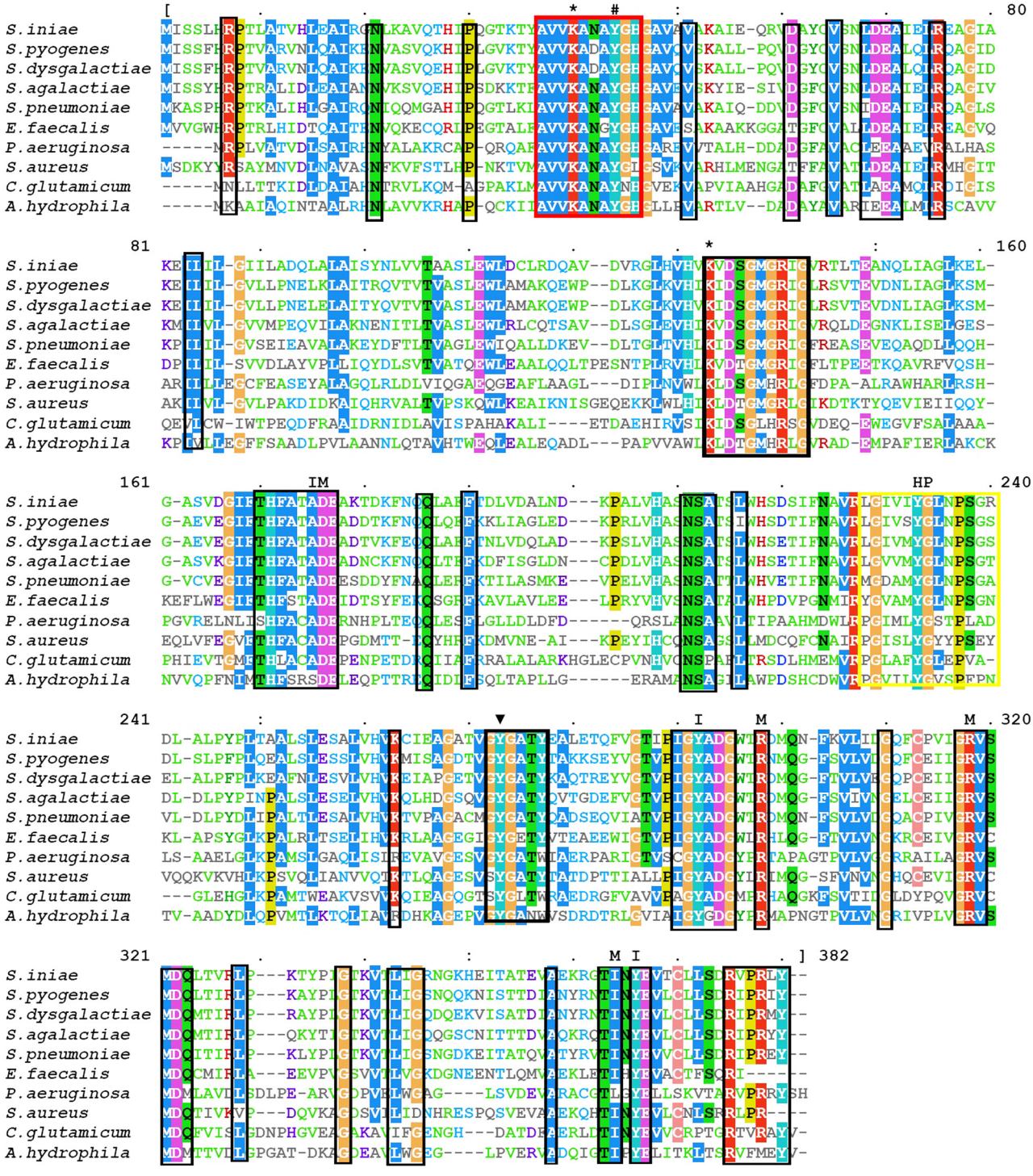

Fig. 2. Structure-based sequence alignment of alanine racemases sequences. The amino acid sequence of Alr from *Streptococcus iniae* was aligned with alanine racemases sequences of *Streptococcus pyogenes* MGAS10750, *Streptococcus dysgalactiae* Kdys0611, *Streptococcus agalactiae* 2603V/R, *Streptococcus pneumoniae* MDRSPN001, *Enterococcus feacalis* D32, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ABFQT, *Corynebacterium glutamicum* ATCC 13032 and *Aeromonas hydrophyla* ATCC 7966. The red box enclosed the conserved PLP-binding sites; Lys40 (*), Tyr44 (#). The catalytic Tyr residue was indicated by (+). Strictly conserved residues were enclosed in the black boxes, while the hydrophobic patch (HP) in the yellow box. Residues of the active site entryway are marked with either I (inner layer) or M (middle layer). Highly conserved residues were indicated by the box and strictly conserved with (*).

Various chemicals and metal ions were reported to inhibit the activity of alanine racemases. The results revealed that the enzyme activity was inhibited by most of the metal ions, but markedly inhibited by Ni^{2+}, Co^{2+} Zn^{2+}, and Fe^{2+} (Fig. 5).

Effect of reducing agents on the activity of SiAlr. Many inhibitors of alanine racemase have been discovered (Wang et al. 2017). The enzyme lost its activity after treatment with 1 or 10 mM hydroxylamine. Addition of 0.1 mM hydroxylamine reduced the activity of

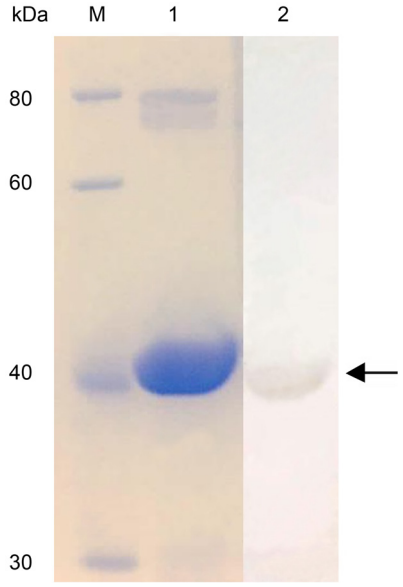

Fig. 3. Purification of *Streptococcus iniae* alanine racemase. The enzyme was purified using Nickel ion affinity chromatography, analyzed by SDS-PAGE and western blotting. M: molecular weight standards; 1; 40 kDa SiAlr. 2: Western blotting analysis of the purified protein.

the enzyme by 80%. Treatment of SiAlr with 1 mM of DTT resulted in a 70% loss of activity and completely inhibition at the concentration of 3 mM (Table II).

We examined the role of PLP in the activity of SiAlr by resolving the enzyme to Apo-enzyme by hydroxylamine treatment. The Apo-enzyme completely lost its activity after treatment with 10 mM hydroxylamine. Addition of 0.01, 0.04, and 0.06 mM of PLP make the enzyme regained up to 56%, 83 % and 96% of its activity, respectively. The result indicated that SiAlr is a PLP-dependent enzyme that requires more than 0.01 mM PLP to maintain its activity (Table II).

Table II
Effect of Hydroxylamine, DTT and PLP on SiAlr Activity.

Chemical	Concentration (mM)	Relative activity (%)
None		100 (0.7)
Hydroxylamine	0.1	21 (1.2)
	1	11 (0.8)
	10	9 (1.4)
DTT	1	27 (3.1)
	3	2 (0.8)
PLP	0.01	56 (2.4)
	0.04	83 (1.5)
	0.06	96 (2.7)

The data were presented as mean (SD) from three independent enzyme reactions

Substrate specificity. Alanine racemase is a highly conserved bacterial enzyme and known to be very specific to its substrate (Patrick et al. 2002). As shown in Fig. 6, the enzyme is highly specific to L-alanine and

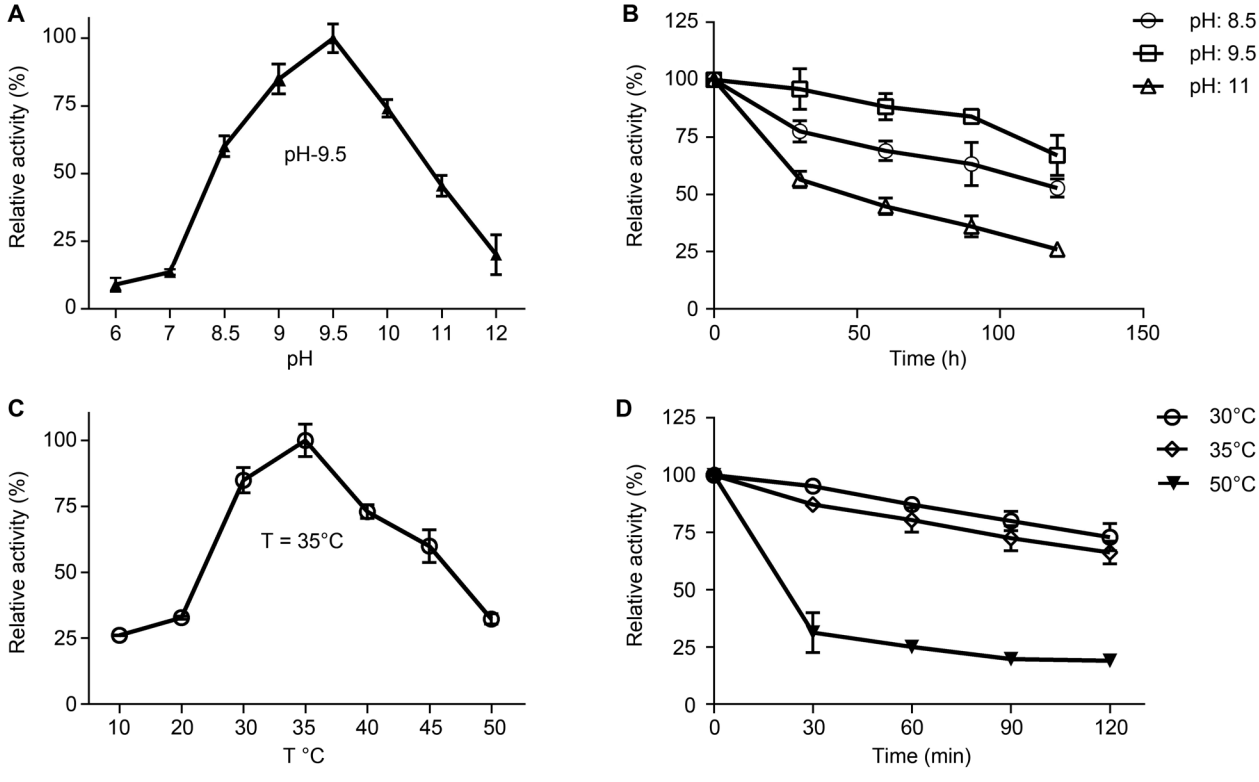

Fig. 4. Effect of pH and temperature on the activity of SiAlr. (A) Optimal pH, (B) pH stability, (C) Optimal temperature, (D) Thermal stability.

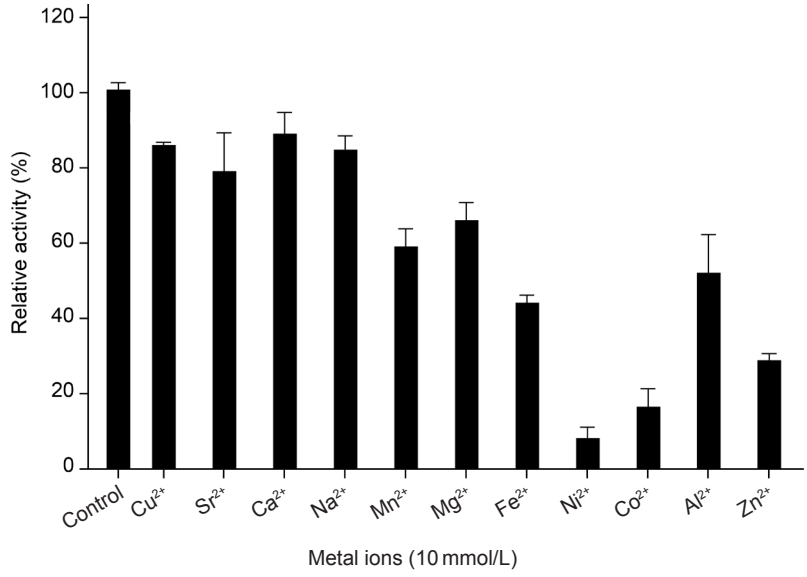

Fig. 5. Effect of metals on SiAlr activity. The metal ions were at a concentration of 10 mM/L. The data were presented as mean ± SD from 3 independent determinations.

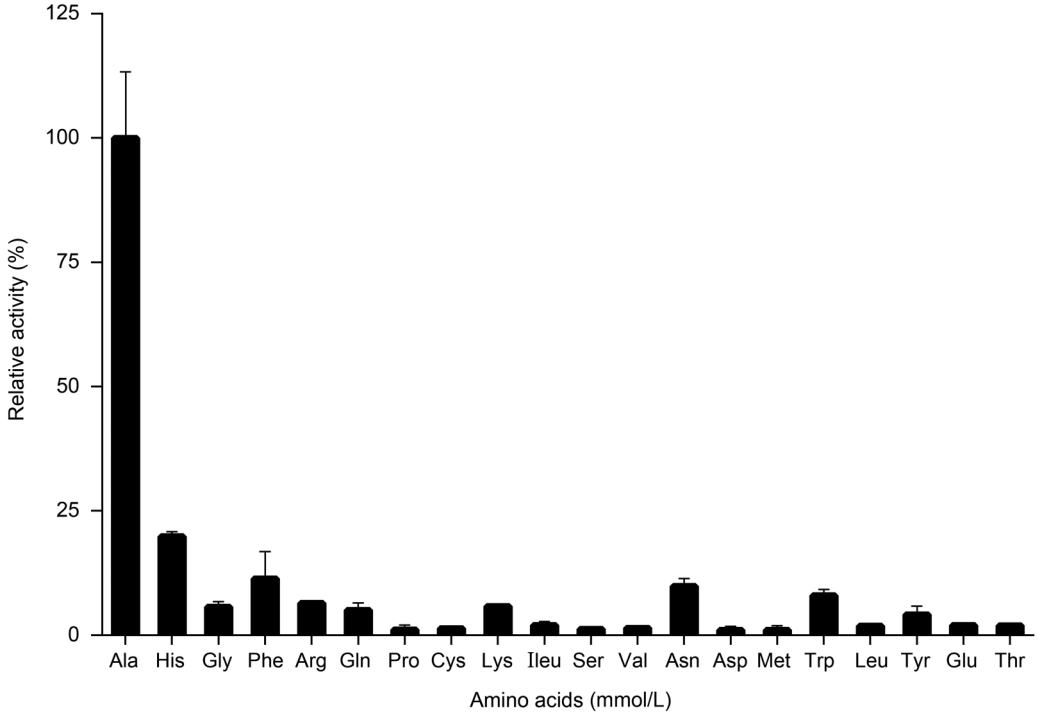

Fig. 6. The substrate specificity of SiAlr. The relative activity of SiAlr for various L-Amino acids was determined at optimum pH and temperature. The data were presented as mean ± SD from 3 independent enzyme assays.

showed weak activity with L-phenylalanine (11%), L-Histidine (20%), and L-Asparagine (10%). This result indicates that SiAlr has strict substrate specificity.

Kinetic parameters determination. Kinetic parameters of SiAlr were determined using HPLC. The substrate affinity constant (K_m) for L-alanine was 33.11 mM with a maximal velocity (V_{max}) of 2426 units/mg, while the D-alanine K_m value was 14.36 mM with a V_{max} of 963.6 units/mg. The V_{max} of L-alanine was more than twice that of its enantiomer. These indicated that the enzyme has a higher binding affinity for L-alanine than for D-alanine, and the conversion of L- to D-alanine was more rapid than the reverse conversion. The equilibrium constant (K_{eq} (L/D)) was 1.09, which is consistent with the reported theoretical equilibrium constant ($K_{eq} = 1$) for alanine racemase (Liu et al. 2015).

IC_{50} determination. In our previous study, we found that homogentisic acid and hydroquinone are two alanine racemase inhibitors with minimal cytotoxicity against mammalian cells and can be utilized as potential

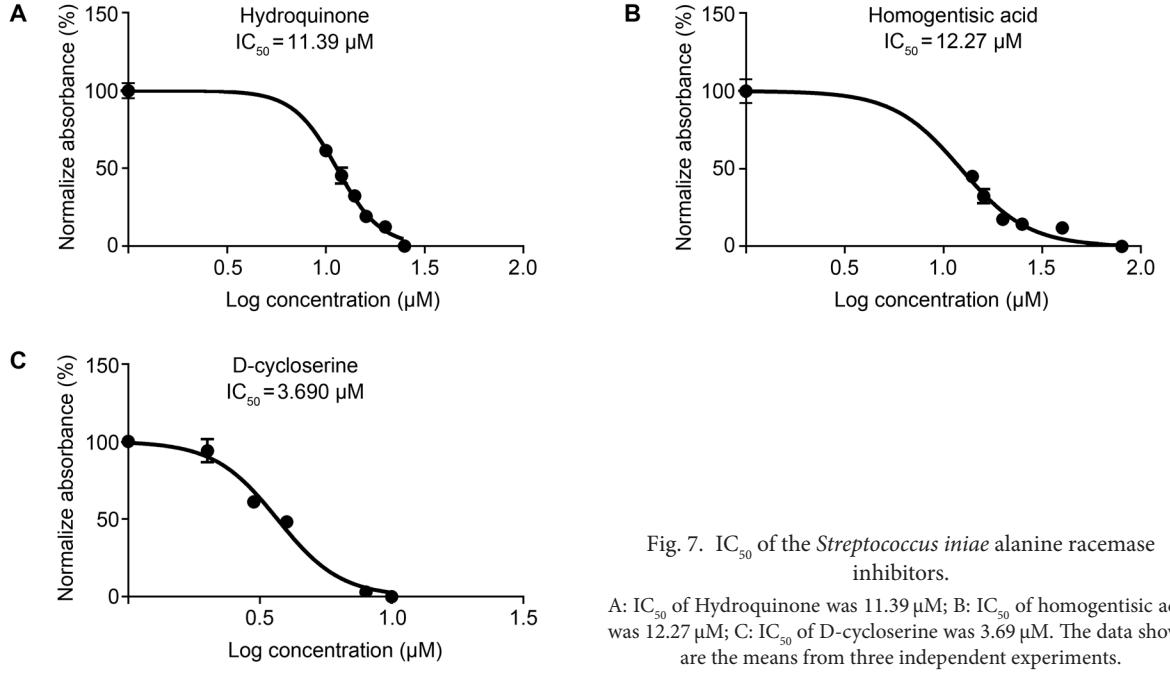

Fig. 7. IC_{50} of the *Streptococcus iniae* alanine racemase inhibitors.

A: IC_{50} of Hydroquinone was 11.39 μM; B: IC_{50} of homogentisic acid was 12.27 μM; C: IC_{50} of D-cycloserine was 3.69 μM. The data shown are the means from three independent experiments.

agents of antibiotics (Wang et al. 2017). In this study, we investigated the inhibitory effects of homogentisic acid and hydroquinone on SiAlr. DMSO was used as the blank control and DCS, a known alanine racemase inhibitor, as the positive control. The results showed that the IC_{50} values of hydroquinone and homogentisic acid were 11.39 μM and 12.27 μM. The IC_{50} values of hydroquinone and homogentisic acid were 3 and 3.3 times higher than that of DCS, respectively (Fig. 7).

Antimicrobial activity of alanine racemase inhibitors. The MIC was assayed to determine the antimicrobial activity of two alanine racemase inhibitors against *S. iniae* HNM-1 and several pathogenic bacteria (Table III). The results showed that hydroquinone and homogentisic acid have broad-spectrum antibiotic activities against both Gram-positive and Gram-negative bacteria. Hydroquinone showed good antibiotic activity against *S. iniae* HNM-1 with MIC value of 25 μg/ml, however, it showed moderate antibiotic activity against other strains with MIC value of 130–210 μg/ml. Homogentisic acid demonstrated moderate antibiotic activity against the bacteria tested, with MIC value of 180–250 μg/ml. Interestingly, hydroquinone and homogentisic acid had no antibiotic activity against *P. aeruginosa*.

Table III
The results of antimicrobial activity of homogentisic acid and hydroquinone inhibitors against numerous isolates of Gram-positive and Gram-negative bacteria.

Organism	[a]MIC (μg/ml)	
	Hydro-quinone	Homogentisic Acid
Streptococcus iniae HNM-1	25 (2.3)	200 (5.6)
Escherichia coli DH5α	130 (7.9)	210 (8.4)
Salmonella typhimurium	150 (8.7)	180 (11.4)
Staphylococcus aureus	210 (13.7)	250 (14.1)
Acinetobacter baumannii	180 (11.5)	210 (12.3)
Pseudomonas aeruginosa	0	0

[a] MIC, average values with standard deviations

Discussion

Streptococcus iniae is one of the pathogenic Gram-positive bacteria that causes morbidity and mortality of farmed and wild fish (Aruety et al. 2016). *S. iniae* HNM-1 strain was isolated from an infected Chinese sturgeon (*A. Sinensis*) after a disease outbreak that caused high morbidity and mortality. The classification of *S. iniae* HNM-1 was confirmed by molecular analysis of 16s rRNA gene sequence. The sequence was deposited at the NCBI Genbank database under accession number KY781829. The bacteria have a single putative alanine racemase (*alr*) gene. As reported earlier, most of the Gram-positive bacteria, including *Lactobacillus plantarum* (Palumbo et al. 2004), *Bacillus anthracis* (Couñago et al. 2009), *Mycobacterium tuberculosis* (Nakatani et al. 2017), and *Mycobacterium smegmatis* (Chacon et al. 2002), appear to possess only one alanine racemase gene.

The optimal pH and temperature of SiAlr were 9.5 and 35°C, respectively, which were similar to alanine racemase from *A. hydrophila* (Liu et al. 2015) and *Bacillus pseudofirmus* OF4 (Ju et al. 2009). Nearly all characterized alanine racemases have optimal pH of more

than 8.0 (Seow et al. 1998; Francois and Kappock 2007). SiAlr is a mesophilic enzyme, stable at a temperature from 0°C to 40°C. Thermal stability of an enzyme is correlated with the host bacteria physiology and environment, thermophilic bacteria Alr are more stable than that of mesophilic and psychrotroph bacteria (Soda and Tanizawa 1990; Yokoigawa et al. 1993). SiAlr was inhibited by metal ions, such as Ni^{2+}, Co^{2+}, Zn^{2+}, and Fe^{2+}, which indicated that some divalent metal ions could influence the enzyme-substrate complex formation in such a way that favor decrease of enzyme activity. Similarly, treatment of SiAlr with 1 mM of DTT resulted in a 70% loss of activity and the complete inhibition at the concentration of 3 mM. However, the activity of alanine racemase from *Tolypocladium inflatum* has increased in the presence of DTT (Di Salvo et al. 2013). DTT reduces cysteine side chains and causes a reduction of intermolecular disulfide bonds, which may affect the enzyme conformation and eventually enhance or inhibit its activity. SiAlr requires exogenous PLP for its activity in contrast to Alr from *B. pseudofirmus* OF4 and *Thalassiosira* sp., which does not require exogenous PLP for optimal activity (Ju et al. 2009). The enzyme showed high substrate specificity to alanine, which is similar to most of the characterized alanine racemases (Kawakami et al. 2018).

Many studies have focused on alanine racemase to develop antibacterial drugs for multiple bacterial species (Scaletti et al. 2012; Shrestha et al. 2017). Although the MIC values of homogentisic acid and hydroquinone were higher than that of D-cycloserine, which is a cyclic analog of alanine and exerted its inhibitory effect through interaction with the enzyme-bound PLP cofactor (Batson et al. 2017), both homogentisic acid and hydroquinone are not structural analogs of Alr as such they are not interfering with other PLP dependent enzymes, but directly interact with the active sites of alanine racemase (Wang et al. 2017). According to the results of antimicrobial activity assay, the two inhibitors are capable of inhibiting both Gram-positive and Gram-negative bacteria with various efficacies, except *P. aeruginosa*. The reason the two compounds showed no antimicrobial activity against *P. aeruginosa* may be because homogentisic acid is a normal product of *P. aeruginosa* and it also contained hydroquinone oxidase that oxidized hydroquinone (Higashi 1958; Hunter and Newman 2010).

Several alanine racemases have been identified and characterized form the *Streptococcus* species. Alanine racemase from *Streptococcus faecalis* NCIB 6459 with the molecular weight of 42 kDa was the first one that was purified and characterized (Badet and Walsh 1985). Strych et al. (2007) isolated and characterized the alanine racemase gene from *S. pneumoniae*. They obtained preliminary crystals of *S. pneumoniae* Alr, and intend to incorporate the enzyme into the structural-based drug design program. Im et al. (2011) solved the structure of *S. pneumoniae* Alr and identified three regions on the enzyme that could be targeted for the structure-based drug design. Qiu et al. (2016) provided the first evidence that D-alanine metabolism is essential for planktonic growth and biofilm formation of *Streptococcus mutans*. It would be possible to take Alr of *S. mutans* as an antibacterial target to screen and optimize the safety and effective specificity of agents. Wei Y. et al. (2016) confirmed that *alr* is an essential factor in maintaining the growth and cell wall integrity of *S. mutans*. A series of *in vivo* and *in vitro* experiments demonstrated that Alr is essential for the cariogenicity of *S. mutans*. Alr might represent a promising drug target to control the prevalence of cariogenic *S. mutans* in a multi-species microbial consortium and be a potential target for the prevention and treatment of caries (Liu et al. 2018). Therefore, Alr is regarded as a drug target for further investigation in the course of development of effective drugs against *S. iniae* and a subject of mutational studies for the growth of mutants with enhanced activity that can be utilized for industrial purpose. D-alanine is also widely used for the production of infusion solutions (Nachbauer et al. 1984), food additive (Awasthy et al. 2012), and in the manufacturing of artificial fibers (Teulé et al. 2009).

Purification and characterization of Alr from both Gram-positive and Gram-negative bacteria is an essential step towards an in-depth understanding of enzyme divers features, design new broad-spectrum antibiotics, and site-directed mutagenesis studies to improve the enzyme catalysis and stability. Hydroquinone and homogentisic acid are promising inhibitors of Alr that are capable of inhibiting the growth of both Gram-positive and Gram-negative bacteria. Future investigation will focus on finding the physiological role of Alr, exploring new novel antimicrobial agents against *S. iniae* and improving their efficacy by designing and analyzing their new derivatives that may have enhanced antimicrobial activity.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Significance of Infections in Implant Loss After Breast Reconstruction in the Course of Breast Cancer Treatment

MARIA SZYMANKIEWICZ[1]*, TOMASZ NOWIKIEWICZ[2, 3] and MARTA BIEDKA[4, 5]

[1] Department of Microbiology, Prof. F. Łukaszczyk Center of Oncology in Bydgoszcz, Poland
[2] Chair and Clinic of Oncological Surgery, Ludwik Rydygier Collegium Medicum UMK in Bydgoszcz, Poland
[3] Clinical Department of Breast Cancer and Reconstructive Surgery, Prof. F. Łukaszczyk Center of Oncology in Bydgoszcz, Poland
[4] Radiotherapy Department, Prof. F. Łukaszczyk Center of Oncology in Bydgoszcz, Poland
[5] Chair and Clinic of Oncology and Brachytherapy, Ludwik Rydygier Collegium Medicum UMK in Bydgoszcz, Poland

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Abstract

The aim of the study was to analyze the reasons for removing implants after breast reconstruction in the course of treatment of breast cancer. The study involved 428 patients, who underwent a total of 648 breast reconstruction procedures using artificial implants. 47 out of 648 cases (7.3%) were identified in which the implant had to be removed. Of the 47 cases, 57.4% had undergone deferred reconstruction, and 42.6% immediate reconstruction; 27.7% had undergone pre-operative chemotherapy and radiotherapy, 27.7% pre-operative chemotherapy, and 2.1% pre-operative radiotherapy; 6.4% were diabetic, 4.3% active smokers, and more than 50.0% had BMI greater than 25 kg/m^2. In 83.0% of the analyzed cases, the reason for removal of the implant was infection, in 8.5% it was local recurrence of breast cancer, in 4.3% it was damage (leakage) of the implant, and in 2.1% it was post-operative pain. About 87.0% of infections appeared within one year of implantation; however, less than a half developed within 90 days of the reconstructive surgery, and up to 30 days only about 13.0% had appeared. Among the etiological agents of infections were: coagulase-negative *Staphylococcus* (31.3%), *Staphylococcus aureus* (18.7%), *Enterococcus faecalis* (9.4%), *Enterobacter cloacae* (18.8%), *Pseudomonas aeruginosa* (12.5%), *Acinetobacter lwoffii* (3.1%), and other Gram-negative fermenting rods accounted for 6.2%. Infections were the most common reason for removing the implant after breast reconstruction. and occurred most often as late infections (> 30 days after surgery). The time of observation for infectious complications should be at least 1 year.

Key words: breast cancer, implants, infections, etiological agents

Introduction

In the last decade, there has been a marked increase in the frequency of breast implantation in patients undergoing surgical treatment for breast cancer. The percentage of patients having breast reconstruction after a mastectomy is as high as 36.4–43.3% (NCIN 2011; Ilonzo et al. 2017). However, this applies to data from specialized treatment centers for patients with breast cancer. In other facilities, the percentage does not usually exceed 20% of the cases of mastectomy (Alderman et al. 2006). The frequency of such treatments in Poland is much lower. However, there is a lack of accurate data on the above problem on a national scale. The previous studies, which are yet not numerous, have shown that a percentage of the patients undergoing mastectomy and breast reconstruction may reach 22.4% (Tarkowski et al. 2017). However, the complications that accompany the introduction of the implant (expander, final prosthesis) remain a major challenge for oncological surgeons. The most frequent complications include infections; it is estimated that up to 29% (mean 5.8%) of breast reconstruction surgery is complicated by infection (Phillips et al. 2013). Infection is cost-intensive and 70–80% of patients ultimately require removal of the implant (Pittet et al. 2005; Seng et al. 2015).

Among the risk factors for infection are associated diseases such as diabetes, renal failure, and skin diseases,

* Corresponding author: M. Szymankiewicz, Department of Microbiology, Prof. F. Łukaszczyk Center of Oncology in Bydgoszcz, Poland; **e-mail: szymankiewiczm@co.bydgoszcz.pl**

but also systemic treatment and radiotherapy. Other factors that increase the risk of infection include obesity and nicotinism. Infections are also triggered by factors associated with the surgery itself: an immediate breast reconstruction with the use of a final prosthesis is more often complicated by infection than deferred reconstruction. The prolonged (> 2 h) duration of surgery and post-operative drainage also have an unfavorable effect (Pittet et al. 2005; Araco et al. 2007).

Among the etiological agents of infections, the most common are skin microbiota: coagulase-negative *Staphylococcus*, *Corynebacterium* spp., *Propionibacterium acnes*, and *Staphylococcus aureus*, but more and more frequently there are reports on the increased proportion of Gram-negative bacteria from the order *Enterobacterales* and anaerobic microorganisms among etiological agents of these infections (Halvorson et al. 2007; Weichman et al. 2013; Seng et al. 2015).

This study aimed to retrospectively analyze the causes of removal of implants after mastectomy in the course of breast cancer treatment and to determine the frequency and time of appearance of implant infections as well as the etiological agents causing them.

Experimental

Materials and Methods

The study involved 428 patients treated in one oncological center in the years 1998–2018, who underwent a total of 648 breast reconstruction procedures using artificial implants.

In each case, the implantation procedure was preceded by the test for MRSA (Methicillin-Resistant *Staphylococcus aureus*) and MSSA (Methicillin-Sensitive *Staphylococcus aureus*). The swabs taken for this purpose came from the nasal vestibule, palms, and axilla on the side of the primary tumor of the operated patients. The materials for microbiological tests were seeded on Columbia Agar supplemented with nalidixic acid and amikacin + 5% sheep blood (bioMérieux, France) as well as chromogenic Brilliance MRSA (Oxoid, UK). After 24 h, the morphology of the colonies was assessed, and a catalase and coagulase assays were performed. Also, the sensitivity to methicillin was assessed following the current recommendations of the National Reference Center for Antimicrobial Susceptibility using the disk diffusion method.

Mupirocin eradication was implemented in cases of MRSA colonization in the nasal vestibule. If screening did not show the presence of MRSA, eradication was not performed. On the day before the treatment, in the evening hours, a whole-body cleansing was recommended with the use of an antiseptic intended for skin decontamination or with the body sponges impregnated with an antiseptic (chlorhexidine 4% soap solution). On the day of treatment, the whole-body cleansing was again recommended with the use of an antiseptic intended for skin decontamination. The preparation of the patient for a surgery proceeded under the standards of nursing practice currently in force at the hospital.

In all patients, perioperative prophylaxis was routinely used (cefazolin in a dose of 1.0 g in an intravenous injection – given every 8 hours for 5 days). The first dose of antibiotics was given up to 30 min before the surgery. If the drain remained longer than 5 days, antibiotic therapy was prolonged until the drain was removed. The procedure for perioperative antibiotic prophylaxis was in line with the procedure adopted in our center, i.e., the prolonged antibiotic prophylaxis in the case of wound drainage. Notwithstanding the currently recommended use of 1 dose of antibiotic (Phillips et al. 2013), the procedure referred to the results of the research presented by Brand et al. (1993), which showed greater effectiveness of longer-term use of antibacterial drugs in the prophylaxis of infectious complications after implantation.

From the group of 428 patients with artificial implants used for breast reconstruction, 44 patients were selected for the analysis when it was necessary to remove the implant, which accounted for 10.3% of the patients that underwent surgery. In two out of the 44 patients (4.5%), this occurred twice (after twice reconstructed breast, with a time interval between subsequent surgical procedures – 26 and 27 months, respectively), in one patient (2.3%), the above situation concerned the reconstruction of both breasts (carried out on two different dates – with an interval of 20 months). In none of these three patients were signs of infection of the surgical site before the second surgery. Thus, the total number of removals of implants concerned 47 cases. This accounted for 7.3% of all surgical procedures associated with generative treatment after implantation of an artificial breast implant. The incidence of infection was found in 39 cases, which accounted for 6.0% of all reconstructive procedures. Further epidemiological analyses were based on the number of the performed procedures, which resulted in the loss of the implant.

In 20 cases (42.6%), the need to remove the implant concerned immediate breast reconstruction surgery, while in the remaining 27 cases (57.4%), deferred reconstruction had been performed. The type of reconstructive implant used during the procedure (expander, expander prosthesis, or final prosthesis) as well as the duration of the procedure (immediate or deferred reconstruction) was the result of the current organizational arrangements for treatment and financing of the surgical procedures. They were not the result of the planned choice of the patients.

In 46 cases, the reconstruction of the amputated breast gland consisted of the insertion of an implant in the large pocket created behind the pectoral muscle. In one case, the patient's tissues were used to cover the implant (pedunculated skin-muscle flap taken from the latissimus dorsi muscle).

In 13 cases (27.7%), the reconstruction was preceded by chemotherapy and radiotherapy, in one case (2.1%) – by radiotherapy (this was the case in most patients who underwent restorative treatment in the deferred mode). In 13 cases (27.7%) included in the study, the patients required pre-operative chemotherapy (regardless of the mode of reconstruction). In the remaining patients, the surgery was not preceded by any other type of anticancer treatment (in most cases, these were immediate reconstruction treatments), as it is presented in Table I.

Data were collected on demographics, histopathological diagnosis, stage of cancer, concomitant diseases, surgery performed, implant characteristics, reasons for implant removal, and etiology of infections.

The reasons for the removal of the breast implants were determined, including the frequency of infectious complications. Infection of the implant was defined as surgical site infection (SSI) on the basis of clinical symptoms of infection when at least one of the following criteria was met: the purulent secretion from the drain placed in the operating space; tissue infection confirmed during reoperation; isolation of microorganisms from aseptically collected exudate/operated space, the presence of local symptoms such as redness and inflammatory infiltration, pain and elevated body temperature, as well as diagnosis of infection given by a doctor. Infection occurring up to 30 days after the implantation procedure was categorized as early surgical site infection, while all other cases, i.e. appearing at 31 days and later, were defined as late surgical site infections. The division into early and late infection was in line with Lankiewicz et al. (2012), Phillips et al. (2016), and Sinha et al. (2017). In our study, with infections defined as late, the endpoint of follow-up was the diagnosis of infection/implant loss. At the same time, within the late infections category, two additional groups of patients were created based on the infection development period; in the first group infection developed within 31 and 90 days, in the second group – after 90 days. All infections were classified as deep surgical site infections.

Table I
Characteristics of pre- and post-implantation treatment of cancer patients.

Characteristic	Number of cases n = 47 n (%)
Pre-implantation treatment:	
– RTH	1 (2.1)
– CHTH	13 (27.7)
– RTH+CHTH	13 (27.7)
– No treatment	19 (40.4)
– No data	1 (2.1)
Post-implantation treatment:	
– RTH	1 (2.1)
– CHTH	4 (8.5)
– RTH+CHTH	1 (2.1)
– No treatment	40 (85.1)
– No data	1 (2.1)

RTH – radiotherapy, CHTH – chemotherapy

The material collected for microbiological tests was seeded on solid media: Columbia agar with a supplement of 5% sheep blood (bioMérieux, France), MacConkey agar (bioMérieux, France), Columbia agar supplemented with nalidixic acid and amikacin with a supplement of 5% sheep blood (bioMérieux, France), D-Coccosel agar (bioMérieux, France), Cetrymide agar (bioMérieux, France), chromogenic medium for the identification of MRSA (Oxoid, UK) as well as the liquid medium Trypticase Soy Broth (bioMérieux, France). Incubation was carried out at 35 ± 2°C for 16–24 h. In the absence of microbial growth on solid media, incubation was continued for a further 16–24 h. In addition, the broth was seeded on solid media: Columbia Agar + 5% sheep blood and MacConkey agar (bioMérieux, France). The identification of microorganisms was carried out using the Vitek 2 Compact system (bioMérieux, US) as well as using conventional tests to identify Gram-positive cocci and Gram-negative bacilli: the assessment of catalase and coagulase production, degradation of esculin in the presence of bile salts, production of pyrrolidonyl peptidase in the PYR test, growth in broth containing 6.5% NaCl and production of oxidase, growth in Trypticase Soy Broth at 42°C, and arginine dihydrolase production. The procedures were developed based on the Clinical Microbiology Procedures Handbook (Isenberg 2004). The susceptibility of staphylococci to methicillin was assessed following the current recommendations of the National Reference Center for Antimicrobial Susceptibility using the disk diffusion method.

The etiology of microorganisms was analyzed concerning the type of surgery (immediate and deferred reconstruction) as well as with the oncological therapy before implantation vs. absence of such therapy.

Statistical analysis of the relationship between the profile of microorganisms and the type of reconstruction and oncological therapy administered before implantation (chemotherapy or radiotherapy) was carried out using Fisher's exact test. Differences were considered statistically significant at $p < 0.05$. The results are also shown as a percentage, median and average of the results recorded.

Results

The average age of patients was 48.4 years (range from 27 to 64 years, median 49.0). The mean body mass index (BMI) was 26.1 kg/m^2 (range from 17.7 to 35.2, median 26.1). In 6.4% of patients, diabetes was confirmed, and 4.3% of them were active smokers. Among the patients, eight (17.0%) were MSSA carriers; none of them was a carrier of MRSA. The average time before removal of the implant was 601 days (range 14–9102, median 113 days). The average follow-up time was 601 days. More than half of the patients have an increased BMI, about 1/3 of them received pre-operative radiotherapy; there were active smokers and people with diabetes. The coexistence of recognized risk factors increased the danger of infection. The detailed clinical characteristics of patients are presented in Table II.

Table II
Clinical and histopathological characteristics of patients with implant loss, and the type of reconstruction.

Characteristics	Number of cases n = 47 n (%)
Type of carcinoma	
Ductal carcinoma	36 (76.6)
Lobular carcinoma	2 (4.3)
Other forms of invasive cancer	3 (6.4)
DCIS	4 (8.5)
No data	2 (4.3)
Clinical stage (cTNM)	
IA	14 (29.8)
IIA	17 (36.2)
IIB	5 (10.6)
IIIA	2 (4.3)
IIIB	1 (2.1)
No data	8 (17.0)
Diabetes	
Yes	3 (6.4)
No	44 (93.6)
Nicotinism	
Yes	2 (4.3)
No	36 (76.6)
No data	9 (19.1)
BMI [kg/m^2]	
< 25	21 (44.7)
≥ 25	26 (55.3)
MSSA carrier	
Yes	8 (17.0)
No	26 (55.3)
No data	13 (27.7)
Type of reconstruction	
Immediate	20 (42.6)
Deferred	27 (57.4)
Type of implant	
Expander	23 (48.9)
Expander prosthesis	8 (17.0)
Prosthesis	5 (10.6)
Expander prosthesis/prosthesis (2nd stage of reconstruction – replacement of implant after earlier implantation of expander)	11 (23.4)

BMI – Body Mass Index
MSSA – Methicillin-Sensitive *Staphylococcus aureus*
DCIS – Ductal Carcinoma *In Situ*
cTNM – Clinical TNM (classification system of malignant tumors – tumor, node, metastasis)

In 83.0% (39/47) of cases, the reason for removal of the implant was an infection, in 8.5% (4/47) local recurrence of breast cancer, and in 4.3% (2/47) damage (a leakage) of the implant. In 2.1% (1/47) of cases, the reason for removal of the implant was post-operative pain, in 2.1% (1/47) the reason was unknown. The reasons for implant removal are shown in Table III.

The only reason for removal of the implant up to 30 days after the reconstructive treatment was an infection, which appeared in six of the cases analyzed (12.8%). The implants were removed on average after 18 days (median 16 days, range 14–27 days) after the reconstructive procedure. In 23.4% (11/47) of cases, the infection was recognized between 31 and 90 days after surgery, in 36.2% (17/47) between 91 and 365 days after surgery, and in 10.6% (5/47) after a period longer than one year. Up to 90 days after surgery, 17/39 (43.6%) infections were identified, up to one year – 34/39 (87.2%) cases, and after this period – 5/39 (12.8%) cases. The late infections (> 30 days) appeared on average after 329 days (median 115 days, range 35–4914 days).

The remaining, non-infectious complications that caused the removal of the implant in seven cases (14.9%) appeared on average after 2465 days (median

Table III
Characteristics of postoperative complications.

Characteristics	Number of cases n = 47 n (%)
Infection	
Early (≤ 30 days)	6 (12.8)
Late (> 30 days)	
31–90 days	11 (23.4)
91–365 days	17 (36.2)
> 365 days	5 (10.6)
– Local recurrence of breast cancer	2 (4.3)
– Local recurrence of breast cancer in the chest wall	2 (4.3)
– Postoperative pain	1 (2.1)
– Leakage of prosthesis/expander prosthesis	2 (4.3)
– No data	1 (2.1)

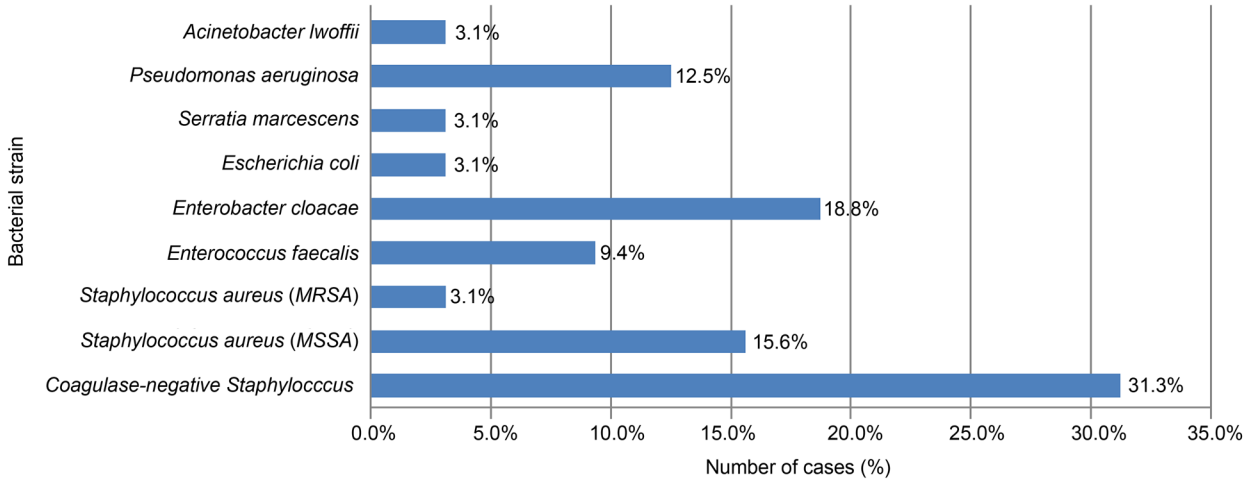

Fig. 1. Bacterial species isolated from the breast artificial implants infections.

1630 days, range 239–9102). The longest time interval between reconstruction and implant removal was 9102 days and it was due to the leakage of the prosthesis. The shortest time interval was 239 days, and the removal of the implant was due to the recurrence of local breast cancer in the chest wall.

In five out of 39 cases (12.8%), the infection was diagnosed only based on clinical symptoms without microbiological diagnosis and no microorganisms were grown in six out of 39 cases of infections (15.4%). In 24 out of 28 (85.7%) microbiologically confirmed cases of infection, bacteria were present in monoculture, in four cases (14.3%) polybacterial infections were found. Among the etiological agents were: coagulase-negative staphylococci (31.3%), *Staphylococcus aureus* (18.7%), *Enterococcus faecalis* (9.4%), *Enterobacter cloacae* (18.8%), *Pseudomonas aeruginosa* (12.5%), and *Acinetobacter lwoffii* (3.1%). Other fermenting Gram-negative rods accounted for 6.2%. The cultured and identified etiological agents of infections are shown in Fig. 1.

There were no statistically significant differences in the etiology of microorganisms (cocci *vs* rods) between the groups of patients after immediate and deferred reconstruction and between cancer patients treated before implantation (radiotherapy and/or chemotherapy) vs. cancer patients that do not receive any treatment ($p > 0.05$).

Discussion

In this study, we show that infections were the most common reason of the removal of the implants after generative treatment in breast cancer and were responsible for the loss of the implant in 83.0% of the cases analyzed. The prevalence of breast implant infection in this study was 6.0% of implantation. Of the infections diagnosed, 87.0% appeared within one year of implantation; however, less than half of the infections developed within 90 days of the reconstructive surgery, while up to 30 days – about 13.0% of infections were diagnosed. Non-infectious complications caused the loss of the implant in about 15.0% of the cases analyzed.

In the available literature, infection after the breast reconstruction surgery appeared with varying frequency and their etiological agents were different. This is related to the adopted definition of infection, the period of time for surveillance, the perioperative prophylaxis applied, and the composition of microbiota inhabiting the operated area (Weichman et al. 2013). The time of observation significantly influences the obtained results. In the studies of Olsen et al. (2017) on a large group of respondents, approximately 17 000 patients undergoing mastectomy, the incidence of SSI up to 90 days after surgery was 3.2–8.9% and was dependent on the type of reconstruction. In the opinion of the authors cited, it is necessary to carry out the infection surveillance up to 90 days after surgery, since the shorter period of observation, limited to 30 days, leads to an underestimation of the rate of infection. Similarly, Lankiewicz et al. (2012) found, however, on a smaller group of patients comprised of 54 women undergoing mastectomy with immediate reconstruction with an implant, that only about 1/2 of the diagnosed SSI developed within 30 days after surgery. Viola et al. (2016), in single-center retrospective studies involving over 3000 patients, found that only 30.0% of all SSIs were diagnosed within the first 30 days after a simultaneous reconstruction using a tissue expander. In our work, we found lower rates than the quoted authors (Lankiewicz et al., 2012; Viola et al. 2016) regarding infection within 30 days after reconstructive surgery. When we limited observation to one month after the procedure we were able to detect only 1/8 of the implant infections confirmed.

The reasons for the variability in the period when the infections appeared were probably related to the eligibility criteria for the reconstructive surgery (the patient's profile), the type of surgery performed, the experience of the center performing the implant treatments, as well as the prophylactic treatment used. In our work, we confirmed that the observation of infections limited to 30 days after surgery was insufficient, as was the 90-days observation. It seems that the optimal time could be a one-year observation. In our study, this allowed the detection of approximately 90% of infections. The necessity to monitor for complications after breast reconstruction during one year after surgery, instead of 30 days, has also been indicated by other researchers (Blough et al. 2018), who used individualized tools to assess the risk of post-surgery complications within one year of breast reconstruction.

The fact that the number of infections within 30 days in the cases we analyzed was lower than the numbers appearing in the studies already published may be related to the strict epidemiological supervision carried out in our hospital and the prophylaxis to prevent perioperative infections.

According to the hospital antibiotic policy, the patients studied in the current work received prophylactically a first generation cephalosporin in therapy extended for five days, or until the drain was removed due to the increased SSI risk associated with drainage (Araco et al. 2007). However, currently, many authors advise against such a procedure, pointing to the side effects that accompany it, namely the increase in resistance among microorganisms responsible for SSI (McCullough et al. 2016). In accordance with the idea of perioperative prophylaxis, a single dose of the first-generation cephalosporin is sufficient to ensure the proper concentration of the antibiotic at the incision site and to prevent infections mainly caused by methicillin-sensitive staphylococci (Pittet et al. 2005). Under the recommendations of scientific societies, it is also not suggested to continue treatment with antibiotics after discharge from the hospital, and the guidelines of the American Society of Plastic Surgeons recommend discontinuation of antibiotics at 24 hours after surgery. Nevertheless, a review of publications (Phillips et al. 2016; Viola et al. 2016) shows that the use of prolonged antibiotic therapy by surgeons, both during hospitalization and after discharge from the hospital, is quite common.

In addition to the prophylactic use of antibiotics, a screening test for *S. aureus* was introduced as a standard in our hospital. When colonization with MRSA strain is confirmed, mupirocin is applied nasally without performing tests confirming the effectiveness of eradication. However, in the cases analyzed in the current work, no MRSA carriers were found. Whereas, concerning the colonization with MSSA strains, we assumed that the perioperative prophylaxis with the first-generation cephalosporin is sufficient protection, with its MSSA spectrum of action. It should be emphasized that screening for *S. aureus* in the group of patients undergoing reconstruction is not recommended. According to the recommendations for microbiological screening tests in hospitalized patients (Fleischer et al. 2017), investigation for the carriage of *S. aureus* is recommended only before cardiac surgery, implantation of joint prostheses and spine procedures, as well as in centers with incidence of SSI caused by *S. aureus* higher than the average reported in literature, mainly in neurosurgery and vascular surgery. Patients qualified for breast reconstruction do not meet the criteria given in these recommendations. Nevertheless, within the framework of the perioperative infections prevention carried out in our center, such examination was undertaken. In the current work, we diagnosed MSSA carriers in 17.0% of the cases. In one case, the host had to remove the implant 14 days after surgery due to infection caused by *S. aureus*. In other cases, the infection caused by MSSA occurred in the late period, on average more than one year after surgery, similarly as in a patient with an MRSA-induced infection.

In the available literature on implant infections, among the etiological agents of infections after breast reconstruction, the microbiota of the skin and at the mouth of the milk ducts were isolated (coagulase-negative *Staphylococcus*, mainly *S. epidermidis* and coagulase-positive *S. aureus*) (Chidester et al. 2016; McCullough et al. 2016; Viola et al. 2016). These species adhere to the smooth surfaces of biomaterials, and may also colonize the skin. They demonstrate the ability to form a biofilm, which protects them against the host's immune system and antibiotics (Vacheethasanee et al. 1998; Costerton et al. 1999; Chessa et al. 2016; Conte et al. 2018).

In our work, staphylococci constituted 50% of the isolated microorganisms. They were in a lower percentage than in the studies by Seng et al. (2015) but similar to the results obtained in Song et al. (2017) (respectively: 71.0%, and 50.0%). In three cases (9.4%), we isolated *E. faecalis* that can adhere, modify the immune response, and form biofilms (Prażmo et al. 2016); however, these species are rarely the etiological agent of implant infections. In the available literature, one can find only a few reports about the participation of these microorganisms in wound infections after immediate breast reconstruction (Abedi et al. 2016).

The incidence of infections caused by Gram-negative rods is varied. In the work of Feldman et al. (Feldman et al. 2010) concerning early infections of breast implants, Gram-negative rods accounted for 6.0% of the microorganisms isolated. Seng et al. (2015) also

confirmed the participation of these bacteria in implant infections: Gram-negative rods were identified in 27.0% of cases, and *P. aeruginosa* was the second most commonly isolated microorganism. In turn, in the work of Chidester et al. (2016) *P. aeruginosa* was the most common causative agent of infections and accounted for 26.8% of them. Research carried out by Song et al. (2017) indicates the involvement of *A. baumanii* in infections alongside with *P. aeruginosa*.

In our work more frequently than in the previous studies (Feldman et al. 2010; Seng et al. 2015; Chidester et al. 2016; Song et al. 2017), Gram-negative bacilli were isolated. They constituted a total of 40.6% of the bacterial isolates. The most commonly isolated rods were *E. cloacae* (18.8%), and *P. aeruginosa* (12.5%). These microorganisms secrete extracellular polymers forming a complex matrix of biofilm. The biofilm matrix plays an important role in survival in tissues and evading the response of the body's immune system, which promotes the development of the infection despite the use of antibiotics (Donlan and Costerton 2002). The occurrence of Gram-negative bacilli, naturally resistant to the first-generation cephalosporin, may be associated with the perioperative prophylaxis. Our work did not show the dependence of the etiology of microorganisms (cocci vs. rods) on the type of reconstruction or oncological treatment.

Polybacterial infections are not associated with implants. However, in this work, in four cases (14.3%) we showed the presence of the polybacterial infections. Similar results were obtained by Seng et al. (2015), as well as Viola et al. (2016), the percentage of polybacterial infections reached 19.0 and 17.0%, respectively, among all diagnosed infections. In the cases analyzed by us, two polybacterial infections related to patients treated with radiotherapy. Radiotherapy is an important risk factor for the occurrence of infection through damage to the skin and other tissues, which may affect the microbiota. In over 1/3 of cases, the adjuvant treatment was radiotherapy (in 14 cases before reconstruction surgery, in 2 cases after surgery). However, in only one case for the reconstruction of the breast beside the implantation, autologous tissues were used (lobe from the latissimus dorsi muscle). According to the current standard of diagnostic and therapeutic procedures, this is the recommended method of treatment (Bocian et al. 2016). This indicates the need to use more complex restorative treatments in the future (especially the use of flap techniques in patients requiring radiation therapy).

The existence of a significant risk of complications (especially infectious) related to radiotherapy was shown by Jagsi et al. (2016) and among the most common adverse effects, they mention an increased risk of wound infection between 7 and 24 months after surgery, and consequently the requirement to remove the implant. Similar observations were also presented by other authors (Momoh et al. 2014; Blough et al. 2018). In our work, the median time to remove the implant after using radiotherapy was 3–4 months after surgery.

In addition to radiotherapy, factors that increase the risk of infection complications according to Warren Peled et al. (2010) also include adjuvant chemotherapy. However, in newer works, this relationship is not shown. The lack of influence of the time of the chemotherapy administration on the risk of SSI (pre-operative vs. post-operative vs. pre- and post- vs. non-chemotherapy) was demonstrated by Chattha et al. (2018).

In approximately 1/6 of the cases discussed by us, there was no confirmation of microbiological infection, despite the presence of clinical symptoms of wound infection. The probable cause of the negative culture results was an occurrence of rare microorganisms, which do not grow on classical microbiological media and also difficulty in culturing microorganisms present in biofilms or the presence of microorganisms with special nutritional requirements (Pajkos et al 2003). This is confirmed by the results of the research made using molecular biology methods. The use of molecular techniques as well as gene sequencing omits the culture and leads to an increase in the recognition of infections (Romero et al. 2017). Management of infections in oncological patients should now include extended microbiological diagnostics to detect hard growing or non-cultivated microorganisms (Pittet et al. 2005; Al-Halabi et al. 2018).

Infections remain the most common reason for removing implants and are a serious complication of reconstruction during the treatment of breast cancer. The existence of risk factors for infection, the diversity of bacterial species that cause the implant infection, their potential for biofilm formation and natural resistance to selected groups of antibiotics, as well as the long time of infection development pose a great challenge for the effective treatment of implant infections, which in turn leads to the removal of the implant. The introduction of standardized data collection protocols in the prospective studies, especially in specialized breast cancer centers, may be helpful in the identification of the factors that increase the risk of surgical site infection after reconstruction, determination the etiology of infections, establishment of the optimal perioperative prophylaxis, in empirical therapy, as well as in post-hospital outpatient care. This should result in lower SSI rates.

Ethical approval

The study proposal was approved by the Bioethics Committee of the Collegium Medicum of Nicolaus Copernicus University in Bydgoszcz, No KB 286/2019.

ORCID
Maria Szymankiewicz 0000-0003-3669-6903

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Characteristics of *Listeria monocytogenes* Strains Isolated from Milk and Humans and the Possibility of Milk-Borne Strains Transmission

KRZYSZTOF SKOWRON[1]*, EWA WAŁECKA-ZACHARKSA[2], KATARZYNA GRUDLEWSKA[1], NATALIA WIKTORCZYK[1], AGNIESZKA KACZMAREK[1], GRZEGORZ GRYŃ[3], JOANNA KWIECIŃSKA-PIRÓG[1], KLAUDIA JUSZCZUK[1], ZBIGNIEW PALUSZAK[4], KATARZYNA KOSEK-PASZKOWSKA[2] and EUGENIA GOSPODAREK-KOMKOWSKA[1]

[1] Department of Microbiology, Nicolaus Copernicus University in Toruń, Collegium Medicum of L. Rydygier in Bydgoszcz, Bydgoszcz, Poland
[2] Department of Food Hygiene and Consumer Health, Wrocław University of Environmental and Life Sciences, Wrocław, Poland
[3] Plant Breeding and Acclimatization Institute – National Research Institute, Bydgoszcz, Poland
[4] Department of Microbiology and Food Technology, UTP University of Science and Technology, Bydgoszcz, Poland

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Abstract

Listeria monocytogenes is the etiological factor of listeriosis. The main source of these organisms is food, including dairy products. The aim was to determine the multiple correlations between the drug susceptibility, virulence genes (VGs), and biofilm formation on silicone teat cups of milk-borne and human *L. monocytogenes* strains. The spread of *L. monocytogenes* via contaminated teat rubbers was assessed. The *L. monocytogenes* strains recovered from milk (18), human blood (10), and the reference strain ATCC®19111™ were used in the study. Penicillin resistance was the most prevalent resistance in the milk isolates (n = 8; 44.4%), whereas among clinical strains erythromycin resistance was predominating – (n = 6; 60%). The most frequent VGs among strains isolated from milk were *hlyA* (100%) and *plcB* (100%) whereas in strains isolated from blood – *hlyA* (100%) and *prfA* (90%). All tested VGs were present in 50% of blood isolates and 11% of milk-borne strains. The strains isolated from milk formed a significantly stronger biofilm. The strains with more numerous virulence genes were resistant to more antibiotics and formed a stronger biofilm. It was shown that contaminated teat cups might contribute to the transmission of *L. monocytogenes* in the herd. It seems reasonable to monitor the occurrence of *L. monocytogenes* biofilm in a dairy processing environment.

Key words: *Listeria monocytogenes,* biofilm formation, drug susceptibility, bacteria transmission, virulence genes frequency

Introduction

Listeria monocytogenes is Gram-positive, facultatively anaerobic, non-spore forming bacteria. *L. monocytogenes* causes listeriosis and is one of the most dangerous food-borne zoonotic pathogens (Schlech and Acheson 2000). Listeriosis mainly occurs in immunocompromised people, neonates, pregnant women, the elderly, and AIDS patients (Lawley et al. 2008; Jamali et al. 2013; Doijad et al. 2015). The major source of human infection is food contaminated with the pathogen, including raw milk and milk products. A recent trend to purchase fresh, unprocessed products from local suppliers has contributed to an increase in the consumption of unpasteurized milk and products thereof (Seremak-Bulge et al. 2013). Such products may be the source of pathogenic bacteria and lead to serious infections (Jamali and Radmehr 2013; Gould et al. 2014).

According to the European Food Safety Authority (EFSA) report in 2017 all tested milk samples included in the RTE (ready-to-eat) products category were in line with Food Safety Criteria (FSC). One of 85 samples (1.2%) from two member countries, in the category "Raw cow's milk for direct consumption" in the

* Corresponding author: K. Skowron, Department of Microbiology, Nicolaus Copernicus University in Toruń, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Poland; **e-mail: skowron238@wp.pl**

retail trade, was positive for *L. monocytogenes* (EFSA 2018). In Poland and other European countries, the number of infections caused by this microorganism is constantly increasing. In 2017 2480 cases of listeriosis were recorded in the EU countries, while in 2013 only 1763 cases were found. The incidence rate per 100 000 inhabitants was 0.26 and 0.32, respectively, in 2016 and 2017 (EFSA 2018). In 2018 124 cases of listeriosis were reported in Poland that is a 20% increase when compared to 2015 (NIZP-PZH 2019). Listeriosis associated with raw milk consumption was reported in 2014 in two patients from California and Florida. The most likely source of *Listeria* spp. was raw chocolate milk (CDC 2016). In Los Angeles County (California) during an outbreak associated with the consumption of the contaminated cheese, the death of 48 out of 142 patients was recorded (Linnan et al. 1988). Another listeriosis outbreaks linked to the contaminated cheese took place in Germany in 2006–2007 (Koch et al. 2010), and in Spain in 2008–2009 (Jackson et al. 2011) where 189 and 8 cases were reported, respectively.

Despite the improvement in the hygiene of the production process, *L. monocytogenes* poses still a serious problem in the food processing plants, including dairies. Such environment favors biofilm formation and bacterial survival (Latorre et al. 2010). Since *L. monocytogenes* multiplies easily and quickly on improperly cleaned dairy appliances, biofilm formation starts within 20 minutes after bacterial contact with the surface (Hayes et al. 1986; Weiler et al. 2013). In dairy plants, *L. monocytogenes* colonizes processing surfaces, floors, equipment, and niches that are difficult to clean (e.g. hard to reach cavities), and becomes the potential source of milk and milk product contamination (Unnerstad et al. 1996; Latorre et al. 2010). Bacteria forming biofilm survive longer and are more resistant to disinfectants and mechanical washing (Frank and Koffi 1990; Walker et al. 1990; Meyer-Broseta et al. 2003). Therefore, it is of great importance to prevent biofilm formation on milking equipment to reduce the risk of milk contamination and, in consequence, human infections (Latorre et al. 2010).

Although most *L. monocytogenes* strains are susceptible to antimicrobial agents used in human and animal medicine, multidrug-resistant strains are increasingly frequently isolated. This is mainly due to the overuse of antibiotics in animal husbandry (Jamali et al. 2013). Also, several virulence factors enable *L. monocytogenes* to infect and spread in the host (Park et al. 2012). *L. monocytogenes* possesses many virulence genes responsible for the invasion of host cells (*inlA*, *inlB,* and *iap*), phagosomal escape (*hlyA*, *plcA*, and *plcB*) and cell to cell spread (*actA*) (Hamon et al. 2006).

This study aimed was to determine the multiple correlations between drug susceptibility, virulence genes and biofilm formation on silicone teat cups from milking machines of milk-borne and human *L. monocytogenes* strains. The spread of *L. monocytogenes* through contaminated teat rubbers was also assessed.

Experimental

Materials and Methods

Materials. Milk samples, obtained in Poland from cows without clinical signs of *mastitis*, were collected in 2015. For each cow, a sample of 100 ml from all the four teats was collected to one sterile container. Of 380 milk samples, 21 (5.5%) were positive for *L. monocytogenes*. Ten genetically different strains (a genetic similarity previously determined for the diagnostic reasons) isolated from the blood of patients by dr. A. Jurasz (University Hospital No. 1 in Bydgoszcz, Poland) were used to evaluate their drug susceptibility and ability to form biofilms. There was no epidemiological link between milk and blood isolate groups. The reference strain *L. monocytogenes* ATCC® 19111™ was included in the study. This strain is widely used as the reference strain in many studies, including biofilm formation assessment.

Isolation of *L. monocytogenes* from milk. Analysis of the intermediate and finished product samples was carried out following the ISO 11290-1 (ISO 2017). To isolate *L. monocytogenes*, 25 ml of milk was added to 225 ml of half-Fraser broth (Merck, Poland) and incubated for 24 h at 30°C. Then 0.1 ml of the culture was transferred into 10 ml of Fraser broth (Merck, Poland) and the secondary selective enrichment was performed at 37°C for 48 h. Finally, the culture was plated onto ALOA agar (ChromoCult Listeria Selective Agar®, Merck, Poland), and OXFORD Agar (Oxoid, United Kingdom), and incubated for 24 h at 37°C.

Identification of the strains isolated from milk. Initial species identification was performed based on morphological traits on the ALOA agar (Merck, Poland). The typical colony of *L. monocytogenes* is a turquoise and blue one surrounded with a turbidity zone.

Then, multiplex PCR was performed. For the genus *Listeria* identification primers (L1, L2) (Oligo.pl, Poland) based on the 16S rRNA sequence were used (Border et al. 1990) whereas primers (LM1, LM2) based on the sequence of the *hly* gene allowed the species identification (Bansal 1996).

DNA was isolated using the Genomic Mini AX Bacteria Spin kit (A&A Biotechnology, Poland), according to the manufacturer's instructions. Amplification was performed in the mixture of 25 µl, containing: 1 × PCR buffer (Promega, United States), 2.0 mM $MgCl_2$ (ABO, Poland), 1.25 mmol dNTPs (Promega, United States),

Table I
Primers used for identification of *L. monocytogenes*.

Primer	Primer sequence (5' – 3')	Size of PCR product (bp)	Amplification conditions
L1	CAG CAG CCG CGG TAA TAC	938	Initial denaturation – 94°C/2 min 30 cycles: denaturation – 94°C/30 s annealing, – 50°C/30 s elongation – 72°C/1 min final elongation – 72°C/5 min
L2	CTC CAT AAA GGT GAC CCT		
LM1	CCT AAG ACG CCA ATC GAA	750	
LM2	AAG CAC TTG CAA CTG CTC		

0.5 µM of each of the primers (Oligo.pl, Poland), 1.0 U Taq DNA polymerase (Promega, United States), ultrapure water (Merck, Poland) and 2.0 µl DNA. The amplification conditions and primer sequences are presented in Table I. *L. monocytogenes* strain ATCC 19111 was used as the reference strain. PCR products were separated on 1.5% agarose gel for 75 min at 80V and stained with a Midori Green dye (NIPPON Genetics EUROPE GmbH, Germany).

Determination of genetic relatedness of *L. monocytogenes* strains isolated from milk. All *L. monocytogenes* isolates were genotyped using Random Amplification of Polymorphic DNA (RAPD) with the nonspecific primer OPA-11 (5'-CA AT CG CC GT-3') (Ozbey et al. 2006). Reactions were carried out in a mixture of 25 µl, containing: 1 × PCR buffer with 2.0 mM $MgCl_2$ (Promega, United States), 1.5 mM $MgCl_2$ (ABO, Poland), 200 µM dNTPs (Promega, United States), 1.0 µM primer OPA-11 (Oligo.pl, Poland), 1.25 U Taq DNA polymerase (Promega, United States), ultrapure water (Merck, Poland), and 3.0 µl DNA. The reaction consisted of six cycles of initial stage: denaturation (94°C/1 min), annealing (30°C/2 min) and elongation (72°C/1 min), followed by 35 cycles of: denaturation (94°C/15 s), annealing (37°C/1 min) and elongation (72°C/45 s), and the final elongation (72°C/10 min). PCR products were separated on 1.5% agarose gel for 150 min at a voltage of 80 V, and stained with a Midori Green dye (NIPPON Genetics EUROPE GmbH, Germany).

To determine the degree of genetic relationship between the isolates, the phylogenetic dendrogram was drawn in the program Phoretix 1D Pro (TotalLab). Data clustering was performed using the UPGMA hierarchic grouping technique with the Dice coefficient.

The frequency of the genes encoding virulence factors in milk and blood isolates. *L. monocytogenes* strains from milk (18) and blood (10) were examined for the multiplex PCR reactions. The study included the following virulence genes: *actA* (actin assembly-inducing protein), *hlyA* (listeriolysin O), *iap* (extracellular protein p60), *inlA* (internalin A), *inlB* (internalin B), *plcA* (phosphatidylinositol-specific phospholipase C), *plcB* (phosphatidylcholine-specific phospholipase C), and *prfA* (positive regulatory factor PrfA).

Two multiplex-PCR reactions were optimized to detect these virulence genes. The first included the detection of *iap*, *hlyA*, *inlB*, and *plcB*, and the second one – *actA, inlA, plcA*, and *prfA* genes. The reaction mixture (25 µl) contained: 1 × PCR buffer with 2.0 mM $MgCl_2$ (Promega, United States), 6.0 mM $MgCl_2$ (ABO, Poland), 1.0 mM dNTPs (Promega, United States), 1.0 µM of each primer (Oligo.pl, Poland) (Table II), 3.0 U Taq DNA polymerase (Promega, United States), ultrapure water, and 3.0 µl DNA. The course of PCR was as follows: the initial denaturation (95°C/2 min), 35 cycles of denaturation (95°C/15 s), annealing (60°C/30 s) and elongation (72°C/1,5 min) and the final elongation (72°C/10 min). The *L. monocytogenes* strain IW41 was used as the reference strain. PCR products were separated on 1.5% agarose gel and stained with a Midori Green dye (NIPPON Genetics EUROPE GmbH, Germany). The Perfect 100 bp DNA Ladder (EurX, Poland) was used.

Evaluation of drug susceptibility of *L. monocytogenes* strains. Drug susceptibility of 18 milk and ten blood isolates was determined using the disk diffusion method on the Mueller-Hinton agar with 5% defibrinated horse blood and β-NAD at a concentration of 20 mg/l (MH-F, bioMérieux, France). For each strain, a suspension of 0.5 McFarland's scale (7.6×10^7 CFU/ml $\pm 9.4 \times 10^6$ CFU/ml) in sterile saline was prepared. The disks with penicillin (1 U), ampicillin (2 µg), meropenem (10 µg), erythromycin (15 µg) and cotrimoxazole (1.25–23.75 µg) (Becton Dickinson, United States) were used. Incubation of antibiograms was conducted in the atmosphere enriched in 5% CO_2 at 35°C for 18 h. The results were interpreted according to the recommendations of EUCAST v. 8.0.

Evaluation of the biofilm formation ability of *L. monocytogenes* strains. Quantitative evaluation of biofilm formation by *L. monocytogenes* strains was performed on irradiated fragments of silicone teat cups from milking machines. The fragments of 1 × 1 cm were used in the study.

The intensity of biofilm formation was determined using a quantitative method by Kwiecińska-Piróg et al. (2011) with some modifications. The study was conducted on one clinical strain, one strain isolated from milk, and the reference strain ATCC 19111. The

Table II
Primers used for the detection of virulence genes in *Listeria monocytogenes* strains (Franciosa et al. 2005; Rawool et al. 2007).

Primer	Primer sequence (5' – 3')	Size of PCR product (bp)
actA – F	CGC CGC GGA AAT TAA AAA AAG A	839
actA – R	ACG AAG GAA CCG GGC TGC TAG	
hlyA – F	GCA GTT GCA AGC GCT TGG AGT GAA	456
hlyA – R	GCA ACG TAT CCT CCA GAG TGA TCG	
iap – F	ACA AGC TGC ACC TGT TGC AG	131
iap – R	TGA CAG CGT GTG TAG TAG CA	
inlA – F	CAG GCA GCT ACA ATT ACA CA	2 341
inlA – R	ATA TAG TCC GAA AAC CAC ATC T	
inlB – F	AGG AGA GGA TAG TGT GAA	1 905
inlB – R	TTA TTT CTG TGC CCT TAA	
plcA – F	CTG CTT GAG CGT TCA TGT CTC ATC CCC C	1 484
plcA – R	ATG GGT TTC ACT CTC CTT CTA C	
plcB – F	GCA AGT GTT CTA GTC TTT CCG G	794
plcB – R	ACC TGC CAA AGT TTG CTG TGA	
prfA – F	CAT GAA CGC TCA AGC AGA AG	706
prfA – R	AAT TTT CCC AAG TAG CAG GA	

sterile fragments of rubber (3 replications for each strain) were placed in tubes containing 3 ml of the bacterial suspensions in the Brain Heart Infusion Broth (BHI) (Merck, Poland) (0.5 of the McFarland scale). Incubation was conducted in the aerobic atmosphere at 37°C for 72 h, and the medium was replaced with a sterile one every 24 h. At each exchange of the medium, the rubber fragments were rinsed with sterile PBS (Phosphate Buffered Saline) (BTL, Poland). The fragments of rubbers incubated in the sterile BHI medium (Merck, Poland) were used as negative controls. After incubation, the samples were rinsed with PBS, placed in a tube containing 3 ml of PBS, and sonicated for 10 minutes (30 kHz, 150W) with the sonicator Ultrasonic DU-4 (Nickel-Electro Ltd.). Then, the samples were shaken for 10 minutes (400 rpm), and the serial 10-fold dilutions were prepared and inoculated on the Columbia Agar medium with 5% sheep blood (Becton Dickinson, United States). After 24-hours incubation at 37°C, the number of colonies per 1 cm^2 of the fragment's surface (CFU/cm-2) was calculated.

The intensity of biofilm formation by *L. monocytogenes* was observed under the confocal microscope. For this purpose, very thin rubber slides were prepared and the biofilms were grown, as described above. The biofilm-forming cells on the rubber slides were then stained with the LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher Scientific, United States), according to the manufacturer's instructions.

The spread of *L. monocytogenes* through contaminated teat rubbers. *Transmission of L. monocytogenes on the skin of the udder and into the milk.* The radiant sterilized, purified from fat, pieces of cow's udder skin (1 × 1 cm) were used in the experiment. Biofilm formation on sterile teat rubbers was assessed as described above (section "Evaluation of biofilm formation ability of *L. monocytogenes* strains"). Two of the strongest and weakest biofilm-forming strains of *L. monocytogenes* derived from both milk and blood as well as the reference strain were selected for the study.

To evaluate the transmission of bacteria from the biofilm formed on the teat rubbers to the udder skin (according to our method), a piece of skin was rubbed with the contaminated rubber in two directions. This simulated the insertion and removal of the teat into the milking cup. For each strain, eight skin fragments were used in six replicates. After swabbing, each piece of skin (1–3 replicates) was placed in sterile PBS (BTL, Poland) and subjected to a 10-minute sonication. Subsequently, the serial 10-fold dilutions were made and plated onto Columbia Agar with 5% sheep blood (Becton Dickinson, United States). After 24 hours at 37°C, the number of bacteria in 1 cm^2 of the udder skin was calculated. Also, to test the proliferation of *L. monocytogenes* on the skin, fragments (repeats 4–6) were left at 25°C for 12 hours. Subsequently, the samples were placed in sterile PBS, sonicated and plated onto Columbia Agar with 5% sheep blood (Becton Dickinson, United States).

To evaluate the transmission of *L. monocytogenes* from the biofilm formed on the teat rubbers to milk, each rubber was flushed with 100 ml of UHT milk. Then, 0.1 ml of milk was plated onto Columbia Agar

medium with 5% sheep blood (Becton Dickinson, United States), incubated at 37°C for 24 hours, and the number of bacteria in 1 ml of milk was determined.

Transmission of L. monocytogenes in udder – teat rubber – udder model. Two of the strongest and weakest biofilm-forming strains of *L. monocytogenes* derived from milk and blood and the reference strain were selected for the study.

The bacterial suspensions in sterile PBS (BTL, Poland) (0.5 McF) were prepared and 50 µl of the suspension (six replicates) was poured on the sterile skin fragments. After drying at room temperature, the sterile teat rubber piece was rubbed in two directions with the contaminated skin. Then, the rubber pieces were placed (repeats 1–3) in sterile PBS (BTL, Poland) and sonicated. The number of bacteria per 1 cm^2 of rubber was determined by plating the sample into Columbia Agar with 5% sheep blood (Becton Dickinson, United States), and incubation at 37°C for 24 hours. The remaining contaminated teat rubber fragments (repeats 4–6) were used to rub six sterile fragments of the udder skin. The number of bacteria transferred to them as well as proliferation on the udder skin was determined as described previously ("*Transmission of L. monocytogenes on the skin of the udder and into the milk*").

Statistical analysis. The statistical analysis of the results was performed using the software Statistica 12 PL (StatSoft).

The frequency of the genes encoding virulence factors in *L. monocytogenes* strains isolated from milk and blood was established. Statistical analysis of the results was carried out using the chi-square test and a Fisher exact test, at the significance level $\alpha = 0.05$. The virulence profiles of the strains tested from both groups were also determined.

The number of bacteria re-isolated from biofilm was averaged separately for both groups of strains and compared with each other and with the reference strain using the analysis of variance ANOVA and the post hoc Bonferroni test, at the significance level $\alpha = 0.05$.

The multiple correlations between antibiotic resistance, virulence genes, and intensity of biofilm formation among clinical strains and milk isolates were tested. Single correlations between antibiotic resistance and prevalence of virulence genes, antibiotic resistance and intensity of biofilm formation, and incidence of virulence genes and the intensity biofilm formation were also evaluated. Correlation coefficients were evaluated according to Guilford's scale.

Results

Of the 380 milk samples tested, 21 (5.5%) were positive for *L. monocytogenes*. Since four isolates were genetically identical, finally 18 unrelated genetically strains were subjected to evaluation of drug susceptibility and the ability to form a biofilm (Fig. 1).

Assessment of the occurrence of virulence genes. The number of virulence genes varied in *L. monocytogenes* strains (Fig. 2). The most prevalent, found in all tested strains, was the *hlyA* gene. The *plcB* gene was

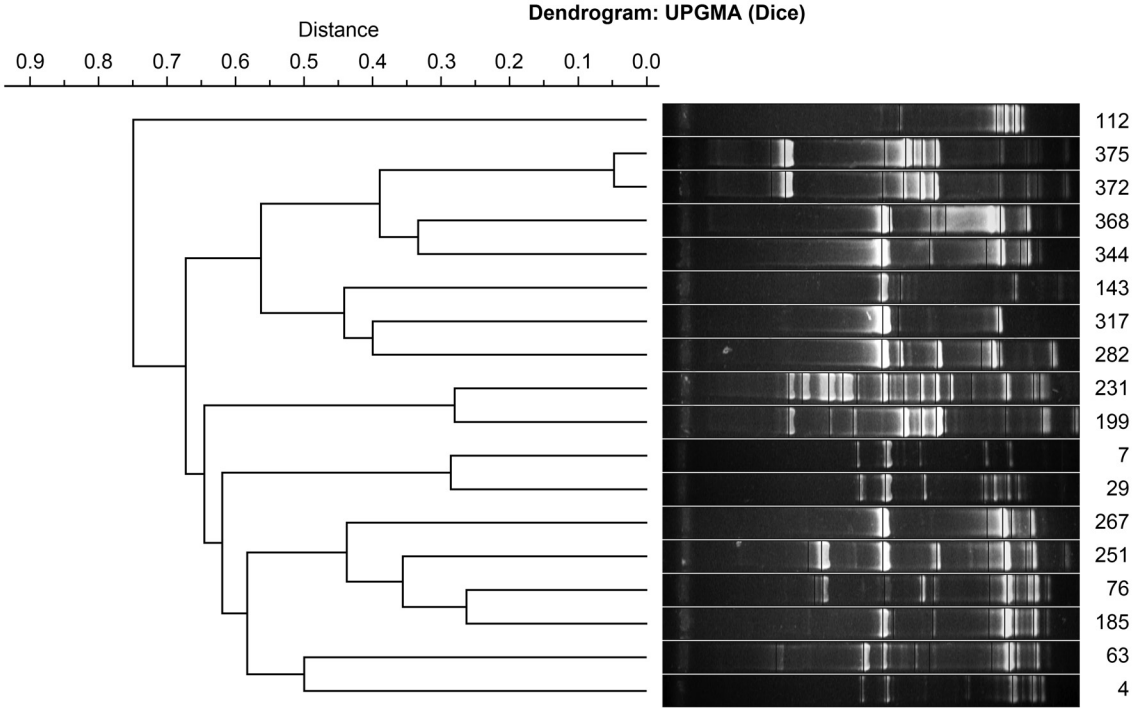

Fig. 1. Genetic similarity of tested *Listeria monocytogenes* strains.

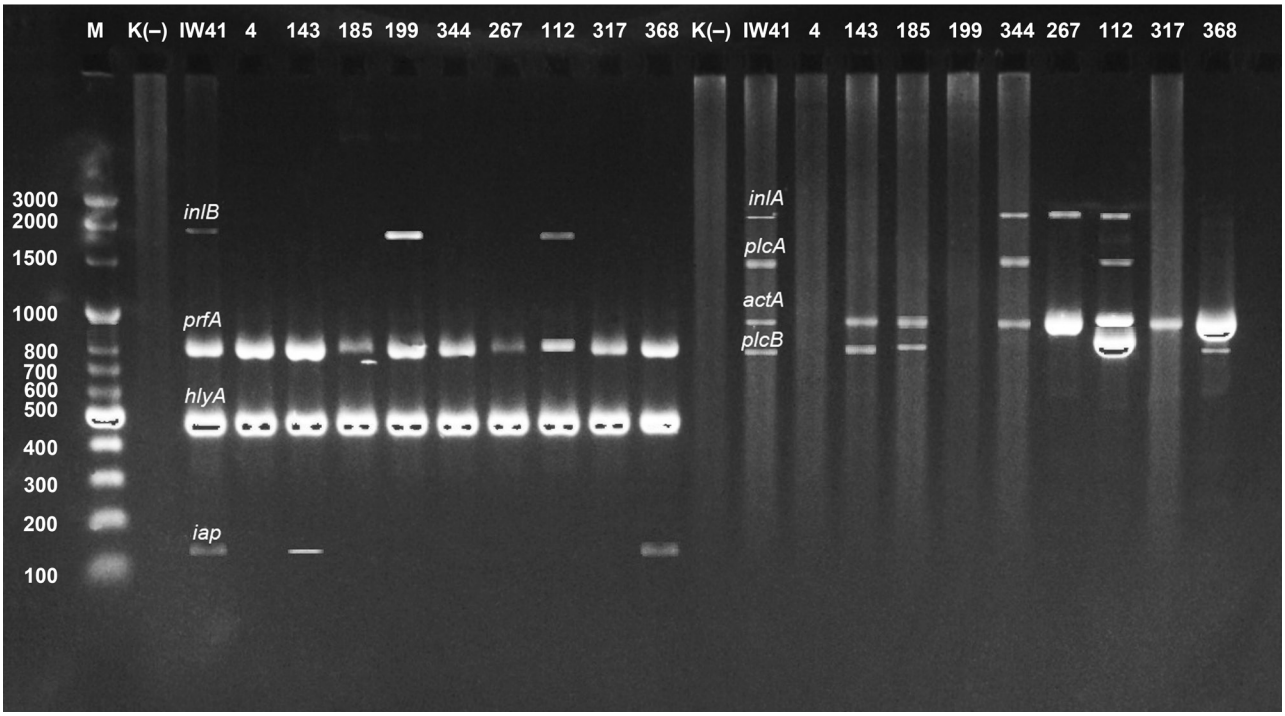

Fig. 2. Electrophoregram presenting the occurrence of virulence genes among the strains tested (M – marker, K+ – positive control (IW41), line 4, 143, 185, 199, 344, 267, 112, 317, 368 – *L. monocytogenes* strains from milk, K (–) – negative control).

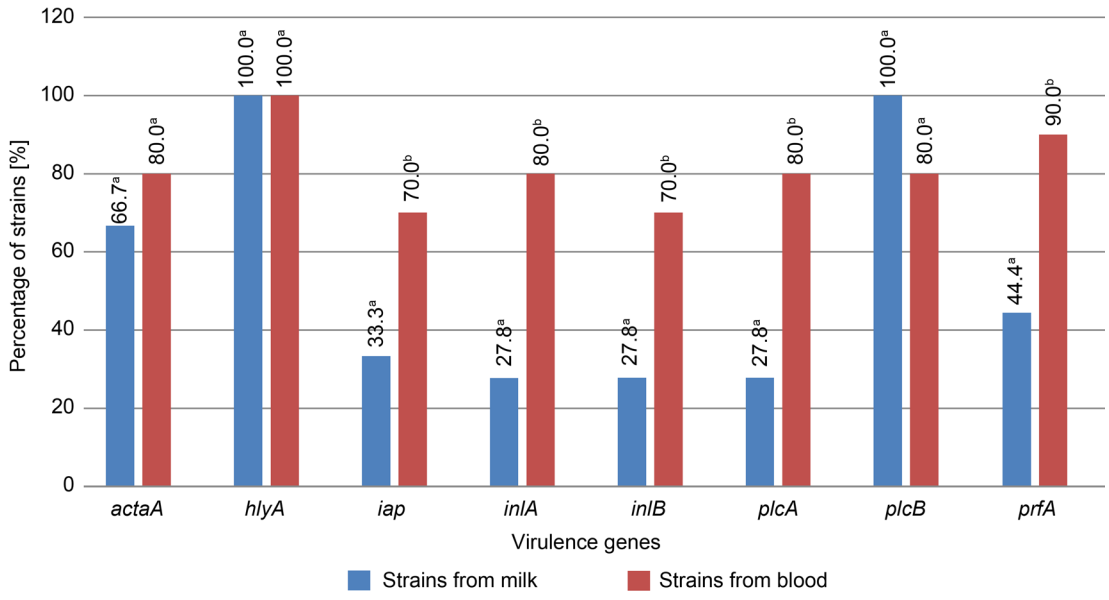

Fig. 3. Prevalence of virulence genes among *L. monocytogenes* strains isolated from milk and blood.

found in all milk isolates, whereas the *prfA* gene was detected in 90.0% of blood strains (Fig. 3). The occurrence of the remaining genes ranged from 27.8% to 80% of the strains tested. The reference strain of *L. monocytogenes* ATCC 19111 had all virulence genes investigated. The occurrence of the genes *iap*, *inlA*, *inlB*, *plcA*, and *prfA* was significantly higher in the strains isolated from the blood than in milk strains ($p \leq 0.05$).

Among *L. monocytogenes* strains isolated from milk, 11 different profiles of virulence genes were detected. None of the profiles was represented by more than 2 strains (Table III). Six (33.3%) of *L. monocytogenes* strains from milk possessed three virulence genes. Two strains with all virulence genes investigated were identified. Among the *L. monocytogenes* isolates from the human blood six gene profiles were identified. The most frequent profile included all virulence genes tested (five strains, 50.0%) (Table III).

Evaluation of drug susceptibility of the strains. It was found that strains derived from milk were the

Table III
The profiles of virulence genes in strains of *L. monocytogenes* isolated from milk and from the blood.

Profile	Virulence genes	Number of strains	The strain identification number
Strains isolated from milk			
I	*hlyA, plcB*	2	4, 63
II	*hlyA, iap, plcB*	2	7, 29
III	*hlyA, inlB, plcB*	2	199, 231
IV	*actA, hlyA, plcB*	2	282, 317
V	*actA, hlyA, plcB, prfA*	2	76, 185
VI	*actA, hlyA, iap, plcB, prfA*	2	143, 368
VII	*actA, hlyA, iap, inlA, inlB, plcA, plcB, prfA*	2	372, 375
VIII	*actA, hlyA, inlA, plcB*	1	267
IX	*actA, hlyA, inlA, plcA, plcB*	1	344
X	*actA, hlyA, plcA, plcB, prfA*	1	251
XI	*actA, hlyA, inlA, inlB, plcA, plcB, prfA*	1	112
Strains isolated from the blood			
I	*actA, hlyA, iap, inlA, inlB, plcA, plcB, prfA*	5	1, 2, 5, 6, 9
II	*actA, hlyA, iap*	1	4
III	*actA, hlyA, iap, prfA*	1	10
IV	*hlyA, inlA, plcA, plcB, prfA*	1	3
V	*hlyA, inlA, inlB, plcA, plcB, prfA*	1	8
VI	*actA, hlyA, inlA, inlB, plcA, plcB, prfA*	1	7

most frequently resistant to penicillin (8; 44.4%) and erythromycin (7; 38.9%). In turn, among strains isolated from blood, four (4, 40.0%) were resistant only to erythromycin, and another two strains (2, 20.0%) were resistant to erythromycin and meropenem (Table IV). Amongst all strains studied eight different profiles of drug susceptibility were found. Profile A was represented by 12 (42.86%) isolates susceptible to all the

Table IV
The profiles of drug resistance/susceptibility of strains of *L. monocytogenes* isolated from milk and the blood.

Profile	Drug resistance/ susceptibility	Number of strains		Total (n = 28)
		Strains from milk (n = 18) (the strain identification number)	Strains from the blood (n = 10) (the strain identification number)	
A	R: – S: P, AM, MEM, E, SXT	8 (44.44%) (7, 29, 63, 199, 231, 282, 317)	4 (40.0%) (3, 4, 8, 10)	12 (42.86%)
B	R: P S: AM, MEM, E, SXT	3 (16.67%) (76, 185, 251)	0 (0.0%)	3 (10.71%)
C	R: E S: P, AM, MEM, SXT	1 (5.56%) (267)	4 (40.0%) (1, 2, 7, 9)	5 (17.86%)
D	R: P, E S: AM, MEM, SXT	1 (5.56%) (143)	0 (0.0%)	1 (3.57%)
E	R: E, SXT S: P, AM, MEM	1 (5.56%) (344)	0 (0.0%)	1 (3.57%)
F	R: P, AM, MEM, SXT S: E	1 (5.56%) (368)	0 (0.0%)	1 (3.57%)
G	R: P, AM, MEM, E, SXT S: – – –	3 (16.67%) (112, 372, 375)	0 (0.0%)	3 (10.71%)
H	R: MEM, E S: P, AM, SXT	0 (0.0%)	2 (20.0%) (5, 6)	2 (7.14%)

R – resistant
S – susceptible
P – penicillin, AM – ampicillin, MEM – meropenem, E – erythromycin, SXT – cotrimoxazole

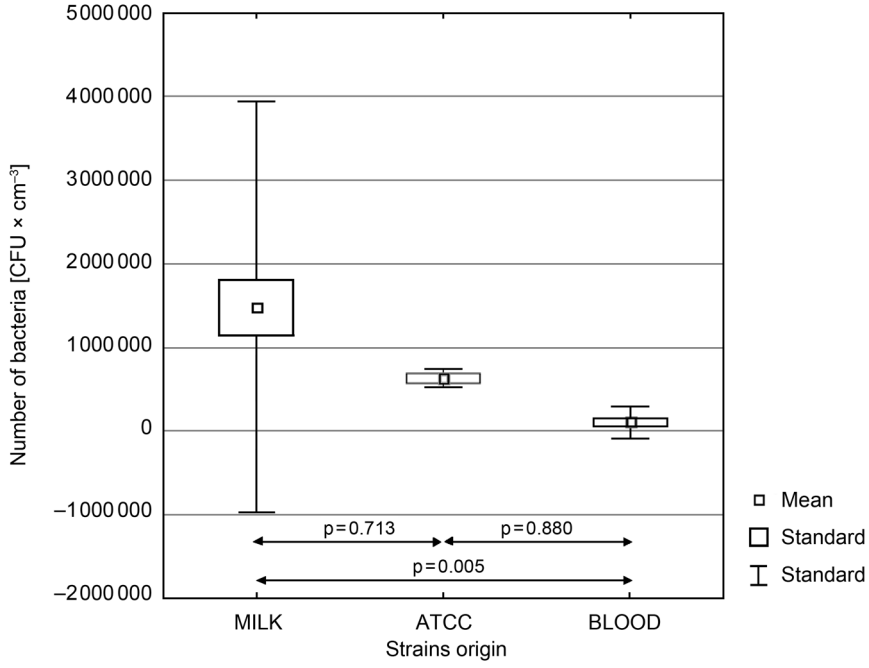

Fig. 4. Differences in biofilm formation among clinical *L. monocytogenes* strains and the strains isolated from milk.

antibiotics tested, eight isolates (44.4%) from milk and four (40.0%) from the blood. We found three (16.7%) strains that were resistant only to penicillin (profile B), and five (17.86%) – resistant only to erythromycin (profile C). At the same time, two (11.1%) strains were resistant to two antibiotics; one of them to penicillin and erythromycin (profile D) and the other to erythromycin and cotrimoxazole (profile E). Further, one strain (5.6%) isolated from milk was susceptible only to erythromycin (profile F), and three (16.7%) strains isolated from milk were resistant to all antibiotics tested (profile G) (Table IV). In turn, among clinical strains of *L. monocytogenes*, the most prevalent was resistance to erythromycin (60.0%). We found also five isolates resistant only to erythromycin (profile C), one of them was acquired from milk and four were isolated from blood. Further, two (20.0%) strains isolated from blood were resistant to meropenem and erythromycin (profile H) (Table IV).

Evaluation of ability to form biofilm by *L. monocytogenes* strains. It was found that isolates derived from milk formed a biofilm with significantly higher efficiency than the clinical isolates from the blood ($p = 0.005$) (Fig. 4). The number of the cells recovered from biofilms formed on fragments of teat cup rubbers ranged from 4.64 to 6.87 log CFU/cm^{-2}, and from 3.61 to 5.70 log CFU/cm^{-2} for the milk strains and the blood strains, respectively (Table V). Seven milk isolates developed more intense biofilms with a higher density of bacterial cell than the reference strain ATCC 19111 (5.80 log CFU/cm^{-2}), whereas all clinical strains were weaker biofilm formers than the reference strain (Fig. 4).

The biofilm formation intensity by *L. monocytogenes* isolates was confirmed under a confocal microscope (Fig. 5).

The correlation between antibiotic resistance, virulence genes, and biofilm formation ability. It was found that the strains with more virulence genes were resistant to a higher number of antibiotics, and their biofilms appeared to be more intense. There was a high positive correlation ($r = 0.956$) and positive correlation ($r = 0.896$) between the efficiency of biofilm formation, the number of virulence genes and the antibiotic resistance level of isolates derived from milk (Fig. 6) as well as clinical strains (Fig. 7), respectively. For the clinical isolates, only the correlation between the number of virulence genes and the efficiency of biofilm formation was not significant.

The spread of *L. monocytogenes* through contaminated teat rubbers. *Transmission of L. monocytogenes on the skin of the udder and into the milk.* Bacteria were transferred to skin pieces by the contact with the contaminated teat rubber (Table VI). The number of bacterial cells from the strains isolated from cow's milk that was detected in eight subsequent fragments of the udder skin in the number ranged from 1.08 log CFU/cm^{-2} (M231) to 6.87 log CFU/cm^{-2} (M372) (Table VI). Only the M63 strain was not isolated from the 8th fragment and its density was 1.59 log CFU/cm^{-2} on the 7th fragment (Table VI). Clinical strains (B5 and B6) that developed strong biofilms were isolated

Table V
The intensity of biofilm formation by *L. monocytogenes* strains derived from cow's milk and the blood.

Strains origin	The strain identification number	Mean number of bacteria [log CFU/cm^{-2}]	STD
Milk	372	6.87	± *6.12**
Milk	375	6.79	± *6.09*
Milk	112	6.71	± *6.13*
Milk	368	6.64	± *6.17*
Milk	344	6.05	± *5.21*
Milk	143	5.95	± *4.99*
Milk	267	5.81	± *5.07*
Milk	251	5.18	± *4.80*
Milk	185	4.99	± *4.06*
Milk	7m	4.94	± *4.00*
Milk	199	4.94	± *3.76*
Milk	317	4.89	± *3.78*
Milk	4m	4.85	± *3.67*
Milk	282	4.81	± *3.81*
Milk	76	4.77	± *3.65*
Milk	29	4.76	± *3.74*
Milk	231	4.74	± *3.96*
Milk	63	4.64	± *3.72*
ATCC	19111	5.80	± *5.04*
Blood	5	5.70	± *4.87*
Blood	6	5.67	± *4.76*
Blood	3	4.18	± *3.83*
Blood	1	4.01	± *2.87*
Blood	8	4.00	± *3.27*
Blood	2	3.97	± *3.61*
Blood	9	3.92	± *3.83*
Blood	7	3.72	± *3.63*
Blood	10	3.71	± *3.72*
Blood	4	3.61	± *3.71*

* – Standard deviation

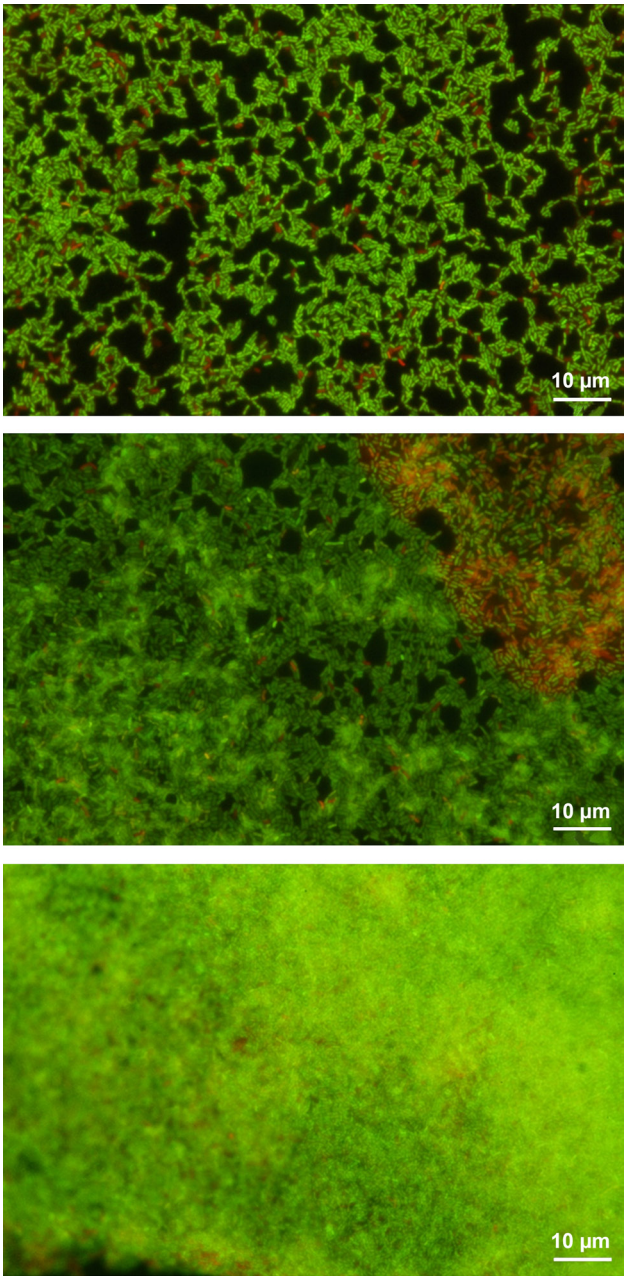

Fig. 5. The intensity of biofilm formation by the selected *L. monocytogenes* strains.
A – weak biofilm, B – moderate biofilm, C – strong biofilm, green – live cells, red – death cells.

from seven consecutive fragments of the skin and the number of bacterial cells was 1.26 log CFU/cm^{-2} for the strain B6 and 1.43 log CFU/cm^{-2} for the strain B5. The clinical isolates that developed weak biofilms were detected in four consecutive fragments and the number of bacterial cells was 1.20 log CFU/cm^{-2} for the strain B4, and 1.49 log CFU/cm^{-2} for the strain B10 (Table VI). Strain ATCC 19111 was transferred from the teat rubber to seven consecutive fragments of the skin and the number of cells on the last one was 1.53 log CFU/cm^{-2} (Table VI). It was also shown that the contamination level of the first skin fragment was higher for the strains that formed a weaker biofilm (Table VI).

The obtained results showed that even if *L. monocytogenes* was not detected on the fragment of the udder skin (the number of bacteria was below the detection threshold), the bacteria were transferred from the teat rubber and their number increased to 1.38–2.92 log CFU/cm^{-2} along with incubation time (Table VI).

All *L. monocytogenes* strains that developed biofilms on teat rubbers also caused contamination of milk flowing through these rubbers. The number of bacteria recovered from milk ranged from 2.71 to 3.37 log CFU/ml for milk strains, and from 2.57 to 2.94 log CFU/ml for clinical strains (Table VII). The reference strain was grown in milk to the density of 2.97 log CFU/ml (Table VII).

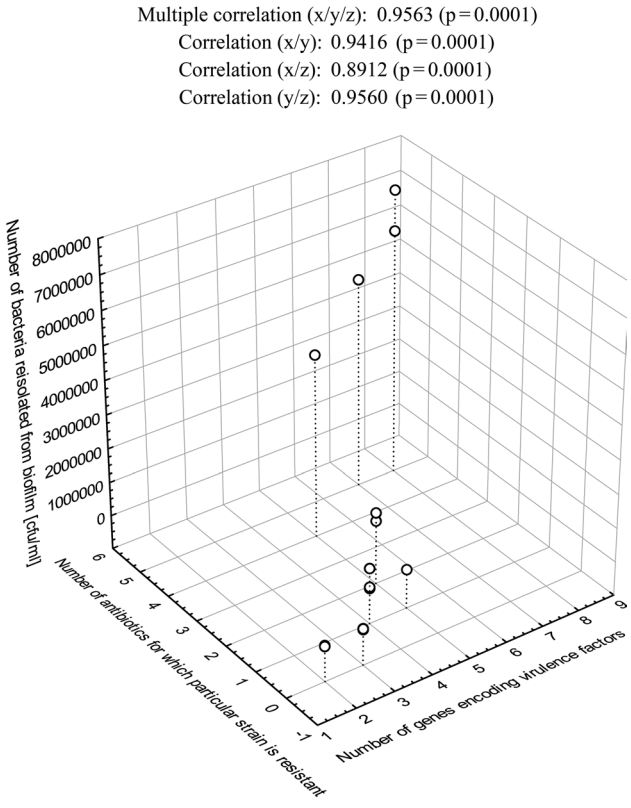

Fig. 6. Multiple correlations between the intensity of biofilm formation, the number of virulence genes, and the drug resistance of *L. monocytogenes* strains isolated from milk.

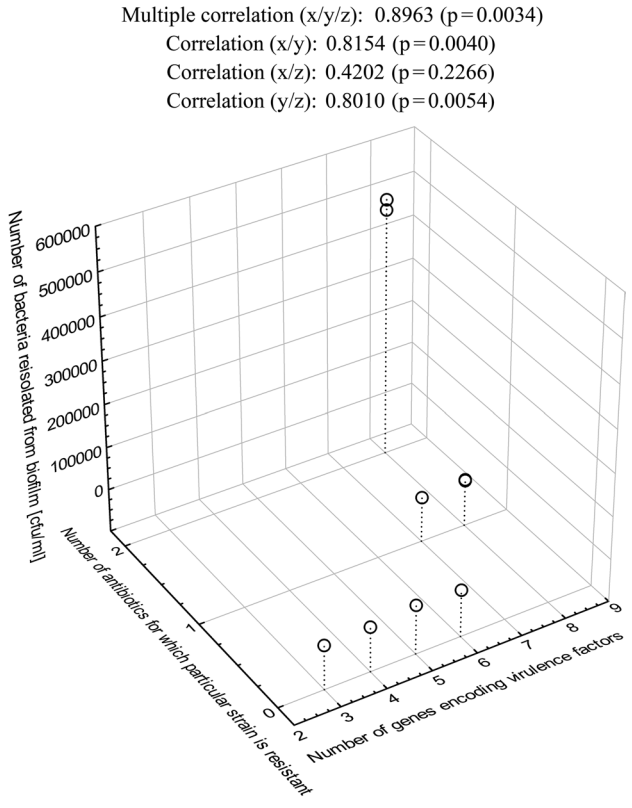

Fig. 7. Multiple correlations between the intensity of biofilm formation, the number of virulence genes, and the drug resistance of the clinical *L. monocytogenes* strains.

Table VI
Transmission of *L. monocytogenes* from a biofilm on silicone teat cups to the cow's udder skin.

Strain	Number of re-isolated *L. monocytogenes* strains [log CFU/cm^2]								
	Silicone teat cup	Udder skin							
		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Regrowth on first negative or last positive sample (12 h/25°C)
M372	6.87* ± *6.12***	4.33 ± *3.98*	3.88 ± *3.77*	3.71 ± *3.34*	3.45 ± *2.88*	2.92 ± *2.71*	2.66 ± *2.35*	2.09 ± *2.31*	1.85 ± *1.84*	2.92 ± *2.74*
M375	6.79 ± *6.09*	4.54 ± *4.37*	3.99 ± *3.82*	3.65 ± *3.63*	3.10 ± *3.45*	2.83 ± *2.69*	2.51 ± *1.96*	1.92 ± *1.82*	1.61 ± *1.31*	2.79 ± *2.62*
M231	4.74 ± *3.96*	3.87 ± *3.72*	3.76 ± *3.79*	3.47 ± *3.55*	2.79 ± *2.71*	2.52 ± *2.33*	1.93 ± *1.20*	1.67 ± *1.47*	1.08 ± *1.54*	1.99 ± *1.85*
M63	4.64 ± *3.72*	3.96 ± *3.82*	3.80 ± *3.33*	3.34 ± *3.62*	2.70 ± *2.58*	2.33 ± *2.32*	1.86 ± *1.78*	1.59 ± *1.48*	n.d.	1.87 ± *1.51*
ATCC 19111	5.80 ± *5.04*	3.64 ± *3.67*	3.02 ± *3.51*	2.79 ± *2.39*	2.62 ± *2.44*	2.14 ± *2.48*	1.79 ± *1.51*	1.53 ± *1.63*	n.d.	2.01 ± *2.31*
B5	5.70 ± *4.87*	3.56 ± *3.71*	2.85 ± *2.70*	2.63 ± *2.20*	2.48 ± *2.23*	1.96 ± *1.86*	1.76 ± *1.61*	1.43 ± *1.01*	n.d.	1.81 ± *1.20*
B6	5.67 ± *4.76*	3.47 ± *3.17*	2.91 ± *2.76*	2.76 ± *1.99*	2.39 ± *2.61*	1.92 ± *1.65*	1.64 ± *1.71*	1.26 ± *1.33*	n.d.	1.72 ± *1.80*
B10	3.71 ± *3.72*	2.85 ± *2.58*	2.57 ± *2.20*	1.88 ± *1.57*	1.49 ± *1.04*	n.d.***	n.d.	n.d.	n.d.	1.63 ± *1.41*
B4	3.61 ± *3.71*	2.92 ± *2.88*	2.73 ± *2.53*	1.84 ± *161*	1.20 ± *1.35*	n.d.	n.d.	n.d.	n.d.	1.38 ± *1.50*

* – Mean, ** – Standard deviation, *** – Not detected

Table VII
Transmission of *L. monocytogenes* from a biofilm on silicone teat cups to milk.

Strain	Number of re-isolated *L. monocytogenes*	
	Silicone teat cup [log CFU/cm^2]	Milk [log CFU/ml]
M372	6.87* ± *6.12***	3.37 ± *3.03*
M375	6.79 ± *6.09*	3.29 ± *3.37*
M231	4.74 ± *3.96*	2.75 ± *2.62*
M63	4.64 ± *3.72*	2.71 ± *2.52*
ATCC 19111	5.80 ± *5.04*	2.97 ± *2.85*
B5	5.70 ± *4.87*	2.94 ± *2.74*
B6	5.67 ± *4.76*	2.84 ± *2.32*
B10	3.71 ± *3.72*	2.63 ± *2.51*
B4	3.61 ± *3.71*	2.57 ± *2.32*

* – Mean, ** – Standard deviation

Transmission of L. monocytogenes in udder – teat rubber – udder model. It was shown that *L. monocytogenes* found on the udder skin may be transferred to sterile teat rubber and then can cause the contamination of sterile udder skin (Table VIII). For all strains tested, the number of bacteria on the skin ranged from 5.13 to 5.91 log CFU/cm^{-2}, irrespective of their origin (milk vs. human) (Table VIII). Bacteria from the skin fragments were transferred to the teat rubber and the number of the bacteria reisolated ranged from 3.58 to 3.81 log CFU/cm^{-2}. In turn, the contact of the teat rubber with sic consecutive sterile pieces of udder skin resulted in the transmission of *L. monocytogenes* to from four (for strains No M63, B4, and B10) to six (for strains M372, M231, ATCC 19111, and B6) skin fragments (Table VIII). The number of bacteria isolated from the last skin fragment examined was from 1.11 to 1.92 log CFU/cm^{-2} (Table VIII). This may suggest the possibility of more than six consecutive udders contamination.

To confirm *L. monocytogenes* proliferation on skin fragments, the fragments were incubated 12 hours at 25°C. It appeared that bacteria were detected in both cases. Moreover, the increase in their numbers on the last tested fragments was observed (Table VIII).

Discussion

Since *L. monocytogenes* is widespread in the environment and survives in harsh environmental conditions, it may easily contaminate food (Wałecka 2011).

Table VIII
Listeria spp. transmission system: udder skin – silicone teat cup – udder skin.

Strain	Number of re-isolated *L. monocytogenes* [log CFU/cm^2]								
	Silicone teat cup	Udder skin	Udder skin						
			Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Recovery of bacteria (12 h/25°C)
M372	5.91* ± *5.81***	3.81 ± *3.61*	3.77 ± *3.73*	3,. 0 ± *2.85*	3.00 ± *2.85*	2.81 ± *2.65*	2.81 ± *2.65*	1.92 ± *1.82*	2.83 ± *2.53*
M375	5.84 ± *5.63*	3.68 ± *3.45*	3.61 ± *3.83*	2.68 ± *2.50*	2.68 ± *2.50*	1.87 ± *1.93*	1.87 ± *1.93*	n.d.***	1.66 ± *1.76*
M231	5.57 ± *5.60*	3.71 ± *3.23*	3.66 ± *3.54*	2.68 ± *2.72*	2.68 ± *2.72*	2.00 ± *1.90*	2.00 ± *1.90*	1.11 ± *1.16*	2.37 ± *2.48*
M63	5.52 ± *5.34*	3.54 *3.63*	3.47 ± *3.55*	1.47 ± *1.66*	1.47 ± *1.66*	1.00 ± *1.69*	1.00 ± *1.69*	n.d	1.26 ± *1.03*
ATCC 19111	5.66 ± *5.54*	3.76 ± *3.57*	3.72 ± *3.27*	2.74 ± *1.99*	2.74 ± *1.99*	2.26 ± *1.98*	2.26 ± *1.98*	1.64 ± *1.47*	2.57 ± *2.32*
B5	5.71 ± *5.61*	3.62 ± *3.20*	3.56 ± *3.47*	2.57 ± *2.42*	2.57 ± *2.42*	1.84 ± *1.03*	1.84 ± *1.03*	n.d.	1.46 ± *1.58*
B6	5.69 ± *5.45*	3.79 ± *3.86*	3.73 ± *3.70*	2.91 ± *2.78*	2.91 ± *2.78*	2.72 ± *2.64*	2.72 ± *2.64*	1.81 *1.71*	2.72 ± *2.63*
B10	5.32 ± *5.68*	3.59 ± *3.02*	3.53 ± *3.37*	1.62 ± *1.48*	1.62 ± *1.48*	1.11 ± *1.46*	1.11 ± *1.46*	n.d.	1.20 ± *1.68*
B4	5.13 ± *5.40*	3.58 ± *3.50*	3.51 ± *3.58*	1.52 ± *1.61*	1.52 ± *1.61*	0.90 ± *0.86*	0.90 ± *0.86*	n.d.	1.08 ± *1.71*

* – Mean, ** – Standard deviation, *** – Not detected

L. monocytogenes is isolated from both unpasteurized and pasteurized cow's milk, dairy products such as soft cheeses, and dairy farms (Fleming et al. 1985; Van Kessel et al. 2004; CDC 2007; Fox et al. 2009; Lomanco et al. 2009; Balandyte et al. 2011; Van Kessel et al. 2011). In recent years, such products have been linked to several outbreaks of listeriosis (Rocha et al. 2013; CDC 2014; CDC 2016). Although milk products have repeatedly proved to be the source of this pathogen, the characteristics of *L. monocytogenes* strains isolated from unpasteurized milk and dairy farms remain unclear.

In the present study, *L. monocytogenes* was isolated from 21 (5.5%) out of 380 milk samples. Similar results were obtained in the Czech Republic (3.2%) (Gelbíčová and Karpíšková 2012a), Ethiopia (4.0%) (Garedew et al. 2015), England, Wales, India (5.1%) (Siegman-Igra et al. 2002; Kalorey et al. 2008), Iran (5.4%) (Jamali and Radmehr 2013), and Spain (6.5%) (Vilar et al. 2007). In contrast, *L. monocytogenes* strains were isolated only three times out of 294 milk samples in Sweden (Waak et al. 2002). In another study carried out in India, the occurrence of *L. monocytogenes* in fresh milk was considerably higher and accounted for 21.73% (Sharma et al. 2012).

Our studies showed the presence of the *hlyA* gene in all strains tested, regardless of their origin (milk vs. human). This is in accordance with the study of Indrawattana et al. (2011) and Aurora et al. (2008). In the present study, we found the *plcB* gene in 100% of the strains isolated from milk and in 80% of clinical isolates. This gene was detected in all strains of *L. monocytogenes* in the studies of Gelbíčová and Karpíšková (2012b), Mureddu et al. (2014), and Wieczorek and Osek (2017). Strains without the *plcB* gene divide but have reduced the ability to escape from phagosome (Bielecki 1994). We showed the presence of the *iap* gene in six (33.3%) strains cultured from milk and seven (70.0%) isolates from blood. In turn, Al-Nabulsi et al. (2015) confirmed the presence of the *iap* gene only among 16.6% of *L. monocytogenes* strains isolated from processed meat whereas Mureddu et al. (2014) demonstrated its presence in 97.1% strains of this species. Spontaneous mutants with reduced secretion of p60 protein do not lose their ability to grow intracellularly and the mutation is easily reversible. In contrast, insertional mutations in the region of the *iap* gene significantly reduce the hemolytic activity of *L. monocytogenes* (Rocourt et al. 2000).

To date, many studies evaluating the drug susceptibility of *L. monocytogenes* have been conducted (Morvan et al. 2010; Rahimi et al. 2010; Dalzini et al. 2016). Since the isolation of the first drug-resistant *L. monocytogenes* strains a systematic growth in resistance of these bacteria to antimicrobial agents has been observed (Poyart-Salmeron et al. 1990; Srinivasan et al. 2005; Morvan et al. 2010; Pesavento et al. 2010; Jamali et al. 2013). Therefore, it is important to monitor drug resistance of this pathogen. In the present study, eight (44.4%) isolates from milk and four (40.0%) isolates from blood were susceptible to all the antibiotics tested. On the contrary, most (98.2%) of *Listeria* spp. strains isolated from cow's milk and products thereof showed resistance to at least one antibiotic in Iran (Rahimi et al. 2010). In our study, the greatest number of *L. monocytogenes* strains from milk was resistant to penicillin (44.4%), followed by erythromycin (33.3%), cotrimoxazole (27.7%), and ampicillin (22.2%). Similar results were obtained by Srinivasan et al. (2005) and Pesavento et al. (2010). A much higher percentage of the resistant strains was reported by Jamali et al. (2013), who isolated *L. monocytogenes* strains from the milk of cows without signs of *mastitis* and from milk of cows with the clinical form of *mastitis*. In our study, four (40.0%) strains isolated from humans were resistant only to erythromycin, whereas two (20.0%) strains were resistant to meropenem and erythromycin. These results are similar to those obtained in other countries (Marco et al. 2000; Vitas et al. 2007; Prieto et al. 2016). In this study, we also found that almost half of the strains examined (eight, 44.4%) isolated from milk are resistant to penicillin, while no penicillin-resistant *L. monocytogenes* isolated from blood samples was observed. It may be due to the recommended empiric use of penicillins in the treatment of contagious disease, including mastitis, in ruminants (EVIRA 2018).

We found also three (16.7%) strains isolated from milk resistant to all of the antibiotics tested. The strain is described as MDR if is resistant to at least three various groups of antibiotic. According to this definition, we found four (22.2%) MDR strains among strains isolated from milk, and none MDR strain was isolated from blood samples. Similar results were shown by Garedew et al. (2015) and Pesavento et al. (2010), who isolated four (16.7%) and 11 (27.5%) multidrug-resistant strains from food, respectively. In contrast, in the study by Jamali et al. (2013) as much as 71.4% of multidrug-resistant strains of *L. monocytogenes* from milk were detected. Charpentier and Courvalin (1999) have shown that the extensive use of antimicrobials in animal production, as well as clinical treatment of animals and humans, contributed to the continuous evolution of antimicrobial-resistant (AMR) bacteria with a diverse pool of genetically-transferred resistance determinants. Thereby, food of animal origin may be a source of AMR *L. monocytogenes* strains. In the food processing environment, *L. monocytogenes* may face many adverse conditions such as heat, high pressure, irradiation, acids, salts, and oxidants which induce the cross-protection against the same or other types of stresses (Wesche et al. 2009). It was shown, that the exposure of *L. monocytogenes* isolates derived from

food to variable pH, cold, heat and salt stress, disinfectants, and low water activity increased their resistance to various antibiotics (Beuls et al. 2012; Al-Nabulsi et al. 2015; Faezi-Ghasemi and Kazemi 2015). Clinical strains usually are not subjected to such stresses. This may explain the difference in AMR between clinical and food isolates.

In Poland, among 471 *L. monocytogenes* strains isolated from different foods and food-related sources from 2004 to 2010, no resistance to ampicillin, amoxicillin, chloramphenicol, erythromycin, gentamicin, rifampicin, sulfamethoxazole, trimethoprim, and vancomycin was reported (Korsak et al. 2012). On the contrary, a Lebanese study assessing AMR in *L. monocytogenes* recovered from traditional dairy products showed that all isolates (n = 30) were resistant to at least one antimicrobial, including the resistance to ampicillin (60%), penicillin (90%), erythromycin (27%), gentamicin (7%), and SXT (17%) (Harakeh et al. 2009).

Monitoring of the occurrence of *L. monocytogenes* in food is a great challenge for food processing plants. The ability of these microorganisms to survive in a moist, cool environment and to form biofilm makes them difficult to eradicate. *L. monocytogenes* presence in milk and milk products may be caused by improper sanitization of surroundings and equipment used during milking, as well as ineffective disinfection in dairies (Tompkin et al. 2002). The present study evaluated the ability of the strains tested to form biofilm on silicone teat cups rubbers. Among *L. monocytogenes* strains derived from milk, seven isolates developed more intense biofilms than the reference ATCC® 19111™ strain. All clinical strains formed biofilm less intense than milk isolates ($p = 0.005$). Latorre et al. (2010) isolated a considerable number of bacteria *L. monocytogenes* from milking machines. Using SEM micrography, they found that the bacteria were particularly visible in scratches on the inside plastic surface of the teat cups. In the study by Doijad et al. (2015), only nine out of 98 strains isolated from different environments formed robust biofilms. It is worthy to note that all strains that developed intense biofilms were isolated from milk and dairy products. In previous studies, it has been proved that *L. monocytogenes* can form biofilm on many surfaces such as polystyrene, polypropylene, glass, stainless steel, quartz, marble, and granite (Silva et al. 2008). Though, studies by Djordjevic et al. (2002) and Harvey et al. (2007) indicated that *L. monocytogenes* formed only weak or moderate biofilm on various surfaces. In turn, the study by Sinde et al. (2000) showed better adherence of *L. monocytogenes* to rubber when compared to stainless steel.

Bacteria within biofilms are much more resistant to antimicrobial agents than floating counterparts (Gong et al. 2013). To understand the relationship between biofilms and antimicrobial resistance in *L. monocytogenes*, we assessed biofilm formation ability of *L. monocytogenes* isolates together with their antimicrobial resistance. Our results showed very high positive correlation (r = 0.96) for isolates from milk and high positive correlation (r = 0.80) for clinical isolates. Such a relationship between biofilm production and antibiotic resistance was demonstrated for *Salmonella* Pullorum and uropathogenic *E. coli* (Adetunji et al. 2008; Gong et al. 2013). Adetunji et al. (2008) showed that strains of *L. monocytogenes* producing intense biofilms were more virulent and drug-resistant. Intrinsic resistance of biofilm bacteria is related to the presence of persister cells or efflux pumps that remove antibiotics from the biofilm environment (Korsak et al. 2005; Sauvage et al. 2008). Bacterial cells from deeper layers of biofilm are protected against antimicrobials by the upper layers. The penicillin resistance among the strong-biofilm forming *L. monocytogenes* strains may be correlated with the presence of PBP5 that binds penicillin G. This protein is a DD-carboxypeptidase related with the membrane fraction and lateral wall growth of *L. monocytogenes* cell (Korsak et al. 2005; Sauvage et al. 2008).

Our study also revealed a positive correlation between the number of virulence genes, drug resistance, and biofilm formation ability. This is in agreement with the study by Soni et al. (2013), which indicated that multi-drug resistant *L. monocytogenes* strains derived from clinical specimens, water, and milk possess a large number of virulence genes (*inlA*, *inlC*, *plcA*, *prfA*, *actA*, *hlyA,* and *iap*).

Our study showed that teat cups, contaminated during milking might contribute to the transmission of *L. monocytogenes* to cow's udders and finally to the milk. It was found that this phenomenon might affect at least several successively milked animals. This supports the study of Benić et al. (2012), which demonstrated that bacteria might be transmitted by utensils used during milking, mainly by the teat cups. It was also confirmed by Azevedo et al. (2016) studies, which found that improper hygiene of teat cups could cause transmission of *Staphylococcus* spp. both between the animals and to the milk tank.

A better understanding of the epidemiology of *L. monocytogenes* infections and the factors affecting their survival, spread and resistance is necessary to prevent contamination in the food industry and transmission of the pathogen. This may help in limiting listeriosis incidence in humans.

Conclusions

In the available literature, there are few data on the bacterial transmission *via* teat cups and the risks posed by this equipment. This study showed that

L. monocytogenes isolates originating from cow's milk are more resistant to antibiotics than the clinical strains. The most frequent virulence genes detected were *hlyA* and *plcB* among milk strains and *hlyA* and *prfA* in strains derived from the blood. The intensity of the biofilm formation was strain-dependent and was significantly higher in the milk strains. The association between biofilm formation the number of virulence genes, and antimicrobial resistance of *L. monocytogenes* strains was high positive for the isolates from milk (r = 0.96) as well as for the clinical isolates (r = 0.90). Our study showed also that teat cups, contaminated during the milking process, might play an important role in the transmission of *L. monocytogenes* to the milk, posing risk to the consumer health. For these reasons, it is reasonable to monitor incidence, susceptibility and biofilm formation by *L. monocytogenes* during milking and milk processing (especially unpasteurized milk).

Author's contributions

Krzysztof Skowron – development of the concept of experiments and methodology of research, collecting the materials for research, participation in the performance of the laboratory part of experiments, collection, and analysis of results, participation in the writing of the manuscript, management of the research team and preparing the publication

Ewa Wałecka-Zacharska – development of the concept of experiments and methodology of research, translation of the manuscript text

Katarzyna Grudlewska – participation in the performance of the laboratory part of experiments and writing of the manuscript

Natalia Wiktorczyk – participation in the performance of the laboratory part of experiments and the collection of materials for research and references

Agnieszka Kaczmarek – participation in the performance of the laboratory part of experiments and the collection of references

Grzegorz Gryń – the collection and analysis of results

Joanna Kwiecińska-Piróg – participation in the performance of the laboratory part of experiments, collection of materials for research, adaptation of the manuscript to the editorial requirements

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All authors contributed to the draft of the manuscript and discussed results. All authors gave final approval for publication.

ORCID

Krzysztof Skowron 0000-0003-0868-864X

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Conflict of interest

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ORIGINAL PAPER

Molecular Diversity of *Staphylococcus aureus* Colonizing the Upper Respiratory Tract of Residents and Staff in a Nursing Home

MARTYNA KASELA*, AGNIESZKA GRZEGORCZYK and ANNA MALM

Chair and Department of Pharmaceutical Microbiology with Laboratory for Microbiological Diagnostics, Medical University of Lublin, Lublin, Poland

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Abstract

Elderly people living in nursing homes are a high-risk population for *Staphylococcus aureus* infection. Multiple comorbidities, a weakened immune system, inadequate hygienic conditions, and crowding might increase the prevalence rates of this opportunistic pathogen. However, the epidemiological aspects, genetic diversity, and transmission of *S. aureus* in nursing homes are still poorly understood, especially in Poland. This study aimed to determine the genetic relatedness and prevalence of colonization of *S. aureus* isolated from the anterior nares and the throat of residents and staff in a nursing home located in Lublin, Poland. The study showed a high *S. aureus* prevalence rate among participants (46.1%), yet there was a low frequency of MRSA strains among residents (1.7%) and staff (0%). The multiple-locus variable-number tandem-repeat fingerprinting (MLVF) analysis demonstrated a high degree of genetic diversity of *S. aureus* strains colonizing the anterior nares and the throat of the participants. The occurrence of simultaneous colonization with more than one unique *S. aureus* strain in any one individual as well as the incidence of colonization with the same genetic variant of *S. aureus* in different individuals was observed. These findings suggest that inter-participant *S. aureus* transmission might contribute to the development of cross-infections.

Key words: *Staphylococcus aureus*, colonization, elderly, genotyping, nursing home

Introduction

Staphylococcus aureus is an opportunistic human pathogen known for its increasing prevalence in both hospitals and the general community. An incremental rise in drug-resistance and high disease-causing capabilities has also been observed (Zhang et al. 2015; Zurita et al. 2016). The most common anatomical location for the commensal carriage of *S. aureus* is the anterior nares (Kluytmans et al. 1997; Pollitt et al. 2018), although recent epidemiological studies highlight the role of pharyngeal carriage in *S. aureus* transmission (Nilsson and Ripa 2006; Hamdan-Partida et al. 2010). The complexity of host-colonizer interactions has led researchers to investigate alternative contexts in pursuit of the mechanism for colonization. Long term care facilities (e.g. nursing homes) constitute communities in which crowding and inadequate hygienic conditions might contribute to higher MSSA (methicillin-sensitive *S. aureus*) and MRSA (methicillin-resistant *S. aureus*) prevalence rates. Chronic diseases, immune system disorders, and frequent hospitalization make elderly people vulnerable to *S. aureus* infection (Ledell et al. 2003; Chuang et al. 2015; Peters et al. 2017).

S. aureus is also one of the most genetically diverse bacteria and the mechanisms by which the predominant strains are selected within each biological niche depends on the geographic location as well as the studied human host (Zurita et al. 2016). MSSA strains are more prevalent than MRSA strains and have a higher genetic diversity, whereas MRSA outbreaks are usually caused by only a few different MRSA clones (Goering et al. 2008). Therefore, analyzing the genetic relatedness of colonizing MSSA strains in nursing homes might result in a better understanding of *S. aureus* epidemiology.

We aimed at determining the genetic diversity and relatedness of MSSA and MRSA strains colonizing the upper respiratory tract of nursing home residents and staff using the MLVF (multiple-locus variable-number tandem-repeat fingerprinting) method. We hypothesized that there would be the same genetic MLVF

* Corresponding author: M. Kasela, Chair and Department of Pharmaceutical Microbiology with Laboratory for Microbiological Diagnostics, Medical University of Lublin, Lublin, Poland; **e-mail: kasela.martyna@gmail.com**

banding patterns of *S. aureus* retrieved from different individuals, and the presence of genetic homogeneity might indicate that bacterial transmission has occurred.

Experimental

Materials and Methods

Bacterial strains. Among 102 participants enrolled in our study, more than three-quarters were female and the mean age of the residents (81.5 years old) was almost twice as high as that of the staff members (43 years old) (Table I). Two anatomical locations (the anterior nares and the throat) were swabbed using sterile cotton swabs (Medlab, Poland) premoistened with sterile 0.9% saline solution (POCH, Poland). The samples were immediately transported to the laboratory and processed within two hours. The swabs were inoculated on 5% sheep blood agar plates (BioRad, USA) and mannitol salt agar plates (BioMaxima, Poland) at 35.5°C for 24 h. The β-hemolytic or/and mannitol-positive colonies were tested for the presence of coagulase (Biomed, Poland) and finally identified as *S. aureus* using the Vitek 2 Compact automated system and GP cards (Biomerieux, France).

Table I
Basic demographic characteristics of participants and the prevalence of *S. aureus* colonization.

	Total (n = 102)	Residents (n = 60)	Staff (n = 42)
Age (years)			
Median (range)	68 (26–97)	81.5 (56–97)	43 (26–67)
Gender n (%)			
Female	78 (76.5)	43 (71.7)	35 (83.3)
Male	24 (23.5)	17 (29.3)	7 (16.7)
S. aureus colonization n (%)	47 (46.1)	28 (46.7)	19 (45.2)
Nares	17 (36.2)	10 (35.7)	7 (36.8)
Throat	11 (23.4)	6 (21.4)	5 (26.4)
Nares and throat	19 (40.4)	12 (42.9)	7 (36.8)

Antimicrobial susceptibility testing was conducted using the Vitek 2 automated system and AST-P644 cards (BioMerieux, France) containing the following antibiotics: ceftaroline, ciprofloxacin, clindamycin, daptomycin, erythromycin, gentamicin, levofloxacin, linezolid, oxacillin, rifampicin, teicoplanin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole, and vancomycin. Additionally, resistance to cefoxitin (30 µg), tobramycin (10 µg), fusidic acid (10 µg) and mupirocin (200 µg) was determined using disc diffusion method. D-test was conducted to determine the mechanism of macrolide, lincosamide and streptogramin resistance (MLS_B). All discs were obtained from the same manufacturer (Becton Dickinson, USA). Disc-diffusion tests were performed and interpreted according to EUCAST recommendations (EUCAST 2018). All *S. aureus* isolates have been stored in freezers (–70°C) for further analysis using a medium of tryptic soy broth (TSB; BTL, Poland) and glycerol (POCH, Poland) in equal proportions (1:1).

Multiple-locus variable-number tandem-repeat fingerprinting (MLVF). The MLVF genotyping was performed to analyze the genetic diversity and relatedness of all 66 identified *S. aureus* strains. The MLVF method involved a multiplex-PCR reaction targeting five variable number of tandem repeats (VNTRs) in the following loci: *sspA*, *spa*, *sdr*, *clfA*, and *clfB*. These five VNTRs are located within the genes that encode the following proteins: serine proteinase V8, protein A, sdrC, sdrD, sdrE fibrinogen binding proteins, clumping factor A, and clumping factor B (Sabat et al. 2003).

Genomic DNA was extracted from the 24-h TSB culture prepared from a single bacterial colony. DNA extraction and purification were conducted according to the manufacturer's instruction (Genomic Mini, A&A Biotechnology, Poland), with the minor alteration of replacing lysozyme with an equal volume of lysostaphin (A&A Biotechnology, Poland). The quality and quantity of the DNA were assessed using BioPhotometer (Eppendorf, Germany) by measuring the absorbance at the wavelengths of 260 and 280 nm.

The PCR program consisted of predenaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min (Biometra, Germany) (Grzegorczyk and Malm 2014). The PCR products and DNA molecular size marker (100 bp DNA Ladder Plus, ThermoScientific, USA) were resolved in 2% agarose electrophoresis gels (Sigma-Aldrich, USA) stained with SimplySafe dye (EURx, Poland). The banding patterns were photographed, exported to BioGene (Vilber Lourmat, France), and analyzed. The dendrogram was constructed using a UPGMA algorithm (unweighted pair group method with arithmetic mean) by applying the Dice Similarity Ratio. A tolerance level of 1% was used in comparing the banding patterns.

Results and Discussion

Prevalence of *S. aureus* colonization. Among all participants, 46.1% (47/102) were colonized with *S. aureus* (Table I). The pattern of colonization was divided into three categories based on the anatomical location. The category that registered the highest number of individuals was the combination of the anterior

nares and the throat (40.4% of colonized individuals), followed by the anterior nares only (36.2%), and the throat only (23.4%).

S. aureus colonizes multiple anatomical locations and the frequency of colonization in various niches might vary depending on the methodology and the context of the study. Our study demonstrated that the anterior nares are colonized more frequently than the throat, which is consistent with the findings of previous studies on the prevalence of both MSSA and MRSA occurring together (Roghmann et al. 2015; Zhang et al. 2015; Peters et al. 2017). Alternatively, some researchers have reported that colonization only in the throat is the most prevalent pattern (Nilsson and Ripa 2006; Hamdan-Partida et al. 2010) or have observed the high prevalence of extra-nasal colonization as well, i.e., in the groin and the perianal regions (Mody et al. 2008).

In the current study, 31.6% (6/19) of the participants colonized with two *S. aureus* strains (one in the anterior nares and one in the throat) registered dissimilar MLVF banding patterns, implying that the two *S. aureus* strains were unrelated. A similar observation has been made by Mongkolrattanothai et al. (2011), who noted that simultaneously carriage of two or more unrelated *S. aureus* isolates was observed in 30.4% of positive swab samples collected from children. Colonization with more than one strain of bacteria entails a risk of heteroresistance, i.e., a situation in which an antibacterial agent is effective against one strain, but not against another strain of the same bacteria. Truly, in cases of co-colonization with MSSA and MRSA both together, heteroresistance might pose a therapeutic challenge.

Antimicrobial resistance profile of *S. aureus*. Table II shows the resistance profiles of *S. aureus* strains with the information about their location in the dendrogram. Among all *S. aureus* strains tested, 10.6% (7/66) were resistant to tetracycline; 6.1% (4/66) to macrolides, lincosamide, and streptogramin; 4.5% (3/66) to tobramycin and/or gentamicin, and 1.5% (1/66) to ciprofloxacin, levofloxacin, and daptomycin. We observed the presence of the MLS_B-constitutive resistance only. The share of *S. aureus* isolates susceptible to all of the antimicrobial agents tested was high (77.3%, 51/66), although the presence of MDR (multi-drug resistant) MRSA strain (Table II) suggests the need of conducting longitudinal studies investigating its transmission and long-term colonization patterns (Sun et al. 2017). The antimicrobial susceptibility testing identified 98.5% (65/66) of *S. aureus* strains as MSSA, whereas only one isolate was identified as MRSA. Consequently, the MRSA prevalence rate in our studies was low when compared to similar studies: 1% among all participants and 1.7% specifically among the residents. The prevalence of MRSA colonization among residents of long term care facilities across the world varies within a wide range: from 0% in nine Swedish nursing homes (Andersson et al. 2012), 3.7% in nine nursing homes in Brasil (da Silveira et al. 2018), 7.2% in 19 long term care facilities in Luxemburg (Mossong et al. 2013), 20.4% in 36 residential care homes for the elderly in Hong Kong (Chuang et al. 2015), to 28% in 13 nursing homes in Michigan and Maryland (Roghmann et al. 2015).

Table II
The antimicrobial resistance profiles of *S. aureus* strains isolated from residents and staff in a nursing home.

Main cluster	Sub-cluster	*S. aureus* strain	Resistance profile
A	IV	R23T	TET
	IV	S123T, S123N	TET
	IV	S133T, S133N	TET
	V	S116T	TOB, GEN
B	VII	R38T	FOX, TOB, CIP, LEV, DAP
	IX	R42T, R42N	TET
	IX	S139N	E, CC
	X	R2T	E, CC
	XI	R33T, R33N	E, CC
	XII	S134N	E, CC
–	–	R41N	TET, TOB, GEN

Note. *S. aureus* strains marked with the same number were retrieved from the anterior nares (N) and the throat (T) of one resident (R) or staff member (S) and had 100% genetic homology according to the MLVF analysis. Tetracycline (TET), tobramycin (TOB), gentamicin (GEN), cefoxitin (FOX), ciprofloxacin (CIP), levofloxacin (LEV), daptomycin (DAP), erythromycin (E), clindamycin (CC).

Multiple-locus variable-number tandem repeat fingerprinting of *S. aureus*. Using molecular typing methods to determine the genetic diversity of human pathogens is crucial in identifying the reservoirs and routes of transmission (Grzegorczyk and Malm 2014). Unlike other bacterial genotyping methods, such as PFGE (pulsed-field gel electrophoresis), MLST (multilocus sequence typing) or spa typing, MLVF has a comparable resolution, but is cheaper, less time- and labour-consuming (Luczak-Kadlubowska et al. 2008; Holmes et al. 2010; Kosecka-Strojek et al. 2016). The high discriminatory power of MLVF is its main advantage, enabling precise detection of single events of transmission between patients (Karynski et al. 2008).

The MLVF of the 66 *S. aureus* strains isolated from 47 individuals resulted in 51 banding patterns: 36 unique banding patterns were represented by only one strain and 15 banding patterns consisted of two identical *S. aureus* strains each. In accordance with our results, a high genetic diversity of MSSA has been reported in other studies (Emaneini et al. 2011; Karynski et al. 2008; Zurita et al. 2016). Zurita et al. (2016) observed 69 MLVA (multiple-locus variable-number

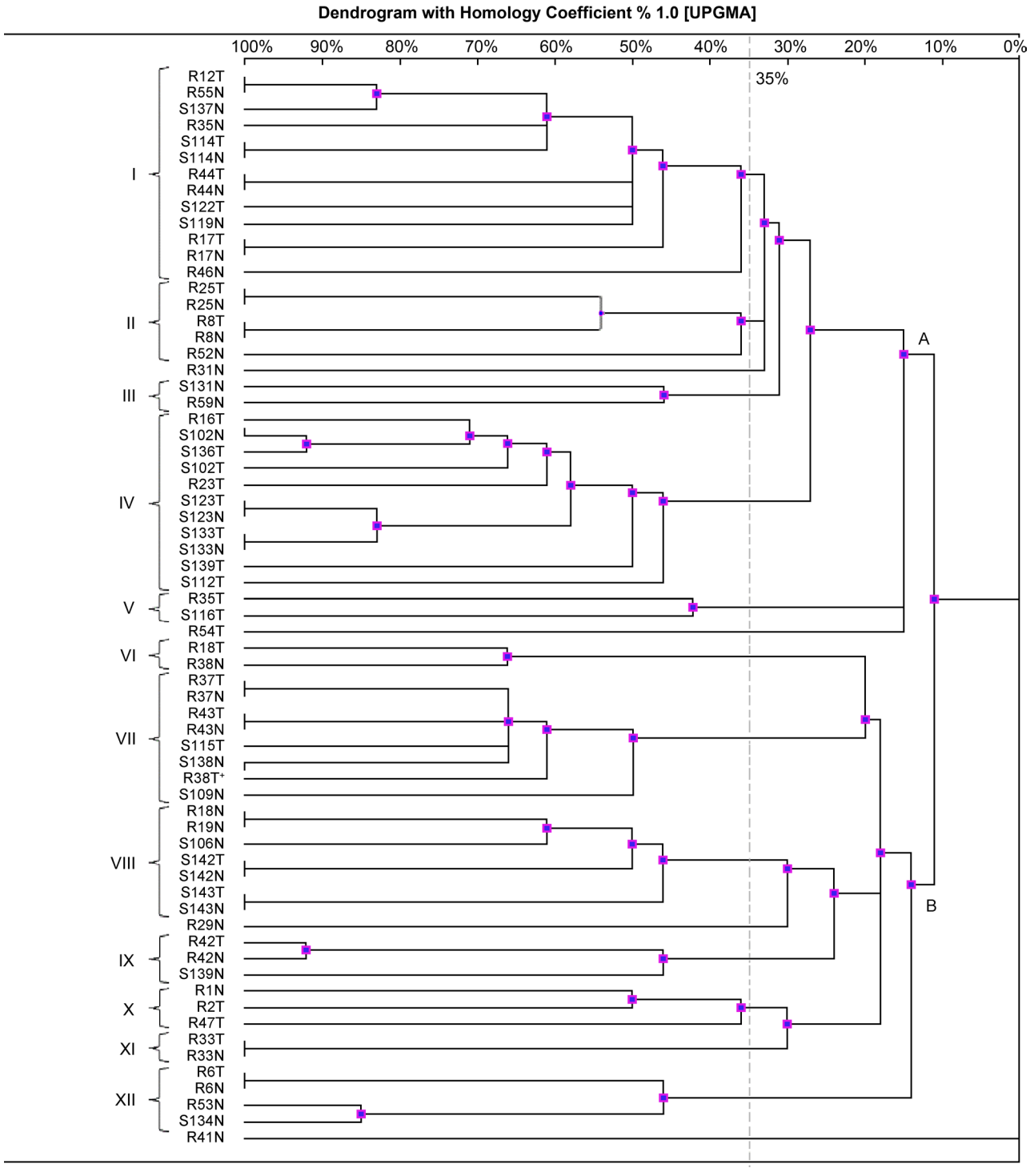

Fig. 1. MLVF dendrogram of *S. aureus* strains isolated from the anterior nares and the throat of residents and staff in a nursing home.

Note. The name of *S. aureus* strain contains the following information: R – collected from resident, S – collected from staff member, number characteristic for each participant, N – nasal swab, T – throat swab. Two main clusters (A and B) and twelve subclusters (I–XII; cut-off > 35%) are marked in the dendrogram. MRSA strain (R38T) is marked with an asterisk.

tandem-repeat analysis) genotypes among 70 tested MSSA strains from various populations.

The MLVF typing allowed us to divide the 66 *S. aureus* isolates into two main clusters, herein designated as A and B (Fig. 1). One *S. aureus* strain (R41N) was not compatible with any other banding pattern. In the dendrogram, it represents a singleton with no association to the main clusters. The main cluster A was divided into five subclusters (herein designated I–V); the main cluster B was divided into seven subclusters (herein designated VI–XII). Altogether, the twelve subclusters included 94% (62/66) of all strains (cut-off

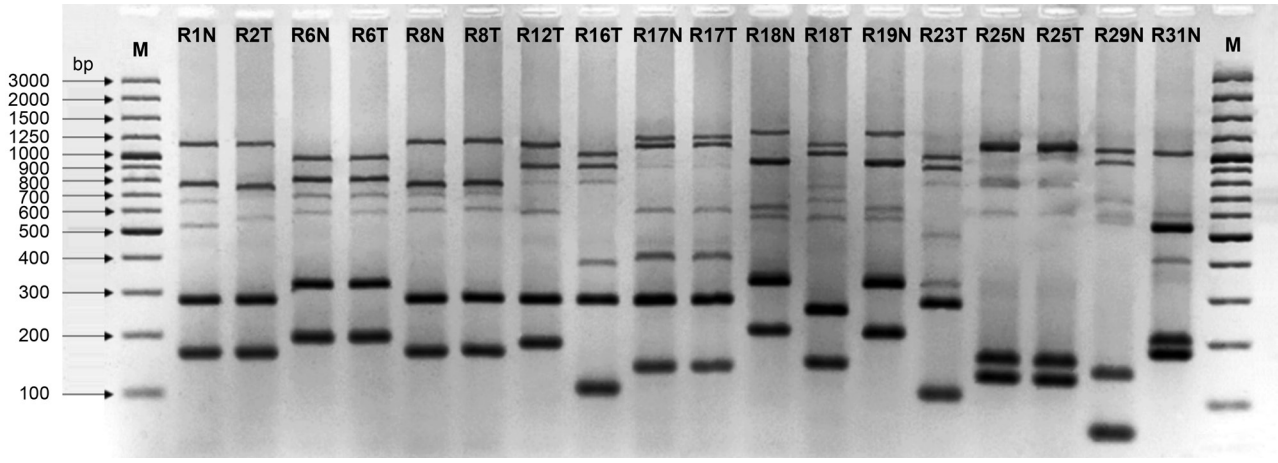

Fig. 2. MLVF of *S. aureus* strains isolated from the nose (N) and the throat (T) of thirteen residents living in a nursing home.

Note. Four residents were simultaneously colonized with the same genetic variant of *S. aureus* (strain R6N and R6T; strain R8N and R8T; strain R17N and R17T; strain R25N and R25T) and one resident was inhabited by two genetically different strains (R18N and R18T). M – molecular marker (100–3000 bp).

> 35%). Comparing the subclusters concerning the size, the two largest (I and IV) both included nine banding patterns, and 13 and 11 *S. aureus* strains, respectively. The six smallest subclusters (III, V, VI, IX, X, and XI) each included between one and three banding patterns and two or three *S. aureus* strains.

Exemplary MLVF banding patterns for *S. aureus* strains are shown in Fig. 2. The number of MLVF bands depends on the variable expression of genes in the *sdr* region of the bacterial DNA. The expression of between one and three genes (*sdrC*, *sdrD* and/or *sdrE*) translates into a range between five and seven MLVF bands (Sabat et al. 2003). In our study, the six-band and seven-band profile were the most numerous, accounting for 56.0% and 36.4% of all isolates, respectively. The less common five-band profile captured only 7.6% of all isolates.

At the level of *S. aureus* isolates, we were able to group 26 isolates in even pairs based on perfect relatedness (one hundred percent homology). Without exception, each related pair of isolates was retrieved from the anterior nares and the throat of one individual, i.e., 13 individuals contributed one pair each. At the level of individuals, this meant that 27.6% (13/47) of the colonized individuals contributed a related pair of isolates. At the group level, i.e., the serendipitous detection of related isolates across the population, we were able to group four individuals in even couples, each couple sharing one bacterial strain. However, the anatomical location and pattern of co-colonization were inconsistent, rendering the similarities imperfect (for details, see subclusters I, VI and VIII in Fig. 1). These findings suggest that inter-participant transmission of *S. aureus* has occurred and might result in cross-infection – especially among immunocompromised individuals living in crowded conditions. Andersen et al. (2002) have also reported that environmental factors, i.e., crowding and insufficient staffing, might increase the risk of *S. aureus* cross-infection.

Although our study provides valuable information about the genetic diversity, prevalence of colonization, and the occurrence of *S. aureus* transmission in a nursing home, it has several limitations. The MLVF method returns its most valid results when applied within a limited geographical area. As a result, making comparisons between studies from various countries or regions is difficult. Future research in the field of active microbial surveillance must include longitudinal aspects by subjecting the study populations to repeated sampling over extended periods. Understanding the temporal aspects of colonization might lead to new insights regarding the routes of bacterial transmission.

Author contributions

M.K. and A.G. collected the samples and performed the experiments. All authors conceived, designed the experiments and wrote the paper.

ORCID

Martyna Kasela 0000-0002-9791-2932

Ethical approval

The study was approved by the Bioethics Committee at the Medical University of Lublin (KE 0254/59/2016). The samples were collected in January 2018 from residents and staff (n = 102) living and working in a nursing home (Lublin, Poland). Written informed consent was obtained from each individual before participation.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Periodontal Status and Subgingival Biofilms in Cystic Fibrosis Adults

TAMARA PAWLACZYK-KAMIEŃSKA[1]*, RENATA ŚNIATAŁA[1], HALINA BATURA-GABRYEL[2], MARIA BORYSEWICZ-LEWICKA[1] and SZCZEPAN COFTA[2]

[1] Department of Paediatric Dentistry, Poznan University of Medical Sciences, Poznan, Poland
[2] Department of Pulmonology, Allergology and Respiratory Oncology, Poznan University of Medical Sciences, Poznan, Poland

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Abstract

The aim of this study was to assess the periodontal status of cystic fibrosis (CF) adult patients and to evaluate whether there is a correlation between the bacterial population of the subgingival biofilm and the health status of the periodontal tissues in this group of adults. The study involved 22 cystic fibrosis adult patients. The periodontal condition was assessed using Plaque Index (PLI), Gingival Index (GI), and Probing Pocket Depth (PPD). The gingival sulcus samples were analyzed by the Real-Time PCR assay (RT-PCR). Majority of patients showed moderate or severe bacterial dental plaque accumulation, but none of them had clinical symptoms of periodontal diseases. RT-PCR showed the presence of periopathogens in 50% of patients. Red complex microorganisms were detected in 9.09%, orange complex in 27.27%, and green complex in 31.82% of the samples analyzed. In cystic fibrosis patients colonized by periopathogens, the periodontal markers were significantly higher in comparison to not colonized by periopathogens patients. Despite the widespread presence of bacterial dental deposits in the cystic fibrosis adult patients examined, none of them has clinical symptoms of periodontal disease; however, the presence of periodontal pathogens in subgingival biofilm may represent a possible risk factor of this disease in the future. An unsatisfactory level of oral hygiene in any patient with cystic fibrosis indicates a need to focus on standards of dental care for such patients.

Key words: cystic fibrosis, periodontal pathogens, periodontitis, Real-Time PCR

Introduction

The role of oral health, including the level of dental hygiene, accumulation of dental bacterial plaque, and periodontal diseases in the pathogenesis of respiratory infections is usually ignored in the medical care of cystic fibrosis (CF) patients. The oral cavity is a reservoir of many microorganisms; to date, more than 700 oral bacterial species have been identified. Over 400 of them form dental bacterial plaque biofilm and can be isolated from gingival sulcus or gingival pocket. The remaining 300 inhabit other areas, such as the tongue, oral mucosa, carious lesions, and teeth with endodontic infection (Haffajee et al. 2008; Dewhirst et al. 2010). Most oral bacteria are saprophytes of the oral cavity; the species composition of the dental plaque microbiome is characterized by stability (homeostasis) that may break down, and, under favorable environmental conditions, the plaque microflora may express their virulence (Haffajee et al. 2008). The oral cavity, especially in individuals with poor oral hygiene or periodontal diseases, can also harbor various important pathogenic microorganisms, including respiratory pathogens (Wise and Williams 2013; Souto et al. 2014; Caldas et al. 2015; Vilela et al. 2015).

Periodontitis is a chronic infection of tooth-supporting tissues. The pathogenesis involves a complex of an immuno-inflammatory reaction of the host to a microbial community in the dental plaque. The severity of the periodontal disease results from the interaction between the dental plaque microbiota and the host's defense mechanisms (Haffajee et al. 2008; Dewhirst et al. 2010). Subgingival plaque bacteria form a specific biofilm complex in which microorganisms become more effectively protected against host defense mechanisms and efficiently use the nutrients (Socransky et al. 1998). The concept of a bacterial complex, introduced by Socransky et al. (1998), is based on the specific plaque hypothesis that assumes that a specific microbiota is associated with gingivitis or chronic or aggressive periodontitis,

* Corresponding author: Tamara Pawlaczyk-Kamieńska, Department of Pediatric Dentistry, Poznan University of Medical Sciences, Poznan, Poland; **e-mail: tpawlaczyk@ump.edu.pl**

and that the pathogenicity of the plaque depends on the presence or increase in the number of specific microorganisms. Based on the bacterial correlations all of the biofilm's periopathogens are divided into four groups (complexes). The most pathogenic for periodontal tissues and considered as a marker of periodontitis is the red complex, which includes *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. The orange complex, directly related to the red one, includes *Fusobacterium nucleatum*, *Prevotella intermedia*, *Peptostreptococcus micros*, and *Campylobacter rectus*. The yellow complex comprises *Streptococcus mitis, Streptococcus oralis*, *Streptococcus sanguis*, and the green complex includes *Capnocytophaga gingivalis, Campylobacter concisus, Eikenella corrodens*, and *Aggregatibacter actinomycetemcomitans* serotype a. Also, there are separate periopathogens such as *A. actinomycetemcomitans* serotype b, *Selenomonas noxia*, and *Actinomyces naeslundii* genospecies, which are not related to other groups. Particular importance in the etiopathogenesis of periodontitis is attributed to (Haffajee et al. 2008) that is considered the dominant etiological factor in the initiation and early stage of periodontal disease. Species of individual complexes are closely related. In most cases, species of a particular group appear together, or they are all absent together. This interaction and cooperation between the bacteria of dental plaque biofilm lead to a form of stabilization in which all species exist in harmony and equilibrium with their environment. This biofilm stabilization is named the climax community (Socransky et al. 1998; Haffajee et al. 2008).

The periopathogens are not only responsible for pathological changes in periodontal tissues, but may also be associated with several systemic conditions, including respiratory diseases (Gomes-Filho et al. 2010; Messika et al. 2018). Oral bacteria may disseminate into the lower respiratory tract, increasing the risk of pulmonary infections (Gomes-Filho et al. 2010). Thereby, to reduce the risk of possible autoinfection especially in patients at risk of such infection, there is a need to control periodontal condition regularly.

The aim of this study was to assess the periodontal status of cystic fibrosis patients and to evaluate whether there is a correlation between the bacterial population of the subgingival biofilm and the health status of the periodontal tissues in this group of adults.

Experimental

Material and Methods

Patients. The research was conducted in accordance with ethical principles, including the World Medical Association Declaration of Helsinki and was approved by an Ethical Committee of the Poznan University of Medical Sciences, Poland (No 427/16). The inclusion criteria were as follows: 1. diagnosis of cystic fibrosis confirmed by positive results of sweat test and a genetic test, 2. age above 18 years, 3. at least 10 teeth present in the mouth. Exclusion criteria included: 1. pregnancy or lactation, 2. diabetes or evidence of any other than CF systemic disease as a risk factor for periodontitis, 3. acute respiratory infections. After explaining the purpose and details of the study, written informed consent was obtained from all the subjects who were willing to participate. Twenty-two CF patients (14 women and 8 men) with an average age of 29.43 ± 6.78 years meetings all the above conditions were included to this study. All of them were patients of the Department of Pulmonology, Allergology and Respiratory Oncology of the Poznan University of Medical Sciences, Poland.

Periodontal examination. Assessment of periodontal condition was conducted following the World Health Organization criteria for epidemiological surveys (WHO 1998). Two professionals examined patients under artificial light using a dental mirror and a dental WHO 621 periodontal ballpoint probe. Prior to the clinical examination, examiners were calibrated and the inter-examiner agreement was determined by Cohen's Kappa values of 0.85.

The clinical examination procedure included the evaluation of dental plaque accumulation, assessment of gingival bleeding and the measure the depth of gingival sulcus. For the presence and quantity of dental plaque recording, the Silness & Löe Plaque Index (PLI) was used (Silness and Löe 1964). The gingival status was assessed using the Löe & Silness Gingival Index (GI) (Löe and Silness 1967). The presence/absence of dental plaque and gingival bleeding were based on the examination of four surfaces (buccal, lingual/palatal, mesial, and distal) of six index teeth: upper first right molar (16), upper right central incisor (11), upper first left premolar (24), lower first left molar (36), lower left central incisor (31), and lower right premolar (44), as recommended by the indices. In the recording of dental plaque accumulation, all four surfaces of teeth were given the score from 0 to 3. The criteria for this index are: 0 – no plaque, 1 – plaque invisible but can be found with the periodontal probe at the gingival margin, 2 – moderate plaque was easily seen without probing, 3 – sample plaque easily seen. Then the scores form four surfaces were summed up and divided by four to give the plaque index for each tooth. The index for the patient was obtained by summing the indices for all six teeth and dividing by six. The PLI score: < 0.1 means no plaque, from 0.1 to 1.0 indicates a small quantity of plaque, from 1.1 to 2.0 a moderate amount and between 2.1 to 3.0 a considerable one.

The bleeding was assessed by probing gently along the wall of the soft tissue of the gingival sulcus. There were given scores from 0 to 3, were 0 means normal gingiva, 1 – mild inflammation – slight change in color and slight edema but no bleeding on probing, 2 – moderate inflammation – redness, edema, glazing, and bleeding on probing, and 3 – severe inflammation – marked redness and edema, ulceration with tendency to spontaneous bleeding. The sites were probed, waiting 10 s to verify the presence or absence of gingival bleeding. The mean index was calculated by dividing the sum of numbers from the scale by the total number of sites scored within the mouth. A score from 0.1 to 1.0 means mild inflammation, 1.1 – 2.0 moderate, and from 2.1 to 3.0 – severe inflammation.

Depth of the gingival sulcus (Probing Pocket Depth, PPD) on six dental sites: distofacial, facial, mesiofacial, distolingual, lingual, and mesiolingual of all teeth except third molar teeth, was examined using the periodontal probe 621. During the examination, the probe was introduced down to the bottom of the sulcus in parallel to the long dental axis with permanent contact with the tooth. The mean individual PPD index was calculated by dividing the sum of the PPD for each tooth by the total number of examined teeth.

Bacterial microflora evaluation. Gingival sulcus samples were collected for quantification of bacterial content and analysis for the presence of nine different microorganisms, using molecular biology tools and specific gene amplification by RT-PCR (Gołyńska et al. 2017). Microbiological analysis was performed to assess the presence and quantity of following periodontal pathogens: 1. *A. actinomycetemcomitans*, 2. *P. gingivalis*, *T. forsythia*, and *T. denticola* from the red complex, 3. *F. nucleatum*, *P. intermedia*, *P. micros* from the orange complex as well as *Eubacterium nodatum* from the complex associated with the orange one, 4. *C. gingivalis* from the green complex. We have used commercially RT-PCR kit (PET Test® plus).

Samples were collected from the gingival sulcus of four teeth – two posterior and two front teeth, two uppers and two lower, so that they were the same teeth (16, 11, 36, 31) in which the clinical indices (PLI, GI) were assessed. All samples were collected following the procedure recommended by the manufacturer. Before collecting the samples, the supragingival bacterial plaque was removed, and the examined area was dried and isolated with sterile swabs from the access of saliva. Using sterile tweezers, a sterile paper point included in the kit was introduced for 20 seconds. Then each of the four samples was loaded into one test tube, placed in a transportation set, and shipped to an MPI Pharma Laboratory.

Statistical analysis. Statistical analysis was done using Statistica, version 12 software (StatSoft Inc., Tulsa, USA). For statistical inference of the collected data, mean values with standard deviation and percentages were adopted. The normalcy of the variable distribution was tested using the Shapiro-Wilk test. Also, the Spearman's rank correlation coefficient was used to determine the correlation between the received values of clinical indices (GI, PLI, PPD) and also between particular clinical index (GI, PLI or PPD) and count of bacteria in the subgingival biofilm. The level of significance was set at 0.05.

Results

Mild dental plaque accumulation (PLI = 0.1–1.0) was detected in 36% of cystic fibrosis patients, moderate (PLI = 1.1–2.0) in 45%, and severe (PLI = 2.1–3.0) in 18%. There was no patient with PLI < 0.1, but this condition is observed only directly after tooth brushing in patients with good oral hygiene. In the vast majority of patients (68%) the GI index ranged between 0.1 and 1.0 indicative of mild inflammation. In 27% of patients, no gingivitis was present (GI < 0.1), and a moderate inflammation (GI = 1.0–2.0) was found in 45%. No patient showed severe gingivitis (GI ≥ 2.1). There was no patient in whom periodontal pocket was recorded (PPD > 4 mm). In 45% of patients, the PPD ranged between 3 and 4 mm, in 18% between 0.5 and 2 mm, and 36% between 3 and 4 mm. There was a very high positive correlation between the following: GI and PLI, GI and PPD, PPD and PLI ($p < 0.0001$). Quantitative microbial analysis of gingival sulcus revealed that the mean total bacteria count in gingival sulcus in CF patients was $1.3 \times 10^7 \pm 3.8 \times 10^7$ CFU/ml. There was a very high positive correlation ($p < 0.0001$) between the number of bacteria and the depth of gingival sulcus (PPD index).

Periopathogens were detected in 50% of CF patients. Based on these results, the patients examined were divided into two groups: 1. CF patients with periopathogens, that is, for whom PCR tests showed the presence of periopathogens, and 2. CF patients without periopathogens, that is, in whom periopathogens were not found in the materials examined (Table I). There was no significant difference in the total bacteria count between the two groups of patients ($p > 0.05$), but in patients with periopathogens, the GI, PLI, and PPD were significantly higher in comparison to patients without periopathogens ($p > 0.05$).

Seven out of nine tested periopathogens were detected in examined patients (Table II). *A. actinomycetemcomitans* and *E. nodatum* were not found in any of them. The average percentage of tested periopathogens within the total bacterial count was 2.42% and ranged from 0.01 to 21.56%. *C. gingivalis* from the green

Table I
Clinical data of adult patients with cystic fibrosis that were colonised or not colonised by periopathogens.

	PLI		GI		PPD		TOTAL BACTERIA COUNT (CFU/ml)	
C	1.76 ± 0.70	$p < 0.01$	0.56 ± 0.36	$p < 0.05$	1.33 ± 0.24	$p < 0.01$	$8.9 \times 10^6 \pm 1.5 \times 10^7$	$p > 0.05$
NC	0.86 ± 0.28		0.21 ± 0.26		0.98 ± 0.12		$1.8 \times 10^7 \pm 5.3 \times 10^7$	
TOTAL	1.31 ± 0.69		0.39 ± 0.36		1.16 ± 0.26		$1.3 \times 10^7 \pm 3.8 \times 10^7$	

Data are presented as mean and standard deviation (SD)
CF – Cystic Fibrosis, PLI – Plaque Index by Silness & Löe, GI – Gingival Index by Löe & Silness, PPD – Probing Pocket Depth,
C – patients colonised by periopathogens, NC – patients not colonised by periopathogens

Table II
The number of bacteria isolated from gingival sulcus and the percentage of the species tested within the total bacterial count in samples.

The periopathogens tested		Number of bacteria (CFU/ml) Mean ± SD	Percentage of the periopathogens tested within the total bacterial count (%)
Aggregatibacter actinomycetemcomitans		0.00	0.00
Red complex	*Porphyromonas gingivalis*	$2.0 \times 10 \pm 9.4 \times 10$	0.01
	Tannerella forsythia	$2.4 \times 10 \pm 1.1 \times 10^2$	0.01
	Treponema denticola	$1.4 \times 10^2 \pm 6.3 \times 10^2$	0.01
Orange complex	*Fusobacterium nucleatum*	$7.3 \times 10^2 \pm 2.5 \times 10^3$	0.93
	Prevotella intermedia	$8.2 \times 10^3 \pm 3.9 \times 10^4$	0.01
	Peptostreptococcus micros	$1.7 \times 10^3 \pm 8.2 \times 10^3$	1.50
	Eubacterium nodatum	0.00	0.00
Green complex	*Capnocytophaga gingivalis*	$6.8 \times 10^4 \pm 3.1 \times 10^5$	3.51

complex was the most frequently detected. Red complex microorganisms (*P. gingivalis*, *T. forsythia*, *T. denticola*) were present in 9.09% of samples, orange complex (*F. nucleatum*, *P. intermedia*, *P. micros*) in 22.73%, and green (*C. gingivalis*) in 31.82% (Fig. 1). Moreover, in 9.09% of patients a significantly increased number of periopathogens (*C. gingivalis*, *F. nucleatum*, *P. intermedia*, and *P. micros*) was detected.

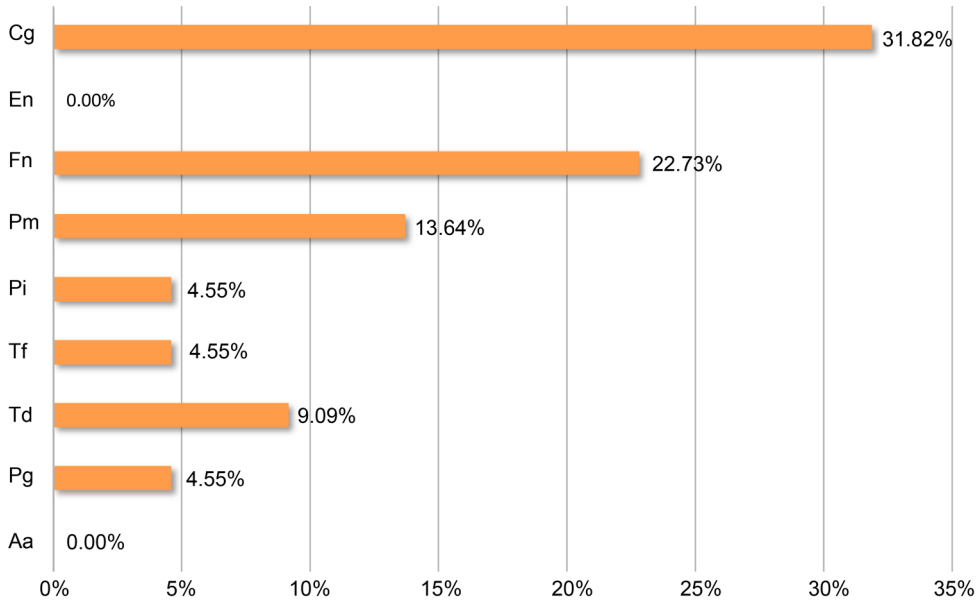

Fig. 1. The percentage of the samples containing specific periopathogens.
Aa – *Aggregatibacter actinomycetemcomitans*, Pg – *Porphyromonas gingivalis*, Td – *Treponema denticola*, Tf – *Tannerella forsythia*, Pi – *Prevotella intermedia*, Pm – *Peptostreptococcus micros*, Fn – *Fusobacterium nucleatum*, En – *Eubacterium nodatum*, Cg – *Capnocytophaga gingivalis*. The mean value of these observations was 10.10% ± 10.85%.

Discussion

Among the many factors influencing the onset and progression of periodontal infection, periopathogens are of key importance, but their presence in the oral cavity is not always manifested. The clinical picture of periodontal disease depends on the balance of the interaction between microbial dental plaque biofilm and host immune response (Haffajee et al. 2008; Dewhirst et al. 2010). In the studied CF patients, there was a high positive correlation between the depth of the gingival sulcus (PPD) and the accumulation of dental plaque (PLI), also between the PPD and gingival bleeding (GI), and between PPD and total number of bacteria in gingival sulcus, confirming the pathomechanism of periodontitis and underscoring the importance of daily removal of dental bacterial plaque, by tooth brushing, as a preventive measure. The dental plaque accumulation index leads to conclusions not only about the current status, but also about the efficiency of hygienic procedures performed by the patient (daily mechano-chemical teeth brushing). However, it does not convey information about the host's immune response to the bacterial infection or the severity of the inflammation. These factors can be analyzed by gingival indices describing the condition of the gums. The available literature regarding the periodontal status of cystic fibrosis patients shows that CF adult patients, compared to healthy individuals, have less gingival bleeding, with a similar bacterial plaque accumulation (Pawlaczyk-Kamieńska et al. 2018). In our study, despite the widespread presence of bacterial dental deposits in cystic fibrosis patients, none of them has clinical symptoms of periodontal disease. This can be explained by frequent and long-term antibiotic therapy, including via inhalation.

Microbiological analysis of bacterial subgingival plaque in CF patients was conducted by Caldas et al. (2015). Their studies included 10 cystic fibrosis patients (five chronically colonized by *Pseudomonas aeruginosa*, and five not colonized) without clinical symptoms of gingivitis or periodontitis. The average age of the chronically colonized patients was 23.8 (17–34 years), and of the not colonized patients, it was age 16.6 (12–27 years). The qPCR PrioAnalyse assay was used for the analysis of bacterial plaque microbiological composition. This test identified nine periopathogens*: A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, and *T. denticola* from the red complex; *F. nucleatum*, *P. intermedia*, *P. micros*, and *C. rectus* from the orange complex; and *E. corrodens* from the green complex.

The analysis of samples revealed seven periopathogens in not colonized patients from the group, and five out of nine analyzed in the chronically colonized group. *A. actinomycetemcomitans* was not found in any patients. The most frequently detected was *F. nucleatum* (orange complex), and the second most frequent was *E. corrodens* (green complex). In our study, the PCR analysis showed seven out of nine the periopathogens tested. Similar to Caldas et al. (2015), no *A. actinomycetemcomitans* were found. *C. gingivalis* (green complex) was the most common, followed by *F. nucleatum* (orange complex).

Reports published to date concerning the presence of oral periopathogens usually relate to generally healthy patients with varying degrees of periodontitis, diagnosed based on history, clinical, and radiological examination (Abiko et al. 2010; Kotslikov et al. 2015; Elamin et al. 2017). The cited studies exclude patients who had used antibiotics in the last three months or anti-inflammatory drugs in the last month. These results report a higher level of periopathogens in individuals with periodontitis compared to patients with healthy periodontium (Abiko et al. 2010). Furthermore, the authors found a positive correlation between the severity of periodontitis and the number of microorganisms, such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, and *T. forsythia* (Abiko et al. 2010; Kotslikov et al. 2015; Elamin et al. 2017). Their numbers and the ratio to other microbes in the periodontal pockets determine the risk of onset and progression of periodontitis. A high titer of periopathogens does not guarantee disease development but informs of the risk (Abiko et al. 2010). The presented research included cystic fibrosis patients in whom the exclusion of systemic drugs application was not possible. Presumably, in those groups of patients, medications used may affect the microbiological oral condition, probably limiting the pathogenicity of the bacterial plaque biofilm. Unsatisfactory oral hygiene levels suggest that the periodontal risk cannot be ruled out.

Knowledge of the pathomechanisms of periodontitis and possible autoinfection by aspiration of oral microbiome should be taken into account in the medical care of CF patients. The dental biofilm cannot be eliminated, but it can be controlled by daily, regular chemo-mechanical oral hygiene practices performed by the patients. There is a necessity to improve the oral health condition in CF patients, and dentists should be a part of a multi-disciplinary cystic fibrosis team. There is also a need to implement effective strategies for care pathways directed toward maintaining oral health in this group of patients.

Conclusions

Despite the widespread presence of bacterial dental deposits in cystic fibrosis adult patients, none of them has clinical symptoms of periodontal disease, but the presence of periodontal pathogens in subgingival biofilm represents a possible risk factor of this disease in the future.

An unsatisfactory level of oral hygiene in any patient with cystic fibrosis indicates a need to focus on standards of dental care for such patients.

Abbreviations used in this paper:

CF – Cystic Fibrosis
PCR – polymerase chain reaction
PLI – Plaque Index by Silness & Löe
GI – Gingival Index by Löe & Silness
PPD – Probing Pocket Depth
Aa – Aggregatibacter actinomycetemcomitans
C – patients colonised by periopathogens
NC – patients not colonised by periopathogens

ORCID

Tamara Pawlaczyk-Kamieńska 0000-0003-3398-8812
Renata Śniatała 0000-0002-3123-7612
Halina Batura-Gabryel 0000-0003-4555-7905
Maria Borysewicz-Lewicka 0000-0001-6391-350X
Szczepan Cofta 0000-0002-8849-5487

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

Antifungal Activity and Physicochemical Properties of a Novel Antimicrobial Protein AMP-17 from *Musca domestica*

LONG-BING YANG[1, 2], GUO GUO[1, 2]*, XIN-YU ZHAO[1, 2], PEI-PEI SU[1, 2], PING FU[1], JIAN PENG[3], JIANG-FAN XIU[1] and BO-YAN LI[2, 4]

[1] School of Basic Medical Sciences, Guizhou Medical University, Guiyang, China
[2] Key Laboratory of Environmental Pollution Monitoring and Disease Control, Ministry of Education, Guizhou Medical University, Guiyang, China
[3] Research Center of Medical Biotechnology Engineering, Guizhou Medical University, Guiyang, China
[4] School of Food Science, Guizhou Medical University, Guiyang, China

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Abstract

Antimicrobial peptides (AMPs) are cationic small peptide chains that have good antimicrobial activity against a variety of bacteria, fungi, and viruses. AMP-17 is a recombinant insect AMP obtained by a prokaryotic expression system. However, the full antifungal activity, physicochemical characteristics, and cytotoxicity of AMP-17 were previously unknown. AMP-17 was shown to have good antifungal activity against five pathogenic fungi, with minimum inhibitory concentrations (MIC) of 9.375–18.75 μg/ml, and minimum fungicidal concentrations (MFC) of 18.75–37.5 μg/ml. Notably, the antifungal activity of AMP-17 against *Cryptococcus neoformans* was superior to that of other *Candida* spp. In addition, the hemolytic rate of AMP-17 was only 1.47%, even at the high concentration of 16×MIC. AMP-17 was insensitive to temperature and high salt ion concentration, with temperatures of 98°C and –80°C, and NaCl and $MgCl_2$ concentrations of 50–200 mmol/l, having no significant effect on antifungal activity. However, AMP-17 was sensitive to proteases, trypsin, pepsin, and proteinase K. The elucidation of antifungal activity, physicochemical properties and cytotoxicity of AMP-17 provided an experimental basis for its safety evaluation and application, as well as indicated that AMP-17 might be a promising drug.

Key words: antimicrobial peptides, AMP-17, antifungal activity, stability, hemolytic activity

Introduction

In recent years, the number of patients presenting with fungal infections has increased, and the emergence of pathogenic fungi has seriously affected human health and safety. Of the several common pathogenic fungi that occur clinically, *Candida* spp., dominated by *Candida albicans*, and non-*Candida* spp., dominated by *Cryptococcus neoformans*, are the most common (Wang et al. 2012; Jensen 2016; Lovero et al. 2016). Candidiasis is mainly an acute, subacute or chronic infection caused by *C. albicans*. It is the most common fungal disease, often invading the skin and mucous membranes, and can also cause visceral or systemic infections. The clinical symptoms are intricate and inconsistent. However, cryptococcal infections are usually seen in patients with low immune function, especially in the case of acquired immunodeficiency syndrome (AIDS) and pulmonary tuberculosis (PTB) (Chopra et al. 2015). Non-candida disease is mainly caused by the invasion of *C. neoformans* spores into the respiratory tract, which subsequently causes hematogenous dissemination, leading to central nervous system infection (Lortholary et al. 2004; Burnik et al. 2007). Patients with cryptococcal lung infection often develop non-specific symptoms such as weight loss, fever, cough, and general weakness. Moreover, clinical manifestations vary from asymptomatic infection to severe pneumonia and respiratory failure (Brizendine et al. 2011; Debenedectis et al. 2013). At present, azoles, polyenes, echinocyclines, and

* Corresponding author: G. Guo, Building Wuben, School of Basic Medical Sciences, Guizhou Medical University, College Town, Gui'an New District, Guiyang, China; **e-mail: guoguo@gmc.edu.cn, guoguojsc@163.com**

fluorocytosines are the most commonly used drugs for the treatment of fungal infections; however, all have certain toxicity and side effects, and drug-resistant strains are constantly emerging. (Bellmann and Smuszkiewicz 2017; Spitzer et al. 2017). Therefore, the development of new antifungal drugs is urgently needed for clinical anti-infection treatment.

Antimicrobial peptides (AMPs), also known as host defense peptides, are biologically active molecules produced by various organisms as important components of their innate immune response (Yan et al. 2018). AMPs not only have strong antimicrobial efficacy but also have activity against viruses, parasites and tumor cells (Ren et al. 2012; Patnaik et al. 2013; Tindwa et al. 2013). In addition to strong antimicrobial efficacy, AMPs have low hemolytic and cytotoxic activities (Edwards et al. 2016). For example, Quintana et al. (2014) found that the AMP subtilosin has antiviral and viricidal effects against herpes simplex virus type 2 (HSV-2). Kovalchuk et al. (2007) showed that a complex of natural cytokines and AMPs (CCAP or Superlymph) inhibits virus proliferation *in vitro*. Therefore, due to their small molecular weight, good thermal stability and broad antimicrobial spectrum, AMPs are expected to replace antibiotics as new and highly effective antibacterial drugs (Presicce et al. 2009; Seydlová et al. 2017).

AMP-17 is encoded by a specific highly expressed gene extracted from the *Musca domestica* transcriptome database constructed after 12 h of microbial infection. The AMP-17 gene has no functional annotation in GeneBank and is of unknown function. To further assess the antimicrobial activity of the protein encoded by this gene, the prediction tools of the Antimicrobial Peptide (APD) and Collection of Anti-Microbial Peptides (CAMP) databases were used (Wang et al. 2004; Thomas et al. 2010; Wang et al. 2016). The two databases used four different algorithms, support vector machines (SVM), random forest (RF), artificial neural networks (ANN), and discriminant analysis (DA) to predict the protein structure, and the scores obtained were 1, 0.944, 1 and 1, respectively. All the results determined that the protein was an AMP. The antifungal function of recombinant AMP-17 protein has been reported by Guo et al. (2017) who showed its strong activity against *C. albicans,* indicating it had good development potential.

In this study, the *in vitro* antifungal activity of AMP-17 protein was assessed against *C. albicans*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, and *C. neoformans*. To further understand the physical and chemical properties of AMP-17 protein, the salt, acid-base, and thermal stabilities were also assayed. In addition, cytotoxicity and hemolytic activity of AMP-17 protein were determined, as a prelude to more extensive safety evaluation.

Experimental

Materials and Methods

Materials. AMP-17 protein was obtained from the Guoguo research team. It was produced in a prokaryotic expression system and purified by a nickel ion metal chelator affinity chromatography (Guo et al. 2017). Fluconazole (FLC), Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were purchased from Solarbio (Beijing, China). Human red blood cells were donated by the Affiliated Hospital of Guizhou Medical University, Guiyang, China.

Microbial strains. *C. albicans* ATCC 10231 was routinely preserved by the Key Laboratory of Modern Pathogenic Biology, Guizhou Medical University, Guiyang, China). *C. krusei* IFM56881, *C. tropicalis* IFM57016, *C. parapsilosis* ATCC 22019, and *C. neoformans* IFM51426 were obtained from the Department of Microbiology, Guizhou Medical University, Guiyang, China. They were stored in 30% glycerol at –80°C.

MIC determination. A single actively growing microbial colony was inoculated into 5 ml sterile SDB medium and incubated overnight at 37°C. The turbidity of the fungal solution was adjusted to $1–5 \times 10^6$ colony-forming units (CFU)/ml using a blood cell counting plate. The fungal suspension was then diluted with SDB to $0.5–2.5 \times 10^3$ CFU/ml. An aliquot of 100 µl of the final suspension was added into each well of a sterile 96-well plate containing 100 µl of medium containing antimicrobial agents at double-diluted concentrations. Phosphate-buffered saline (PBS) was used as a negative control and fluconazole as a positive control. The plate was assessed for MIC values of AMP-17 after 24 h or 48 h of incubation at 37°C. The MIC value was determined to be the minimum concentration at which microscopic growth could not be observed by the naked eye, as recommended by the Clinical Laboratory and Standards Institute CLSI (2008) methods (Cantón et al. 2008; Fothergill 2012). The experiment was repeated three times, three biological replicates at a time.

MFC determination. Based on the MIC value, the criteria for fungal MFC values were slightly adjusted. Briefly, samples from each well of a 96-well plate, prepared as described above, were used to determine MFC. Ten µl samples were plated on SDA, with CFUs being counted after incubation at 37°C for 24 h (*C. albicans, C. krusei*, *C. tropicalis*, *C. parapsilosis*) or 48 h (*C. neoformans*). The experiment was repeated three times, three biological replicates at a time.

Time-kill curves. Overnight cultures of fungi were diluted in sterile SDB medium and adjusted to $1–5 \times 10^6$ CFU/ml. An aliquot of 100 µl of this fungal suspension was added to a 96-well plate containing 900 µl of a specific concentration of AMP-17 and then

incubated at 37°C. The optical density of the culture was recorded every two hours at a wavelength of 562 nm. With different sampling time (h) as the abscissa, the A_{562} value of each plate well was plotted on the ordinate, generating a time-kill curve. PBS and fluconazole were used as negative and positive controls, respectively. The experiment was repeated three times, three biological replicates at a time.

Determination of salt ion strength stability. An overnight culture of *C. albicans* was diluted in sterile SDB medium and adjusted to $1.0 – 2.5 \times 10^3$ CFU/ml. One hundred µl of this fungal suspension was added to a 96-well plate, where each well contained 100 µl of AMP-17, at 1–6 × MIC, and NaCl or $MgCl_2$ at final concentrations of 0, 50, 100, and 200 mmol/l. The MIC value of AMP-17 against *C. albicans,* under the influence of different concentrations of NaCl or $MgCl_2$, was determined as recommended by the CLSI (2008) methods. The experiment was repeated three times, three biological replicates at a time.

Determination of heat stability and freeze-thaw resistance. An overnight culture of *C. albicans* was diluted in sterile SDB and adjusted to $1.0 – 2.5 \times 10^3$ FU/ml. One hundred µl AMP-17, at 1 × MIC, was maintained at 98°C in a water bath for 5, 20, 30, 60, 90, and 120 minutes (heat-resistant group). One hundred µl AMP-17, at 1 × MIC was also repeatedly frozen at –80°C, 1, 2, 4, 6, 8, and 10 times (freeze-thawed group). One hundred µl aliquots of the fungal suspension were added to a 96-well plate containing the heat-resistant group, the freeze-thawed group, and a negative control group, and incubated at 37°C for 24 hours. Ten µl samples from the heat-resistant group plate were then directly inoculated onto SDA, and CFU counts were taken after incubation at 37°C for 24 h. Samples taken from the freeze-thaw group and the negative control group were diluted 1:10 000, and then 100 µl was plated on SDA. CFU counts were taken after incubation at 37°C for 24 h. ddH_20 was used as a negative control. The experiment was repeated three times, three biological replicates at a time.

Stability to treatment with trypsin, pepsin and proteinase K. An overnight culture of *C. albicans* was diluted in sterile SDB and adjusted to $1.0 – 2.5 \times 10^3$ CFU/ml Fifty µl 0.1 mg/ml proteases (the pH values of trypsin, pepsin, and protease K solutions were: 7, 5, and 7, respectively) and 50 µl 37.5 µg/ml AMP-17 (2 × MIC) were mixed in a 96-well plate and placed in a 37°C water bath for 2 min, 20 min, 40 min, 60 min, 80 min, 100 min, and 120 min. One hundred µl of the fungal suspension was then added to the 96-well plate, followed by incubation at 37°C for 18 h. Subsequently, 10 µl of the fungal suspension was diluted 1:10 000, and 50 µl of this was incubated on SDA for 24 hours, followed by CFU counting. PBS was used as a negative control. The experiment was repeated three times, three biological replicates at a time.

Determination of hemolytic activity. Hemolytic activity of AMP-17 was evaluated using the method of Souza et al. (2013). Briefly, freshly isolated and washed human erythrocyte suspensions were diluted with 0.01 M PBS, pH 7.4, and then centrifuged at 125 × g and 4°C for 10 min. This process was repeated three times. The final pellets were resuspended in PBS at a concentration of 4% (v/v). One hundred µl of different concentrations of AMP-17 (2.344–300 µg/ml) was added to a 96-well plate containing 100 µl 4% human red blood cell suspension, and this was incubated at 37°C for 1 h and then centrifuged (225 × g, 4°C, 10 min). The absorbance of the supernatants at 450 nm was then measured using an ultra-microplate spectrophotometer (Model: US Biotekepoch 2). PBS (0.01 M) was used as a negative control and 0.1% Triton X-100 was a positive control. The experiment was repeated three times, three biological replicates at a time. Percentage hemolysis was determined by the formula:

$$\text{Hemolysis (\%)} = [(Abs_{450} \text{ in the peptide solution} - Abs_{450} \text{ in PBS}) / (Abs_{450} \text{ in 0.1\% Triton X-100} - Abs_{450} \text{ in PBS})] \times 100$$

Statistical analysis. Statistical differences were analyzed using the GraphPad Prism 6 software. The comparison between groups was carried out by using a Student's t-test. The p-value < 0.05 was statistically significant. All data for the experiment were expressed as mean ± SD.

Results

Antifungal activity of AMP-17. In a previous study, AMP-17 was shown to have strong antifungal activity against *C. albicans* ATCC76615, with a MIC of 2 µg/ml (Guo et al. 2017). To further validate its antifungal effect against several common clinical pathogens, actively growing fungal cells of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. neoformans*) were treated with purified AMP-17 protein. Notably, 100 µg/ml AMP-17 could inhibit or kill *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. neoformans* in SDB medium. Compared to the PBS negative control, AMP-17 showed antifungal activity against each fungus from 2 to 24 h of culture. When fungi were cultured for 24 h, the fungicidal effect of AMP-17 was slightly different between species. The fungicidal curves of *C. albicans* and *C. tropicalis* increased slightly, but were lower than the PBS control group, while the fungicidal curves of *C. krusei*, *C. parapsilosis,* and *C. neoformans* were linear. Among them, AMP-17 had the strongest antifungal effect on *C. neoformans*. The efficiency with which 100 µg/ml AMP-17 inhibited the growth of several common clinical pathogens was comparable to that of the clinical fungicide fluconazole at 100 µg/ml,

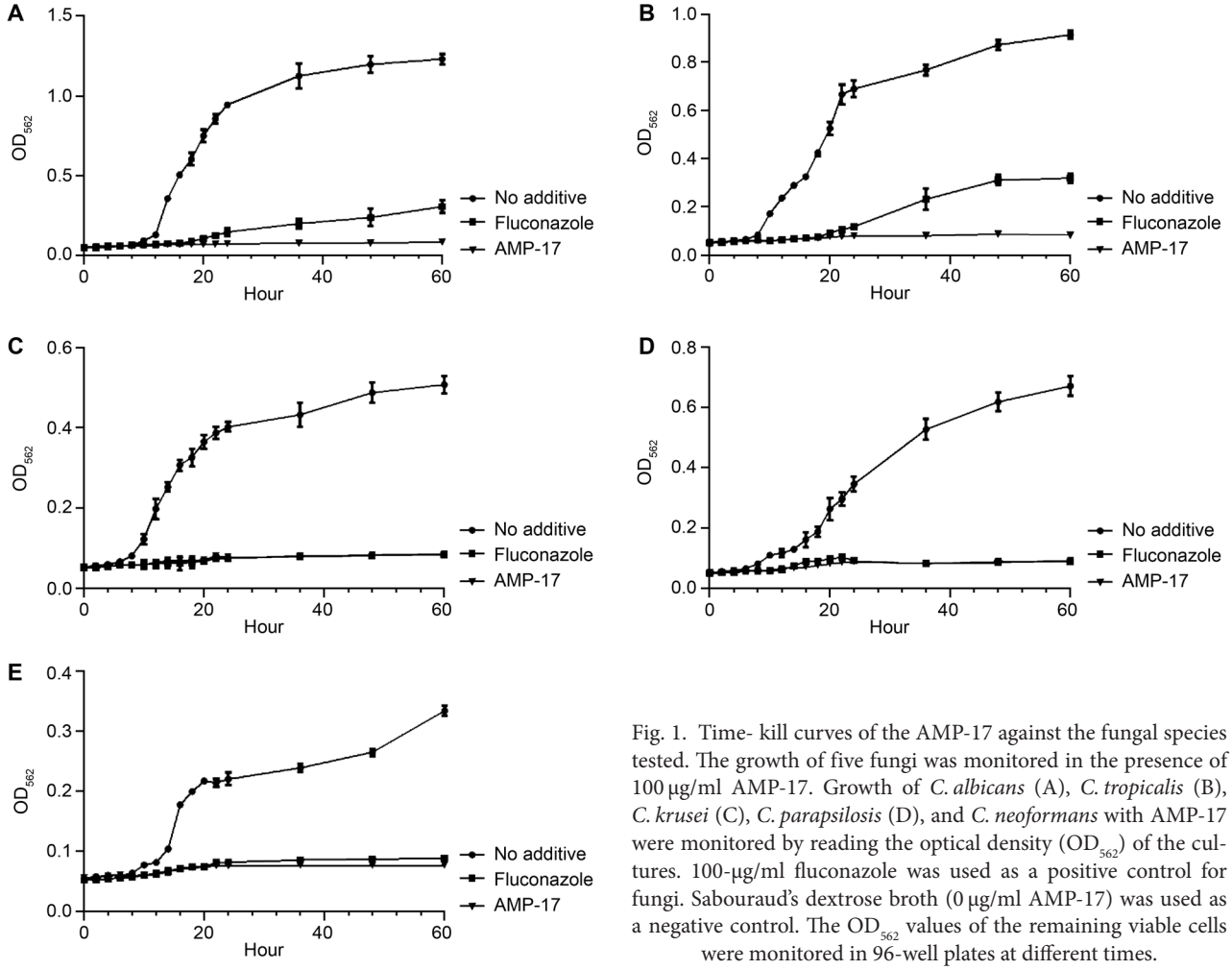

Fig. 1. Time- kill curves of the AMP-17 against the fungal species tested. The growth of five fungi was monitored in the presence of 100 μg/ml AMP-17. Growth of *C. albicans* (A), *C. tropicalis* (B), *C. krusei* (C), *C. parapsilosis* (D), and *C. neoformans* with AMP-17 were monitored by reading the optical density (OD_{562}) of the cultures. 100-μg/ml fluconazole was used as a positive control for fungi. Sabouraud's dextrose broth (0 μg/ml AMP-17) was used as a negative control. The OD_{562} values of the remaining viable cells were monitored in 96-well plates at different times.

and the fungistatic efficiency of AMP-17 was slightly stronger than that of fluconazole (Fig. 1).

To better evaluate the antifungal activity of AMP-17, the MICs and MFCs for several common clinical pathogens were determined. MIC and MFC values of AMP-17 against *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* were 18.75 μg/ml and 37.5 μg/ml, respectively. For *C. neoformans,* MIC and MFC values of AMP-17 were 9.375 μg/ml and 18.75 μg/ml, respectively (Table I–II). These values demonstrated that AMP-17 exhibited significant antifungal activity against several common clinical pathogenic yeasts.

Physicochemical properties of AMP-17. Physical and chemical factors such as ionic strength, temperature, protease, and pH were some of the most important factors that might affect the AMP activity. In the

Table I
MIC values of AMP-17 against the fungi species tested.

Peptide	MIC (μg/ml)				
	C. albicans ATCC10231	*C. krusei* IFM56881	*C. tropicalis* IFM57016	*C. parapsilosis* ATCC22019	*C. neoformans* IFM51426
AMP-17	18.75	18.75	18.75	18.75	9.375

Table II
MFC values of AMP-17 against the fungi species tested.

Peptide	MIC (μg/ml)				
	C. albicans ATCC10231	*C. krusei* IFM56881	*C. tropicalis* IFM57016	*C. parapsilosis* ATCC22019	*C. neoformans* IFM51426
AMP-17	37.5	37.5	37.5	37.5	18.75

Table III
Influences of temperature and the number of freeze-thaw times on the antifungal activity of AMP-17.

Temperature (98°C) (min)	Colony count ($\bar{x} \pm SD$)	Temperature (–80°C) (Times)	Colony count ($\bar{x} \pm SD$)
5	69.50 ± 4.95**	1	(240.50 ± 3.54**) × 10^2
20	6.00 ± 2.83**	2	(273.50 ± 3.54**) × 10^2
30	4.50 ± 2.12**	4	(342.00 ± 8.49**) × 10^2
60	6.50 ± 0.71**	6	(357.00 ± 1.41**) × 10^2
90	9.50 ± 3.54**	8	(351.50 ± 6.36**) × 10^2
120	3.50 ± 0.71**	10	(370.50 ± 3.54**) × 10^2
Negative control (ddH_2O)	565900.00 ± 17112.00	Negative control (ddH_2O)	(5507.00 ± 147.08) × 10^2

Note: Compared with ddH_2O as a control, ** $p < 0.01$

presence of NaCl and $MgCl_2$ at a concentration from 0 to 200 mmol, the MIC value of *C. albicans* increased with an increase in the metal ion concentration, indicating a decrease in antifungal activity for AMP-17. The effect of NaCl on the antifungal activity of AMP-17 was stronger than that of $MgCl_2$ (Fig. 2). By contrast, AMP-17 did not show any loss of activity when exposed to temperatures up to 98°C for 120 min and was enhanced. AMP-17 also still had a strong antifungal activity after 10 cycles of freezing and thawing at –80°C. Therefore, the antifungal activity associated with AMP-17 protein was both heat tolerant and freeze-thaw resistant (Tables III). However, the antifungal activity of AMP-17 was still significant after treatment with trypsin, pepsin, and proteinase K for 20 min ($p < 0.05$). The antifungal activity of AMP-17 was significantly decreased after treatment with trypsin, pepsin and proteinase K for 40 min ($p < 0.05$). This phenomenon revealed that the anti-*C. albicans* activity of AMP-17 decreased significantly after long treatment with trypsin, pepsin and protease K, indicating that AMP-17 was sensitive to trypsin, pepsin and protease K (Fig. 3).

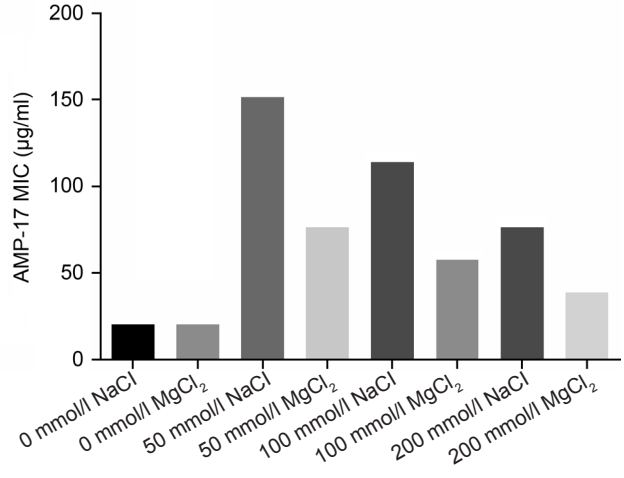

Fig. 2. Influences of various NaCl or $MgCl_2$ concentrations on the antifungal activity of AMP-17. The MIC value of AMP-17 against *C. albicans* was determined under the influence of different concentrations of NaCl or $MgCl_2$ according to the conditions recommended by CLSI (2008).

Hemolytic assay of AMP-17. The toxicity of the new AMP, AMP-17 of *M. domestica,* was evaluated with human red blood cells. The human red blood cells were incubated with AMP-17 at 37°C for 1 h, and the level

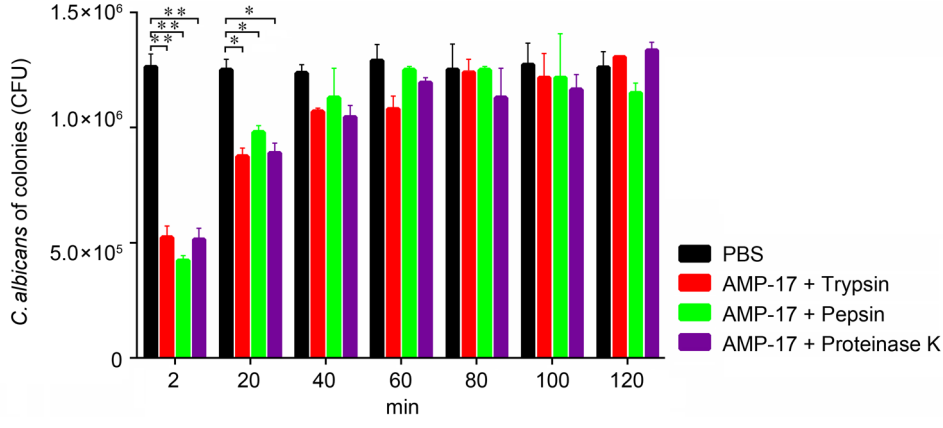

Fig. 3. Influences of trypsin/pepsin/proteinase K on AMP-17 when used against *C. albicans.* Fifty μl of the proteases (at a concentration of 1 mg/ml) and 50 μl AMP-17 (at a concentration of 37.5 μg/ml) were mixed in a 96-well plate and placed in a 37°C water bath for 2, 20, 40, 60, 80, 100, and 120 min. Then, it was subjected to CFU enumeration after being treated with 100 μl of the fungal suspension for 18 hours. PBS was used as a negative control. Compared with PBS control, *$P < 0.05$; **$P < 0.01$.

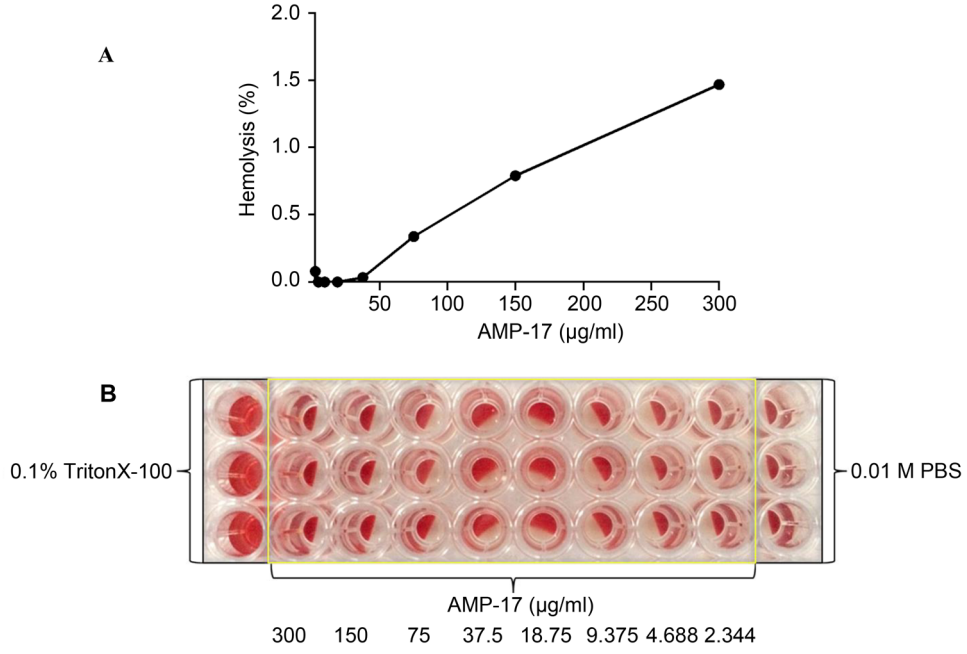

Fig. 4. Hemolytic activities of AMP-17 *in vitro.* The diluted human red blood cells (100 μl) were incubated with AMP-17 at 37°C for 1 h and spun at 225×g for 10 minutes. The supernatants (100 μl) were transferred to a 96-well plate for measurement of the absorbance of the supernatants at 450 nm by the ultra-microplate spectrophotometer. 0.1% Triton-X 100 and 0.01M PBS were used as a positive control and a negative control, respectively. Fig. 4A is based on Fig. 4B calculated by the hemolysis rate formula. Fig. 4B was the original experimental diagram.

of hemolysis was determined. Notably, the hemolysis rate of AMP-17 at 16 × MIC was only 1.47%, which was far below that of the prescribed drug standard (5%). As AMP-17 was not hemolytic to human red blood cells, it may lead to the conclusion that it has great drug development and application potential (Fig. 4).

Discussion

With the increase in the incidence of pathogenic fungal infections, antifungal drugs are increasingly being used in clinical practice; however, drug-resistant strains are also gradually increasing, posing a serious health problem (Loeffler and Stevens 2003). Therefore, the development of new natural antifungal drugs has become a hot topic of current research, with research on AMP functions from *M. domestica* being carried out in-depth. In addition to traditional AMPs, more new classes of high-efficiency molecules have been discovered. For example, Liu et al. (2016) isolated the antifungal peptide termicin from the salivary glands and blood cells of the termite *Pseudacanthotermes spiniger*. Iijima et al. (1993) isolated an antifungal peptide (AFP) from the hemolymph of *Sarcophaga peregrina* larvae, which inhibits the growth of *C. albicans,* causing fungal cytoplasmic leakage and fungal death.

Guo et al. (2017) identified AMP-17 protein as a new AMP, which has a strong antifungal activity against *C. albicans*. In the current study, these findings were advanced by evaluating antifungal spectrum, time-kill curves, physicochemical properties, and cytotoxicity of AMP-17. *In vitro* antifungal test results showed that AMP-17 protein had strong antifungal activity against several common clinical pathogenic fungi, with MICs and MFCs ranging from 9.375 μg/ml to 18.75 μg/ml and from 18.75 μg/ml to 37.5 μg/ml, respectively. Among them, AMP-17 had better antifungal activity against *C. neoformans* than other *Candida* species. These results indicated that different organisms had different biological effects, suggesting that the antifungal activity of AMP-17 was selective. This might be related to the surface structure of the fungal cell, the type, and content of the membrane proteins or differences in physiological metabolism. Klotz et al. (1985) observed the effects of environmental factors on cell surface hydrophobicity (CSH), and found that it changed rapidly on the surface of *C. albicans* cells after changing the culture environment, while a change for *C. glabrata* was not obvious. In addition, the results of a time-sterilization dynamic curve showed that the fungus began to grow after 6 h of incubation in the PBS group, and this increased in an upward trend. The positive control group (FLC) and the experimental group (AMP-17) were almost inhibited by several common pathogenic yeasts within 0–60 h, which showed the phenomenon of fungistasis after sterilization; however, the effect had some differences. The antifungal effect of AMP-17 on *C. albicans*

and *C. tropicalis* was almost completely inhibited within 0–60 h, while the antifungal effect of FLC on both was gradually reduced after 20 h. The antifungal effects of AMP-17 and FLC on *C. krusei*, *C. parapsilosis* and *Cryptococcus neoformans* were almost completely inhibited within 0–60 h. Therefore, this phenomenon suggested that in addition to the membrane-bound fungicidal mode of conventional cationic AMPs, AMP-17 might also bind target sites in different antifungal mechanisms in the cytoplasm, though this needs further verification.

Although AMP-17 has good antifungal activity against several common clinical pathogenic fungi, its physicochemical properties were not known, which prompted us to carry out the experiments in the current study. It was found that AMP-17 protein had good thermal stability, antifreeze and salt stability; however, its antifungal activity was easily destroyed by protease. Under the action of proteases (trypsin, pepsin and proteinase K), the antifungal activity of AMP-17 protein decreased with time of treatment, to the point when it was lost. Tang et al. (2015) also found that an AMP was weakened by protease action. It was suggested that under certain temperature conditions, a variety of proteases could hydrolyze the carboxyl-terminal peptide bond of AMP-17 protein, destroying the spatial structure of the protein and leading to loss of antifungal activity; however, the specific mechanisms need further verification. In this study, it was also found that on exposure to a high-temperature environment (98°C) for 5 to 120 minutes, the antifungal activity of AMP-17 did not decrease, but increased. This was in contrast to Zhang et al. (2017). The reason for the increase in activity of AMP-17 protein against *C. albicans* might be that the spatial structure of the AMP-17 protein changes after high-temperature treatment; however, the specific reasons need further verification.

Cytotoxicity, as measured by human red blood cell hemolysis, is an important factor in new drug development. Hemolysis concentration-50 (HC_{50}), as one of the most commonly used indicators of cytotoxicity, provides strong technical support in the development of new drugs (Konai et al. 2014). At present, although AMPs are expected to be the best substitute for antibiotics, most AMPs still show cell hemolysis, which limits their use as drugs. For example, Chang et al. (2017) found that the AMP TP4 residue A12I/A15I had a higher hemolytic activity, which may be due to the increased hydrophobicity in the main helix caused by the A12I/A15I mutation, which reduces bacterial outer membrane target protein, OprI, binding, and bactericidal activity, but increases hemolytic activity. The phenomenon is similar to that of Chen et al. (2005), who also found that the hemolysis of AMP was related to the content of hydrophobic residues. As the hydrophobicity increased, the helicity and self-assembly ability of the AMP also increased. As a new type of potential AMP, the cell hemolysis characteristics of AMP-17 could be key in drug design. Surprisingly, hemolysis by AMP-17 protein was much lower than the prescribed drug hemolysis standard (5%), making it a promising antifungal agent.

In conclusion, AMP-17 protein has extensive antifungal activity against several common pathogens. Besides, it has strong stability and low hemolytic activity. These characteristics make AMP-17 protein an attractive molecule for development and application in medicine.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Warszawa, 12.09.2019 r.

Szanowni Państwo,

Polskie Towarzystwo Mikrobiologów organizuje w przyszłym roku **XXIX Ogólnopolski Zjazd PTM w Warszawie w terminie: 15–18 września 2020 r.** Jest to okazja do zdobycia wiedzy mikrobiologicznej, podzielenia się swoimi osiągnięciami badawczymi oraz do spotkania merytorycznego i towarzyskiego osób zainteresowanych wspólną tematyką naukową.

PTM zostało powołane 92 lata temu i Ogólnopolskie Zjazdy Towarzystwa organizowane są co 4 lata (www.microbiology.pl).

Jest to największe wydarzenie naukowe związane z mikrobiologią i najliczniejsze spotkanie polskich mikrobiologów w tym okresie. Zwykle bierze w nim udział ponad pół tysiąca mikrobiologów: naukowców, nauczycieli akademickich, lekarzy, diagnostów laboratoryjnych, specjalistów pracujących w służbie zdrowia, weterynarii, instytutach naukowych i uczelniach, a także w przemyśle, rolnictwie oraz wykonujących mikrobiologiczne badania kontrolne w rozmaitych obszarach. W Zjazdach PTM udział biorą firmy diagnostyczne, chemiczne, farmaceutyczne, kosmetyczne, wytwarzające żywność, środki przeciwdrobnoustrojowe, odczynniki, testy oraz aparaturę kontrolną i badawczą stosowaną w rozmaitych działach mikrobiologii.

Wiele mikrobiologicznych badań naukowych, rozwojowych i użytkowych dotyczy aktualnych problemów: narastającej lekooporności drobnoustrojów chorobotwórczych, doskonalenia diagnostyki mikrobiologicznej, badań genetycznych drobnoustrojów, badań mikrobiologicznego zanieczyszczenia środowiska, GMO, poprawy systemu jakości i metod kontroli żywności, leków, wyrobów medycznych, produktów biobójczych i kosmetycznych.

Planujemy, że obrady będą odbywały się w sesjach naukowych:
* Mikrobiom człowieka i probiotyki; * Zakażenia układu pokarmowego; * Zakażenia układu oddechowego;
* Zakażenia układu moczowo-płciowego; * Oporność bakterii na antybiotyki – mechanizmy lekooporności;
* Genetyka drobnoustrojów; * Dochodzenie epidemiologiczne z zastosowaniem metod molekularnych;
* Nowe terapie przeciwbakteryjne – kandydaci na leki; * Bakteriofagi i peptydy – jako nowe terapie alternatywne;
* Aktualne problemy w wakcynologii; * Immunologia zakażeń;
* Nowości w obszarze sterylizacji, dezynfekcji i antyseptyki; * Patomechanizmy zakażeń; * Mykologia;
* Wirusologia; * Mikrobiologia środowiskowa naturalnego, bioróżnorodność i bioremediacja;
* Mikrobiologia środowiska przemysłowego i biotechnologia; * Mikrobiologia żywności;
* Mikrobiologia weterynaryjna; * Varia.

Planujemy również prezentacje najnowszych prac habilitacyjnych i doktorskich z obszaru mikrobiologii, a także sesje sponsorowane przez firmy.

Zachęcamy wszystkie zainteresowane osoby do zarezerwowania sobie terminu 15–18.09.2020 na uczestnictwo w tym wielkim wydarzeniu w świecie mikrobiologii polskiej. Zachęcamy również wszystkich mikrobiologów, a zwłaszcza młodych adeptów tej nauki do przygotowania interesujących wystąpień i podzielenia się wynikami swoich badań naukowych, jak również rutynowych.

Sekretarz Komitetu Organizacyjnego
XXIX Zjazdu PTM

SEKRETARZ
Polskiego Towarzystwa Mikrobiologów
A. Laudy
dr hab. n. farm. Agnieszka E. Laudy

Przewodniczący Komitetu Organizacyjnego
XXIX Zjazdu PTM

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski

Sprawy Logistyczne i Marketingowe Zjazdu:
Global Congress Sp. z o.o.
ul. Mokotowska 4/6 lok. 103, 00-641 Warszawa
info@globalcongress.pl, www.globalcongress.pl

Główny Organizator Zjazdu:
Polskie Towarzystwo Mikrobiologów
ul. S. Banacha 1b, 02-097 Warszawa
ptm.zmf@wum.edu.pl, www.microbiology.pl

KONFERENCJA POD PATRONATEM PTM

„PIERWSZE MAZOWIECKIE SPOTKANIE MIKROBIOLOGÓW I EPIDEMIOLOGÓW"

Warszawa, 30.09.2019 r.

Patronat Honorowy

Prof. Katarzyna Dzierżanowska-Fangrat
Konsultant krajowy w dziedzinie mikrobiologii lekarskiej

Kierownik Programowy

Prof. Ewa Augustynowicz-Kopeć
Konsultant wojewódzki w dziedzinie mikrobiologii lekarskiej

Patronaty Naukowe

Polskie Towarzystwo Zakażeń Szpitalnych
Polskie Towarzystwo Mikrobiologów
Polskie Towarzystwo Wakcynologii

Polskie Towarzystwo Wakcynologii

Tematy wiodące

- Odra – sytuacja epidemiologiczna w Polsce i województwie mazowieckim. Czy grozi nam epidemia?
- Krztusiec – nadal groźny pomimo kilku dekad szczepień.
- Borelioza – drogi zakażenia. Jaka skuteczna terapia?
- Grypa i paragrypa – infekcje sezonowe, zakażenia górnych dróg oddechowych.

Lokalizacja	Termin	Opłaty
Instytut Biocybernetyki i Inżynierii Biomedycznej PAN ul. Księcia Trojdena 4, 02-109 Warszawa	30 września 2019 r. (poniedziałek)	Konferencja bezpłatna. Uczestnictwo na podstawie rejestracji

Szczegółowe informacje oraz bezpłatna rejestracja na stronie internetowej:
smie.mavipuro.pl

Za udział w konferencji uczestnikom przysługują **4 punkty edukacyjne.**
Zgodnie z Rozporządzeniem Ministra Zdrowia z dnia 24 lipca 2017 r. w sprawie ciągłego szkolenia diagnostów laboratoryjnych (Dz.U.2017, poz. 1510)

Organizator

MAVIPURO

Patronat medialny

ZAKAŻENIA XXI WIEKU

KONFERENCJA POD PATRONATEM PTM

„MEDYCYNA LABORATORYJNA – INTERDYSCYPLINARNA ROLA MLD W SYSTEMIE OCHRONY ZDROWIA"

Bydgoszcz, 10.10.2019 r.

Tematyka konferencji obejmie różne aspekty pracy w medycznym laboratorium diagnostycznym (m.in. normy jakości, benchmarking) i zawodu diagnosty laboratoryjnego (m. in. aktualne wymagania prawne, szkolenia podyplomowe). Podczas konferencji przedstawione będą również zagadnienia dotyczące choroby Alzhaimera, patomechanizmu i diagnostyki zaburzeń nerek, czy oznaczania alkoholu etylowego.

Lokalizacja:

Budynek Farmacji Collegium Medicum w Bydgoszczy Uniwersytetu Mikołaja Kopernika w Toruniu sala nr 35

Organizatorzy:

Krajowa Izba Diagnostów Laboratoryjnych – przedstawiciele województwa kujawsko-pomorskiego w Krajowej Radzie Diagnostów Laboratoryjnych

Patronat honorowy: Minister Zdrowia

Partnerzy:

Polskie Towarzystwo Mikrobiologów Oddział w Bydgoszczy,
Polskie Towarzystwo Diagnostyki Laboratoryjnej Oddział w Bydgoszczy,
Wydział Farmaceutyczny CM UMK, Uniwersytet Medyczny w Łodzi,
Narodowy Fundusz Zdrowia, Roche Diagnostics

Kontakt:

d.krawiecka@kidl.org.pl, a.meyer-stachowska@kidl.org.pl, biuro@kidl.org.pl

Informacje o programie spotkania dostępne są na stronie www.kidl.org.pl

Konferencja pod patronatem PTM

„MAKRO-kierunki w MIKRO-biologii"

Warszawa 02.12.2019 r.

Zapraszamy do udziału w ogólnopolskiej konferencji historyczno-naukowej pn. „MAKRO-kierunki w MIKRO-biologii", która odbędzie się dnia 2 grudnia 2019 r. na Wydziale Biologii Uniwersytetu Warszawskiego.

Konferencja organizowana jest przez Zarząd Oddziału Warszawskiego Polskiego Towarzystwa Mikrobiologów dla uczczenia 70 lat działalności Oddziału Warszawskiego PTM. W trakcie konferencji odbędą się dwie sesje. W sesji historycznej wspominani będą główni animatorzy i założyciele Oddziału. Sesja naukowa, pomyślana jako seria krótkich, eksperckich wykładów, przybliży osiągnięcia i postępy mikrobiologii w jej kluczowych dziedzinach – tytułowych makro-kierunkach.

Zgłoszenia na konferencję będą przyjmowane do dnia 15 listopada br.,
poprzez formularz rejestracyjny dostępny pod adresem:
http://ptm.waw.pl/rejestracja/.

Szczegółowe informacje o wydarzeniu znajdą Państwo na stronie internetowej:
http://ptm.waw.pl/.

Od ostatniej informacji o działalności Zarządu Głównego Polskiego Towarzystwa Mikrobiologów, zamieszczonej w zeszytach nr 2 z 2019 r. kwartalników Postępy Mikrobiologii i Polish Journal of Microbiology, Prezydium ZG PTM zajmowało się następującymi sprawami:

1. Podjęło **Uchwałę nr 18–2019** w sprawie przyjęcia 9 nowych członków zwyczajnych PTM.
2. Podjęto **Uchwałę nr 19–2019** w sprawie objęcia patronatem konferencji „Pierwsze Mazowieckie Spotkanie Mikrobiologów i Epidemiologów" organizowanej przez czasopismo „Zakażenia XXI wieku" oraz Zakład Mikrobiologii IGiChP Krajowego Referencyjnego Laboratorium Prątka w Warszawie, które odbędzie się w Warszawie 30 września 2019 r.
3. Podjęło **Uchwałę nr 20–2019** w sprawie przyjęcia 5 nowych członków zwyczajnych PTM.
4. Podjęto **Uchwałę nr 21–2019** w sprawie wykorzystania logo PTM w materiałach konferencyjnych oraz objęcia patronatem konferencji „Medycyna laboratoryjna – interdyscyplinarna rola MLD w systemie ochrony zdrowia" organizowanej przez Krajową Izbę Diagnostów Laboratoryjnych Oddział Kujawsko-Pomorski i Polskie Towarzystwo Mikrobiologów Oddział w Bydgoszczy, która odbędzie się w Bydgoszczy w dniu 10 października 2019 r.

4. Podjęto **Uchwałę nr 22–2019** w sprawie wykorzystania logo PTM w materiałach konferencyjnych oraz objęcia patronatem konferencji „MAKRO-kierunki w MIKRO-biologii" organizowanej w Warszawie w dniu 2 grudnia 2019 r. dla uczczenia 70-lecia istnienia Oddziału Warszawskiego PTM.

5. Informujemy, że podczas FEMS Council 6–7.09.2019 r. w Mediolanie, **profesor Hilary Margaret Lappin-Scott** z Uniwersytetu Exeter, Exeter, Devon, South West England, Wielka Brytania **została powołana na Prezydenta FEMS**. Jej głównym obszarem badawczym jest biofim. W latach 2009–2012 prof. Hilary sprawowała funkcję prezydenta Society for General Microbiology. W latach 2006–2010 była prezydentem International Society for Microbial Ecology. W 2018 r. została odznaczona Orderem Imperium Brytyjskiego. (OBE).
   Na Sekretarza Generalnego FEMS wybrana została profesor Branka Vasiljevic, z Institute of Molecular Genetics and Genetic Engineering, z Belgradu, Serbia.

6. Informujemy również, że Komitet Organizacyjny **XXIX Zjazdu PTM** intensywnie pracuje nad organizacją zjazdu w tym pozyskiwaniem sponsorów. Spodziewamy się licznej obecności na naszym Zjeździe członków PTM a także mikrobiologów nie zrzeszonych w naszym Stowarzyszeniu.

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CZŁONKOWIE WSPIERAJĄCY PTM

Członek Wspierający PTM – Złoty
od 27.03.2017 r.

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

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

Członek Wspierający PTM – Srebrny
od 12.09.2017 r.

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

Członek Wspierający PTM – Srebrny
od 12.12.2017 r.

Od ponad 100 lat siedziba Wodociągów Krakowskich mieści się przy ul. Senatorskiej. Budowę obiektu ukończono w 1913 roku. W 2016 r. do sieci wodociągowej wtłoczono ponad 56 mln m^3 wody. Szacuje się, że ponad 99,5% mieszkańców Gminy Miejskiej Kraków posiada możliwość korzystania z istniejącej sieci wodociągowej.

Członek Wspierający PTM – Zwyczajny
od 12.09.2017 r.

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

Członek Wspierający PTM – Zwyczajny
od 06.06.2019 r.

BART Spółka z o.o. Sp. K
ul. Norwida 4, 05-250 Słupno
NIP: 1180741884, KRS: 0000573068
https://bart.pl/, email: info@bart.pl

Firma BART jest producentem i dystrybutorem surowców oraz dodatków dla przemysłu spożywczego i farmaceutycznego. Specjalizujemy się w probiotykach oraz surowcach uzyskiwanych metodami biotechnologicznymi.
Współpracujemy z renomowanymi producentami: Probiotical, Gnosis, Lesaffre