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ASSESSMENT OF DRUG SUSCEPTIBILITY AND BIOFILM FORMATION ABILITY OF CLINICAL STRAINS OF *LISTERIA MONOCYTOGENES*

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ABSTRACT

INTRODUCTION: *Listeria monocytogenes* is a cause of listeriosis, which is dangerous especially for the elderly, immunocompromised people, and pregnant women. The ability of these pathogens to colonise biotic and abiotic surfaces and form biofilm poses a serious threat for hospitalised, catheterised patients.

MATERIAL AND METHODS: The study was conducted on 29 *L. monocytogenes* strains isolated from clinical materials (blood, cerebrospinal fluid, swabs from vagina) and the reference strain *L. monocytogenes* ATCC 1911. The ability of the tested strains to form biofilm in 96-well plates and their drug susceptibility (disk diffusion method) was determined.

RESULTS: All strains formed biofilm, but its intensity was correlated with the source of isolation. A strong biofilm formed in 72.73% of isolates from cerebrospinal fluid [(A570 0.421–1.3), 75.0 % of blood isolates 9 (A570 0.389–1.063), and 50.0 % of isolates from vaginal swabs (A570 0.457–0.487)]. The strongest biofilm was formed by strains derived from cerebrospinal fluid whereas isolates from vaginal swabs, which strongly formed a biofilm, accounted for 50.0% of the studied population (absorbance 0.457–0.487).
It was found that 93.1% (n = 27) of strains were susceptible to all drugs tested. Two strains (6.9%) were resistant to cotrimoxazole and one strain (3.45 %) to erythromycin.

CONCLUSIONS: The diverse ability of clinical *L. monocytogenes* strains to form biofilm is an important aspect in the prophylaxis of catheterised patients.

KEY WORDS: *Listeria monocytogenes*; biofilm; crystal violet; drug susceptibility

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INTRODUCTION

L. monocytogenes is a Gram-positive, relatively anaerobic, widely distributed in nature (water, soil, wastewater), bacterium causing listeriosis [1]. The most susceptible to listeriosis are: elderly and immunocompromised people as well as pregnant women and newborns. *L. monocytogenes* has the ability to cross natural human barriers: the blood-brain, intestinal, and placental barrier [2]. *L. monocytogenes* may cause mild infections such as gastroenteritis or contribute to more severe infections affecting the central nervous system (CNS) [3]. Clinical symptoms include fever, nausea and vomiting, diarrhoea, and in the case of invasive forms: bacteraemia and meningitis [4]. In recent years, there have been numerous cases of meningitis caused by *L. monocytogenes*

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worldwide. The incidence is estimated at 0.05 and 0.2 cases per 100,000 population [5].

Bacterial biofilms are found on almost every surface in the natural, medical, and industrial environment. In the hospital environment, bacterial biofilms occur, among others, on catheters and implants (e.g. heart valves) leading to difficulties in treatment and chronic infections [6]. *L. monocytogenes* colonises and forms biofilm both on biotic and abiotic surfaces. The structure of the biofilm increases bacterial resistance to antibiotics as well as the immune response of the host [7, 8]. In addition, sub-lethal doses of antibiotics may increase the formation of bacterial biofilm, which hinders the treatment process [9, 10]. Microorganisms that form biofilms often cause infections that are difficult to detect using conventional culturing methods [11].

The objective of this study was to assess the ability of biofilm formation using crystal violet and drug susceptibility of *L. monocytogenes* strains isolated from clinical materials.

MATERIAL AND METHODS

Material

The material for the study consisted of 29 strains of *L. monocytogenes* isolated from clinical materials [blood (BL), cerebrospinal fluid (CSF), vaginal swab (VS)] and the reference strain *L. monocytogenes* ATCC 1911. The tested strains are the part of collection of Department of Microbiology, L. Rydygier Collegium Medicum in Bydgoszcz of the Nicolaus Copernicus University in Toruń.

The species identification of the cultured strains was carried out using the MALDI TOF MS apparatus (Bruker), according to the manufacturer's instructions.

Assessment of biofilm formation using crystal violet (CV)

Bacteria were grown for 24 hours, and suspensions of 0.5 McFarland scale density in Mueller Hinton broth (MHB, Becton Dickinson) were prepared. Then, 20 μl of each suspension was added, in triplicate, to 96-well plates containing 180 μl of sterile MHB medium. The negative control was 200 μl of sterile MHB medium. The positive control was the strain *Staphylococcus aureus* ATCC 35556, for which intensive biofilm formation has been proven. The culture plates were incubated for 24 hours (37°C) in a humid chamber. Next, the bacterial suspension was removed, and each well was rinsed three times with sterile distilled water and air-dried (20 minutes, 37°C). Then methanol (200 μl, Avantor) was added to the wells and the plates were shaken (400 rpm, 20 minutes, 25°C). The methanol was removed and the wells were air-dried. A water solution of 0.1% crystal violet (200 μL, Merck) was then added and plates were shaken (400 rpm, 20 minutes, 25°C). Crystal violet was subsequently removed, and the plates were rinsed with water to obtain colourless washings and allowed to evaporate (20 minutes, 37°C). After drying, methanol (200 μl) was added to each well and plates were shaken (400 rpm, 5 minutes, 25°C). Absorbance was measured at 570 nm wavelength (BIO-TEK spectrophotometer, Synergy HT Multi-detection) using the KC4 v3.4 and KC4 Signature program.

Evaluation of drug resistance of *L. monocytogenes* strains

Antibiotic susceptibility assessment was made using the disk-diffusion method. Bacteria from 24-hour Columbia Agar with 5.0% sheep blood plates (CAB, bioMérieux) were used to prepare suspensions (0.5 McF) in 0.9% saline (Avantor). The suspensions of 100 μL were spread on Mueller Hinton Agar with the addition of 5.0% equine blood and 20 μg/ml β-NAD (MHF, bioMérieux). Sensitivity of isolates to: penicillin (1 μg), ampicillin (2 μg), meropenem (10 μg), erythromycin (15 μg), and cotrimoxazole (1.25–23.75 μg) were assessed. Antibiograms were incubated at 35°C for 20 hours. After the incubation period, growth inhibition zones were measured. Interpretation of the results was made in accordance with the EUCAST v.8.0 recommendations [12].

Statistical analysis

The intensity of biofilm formation (CV method) was determined based on the measured absorbance values. The studied strains were divided, depending on the strength of biofilm formation and metabolic activity in the biofilm, into three groups: weak biofilm (T–2T), medium biofilm (> 2T–4T), and strong biofilm (> 4T). The value of T, calculated from the formula $T = x\,nc + 3\delta$, for CV is 0.091.

The arithmetic mean for the absorbance values was calculated for each *L. monocytogenes* strain tested. The obtained results were subjected to statistical analysis in the Statistica 12 PL program (StatSoft). ANOVA with the Tukey post-hoc test was used to determine significant differences between strains in the ability to form biofilm.

Based on the chi-square test and Fisher's exact test, differences in the frequency of individual drug resistance profiles between strains of different origin were checked. The correlation between the number of antibiotics that the strain was resistant to and the intensity of biofilm formation was also assessed. Significance was set at $p < 0.05$.

RESULTS

Biofilm formation intensity

It was shown that all tested strains formed a biofilm, but the intensity of its formation varied.

Among the tested strains, 66.67% (n = 20) were characterised by strong biofilm formation ability, whereas 33.33% (n = 10) of strains were classified into the group of medium biofilm formation intensity. There were no strains with weak biofilm formation ability. The intensity of biofilm formation correlated with the origin of isolates. Strong biofilm formation ability was found in eight (72.73%) strains from cerebrospinal fluid (absorbance 0.421–1.3), nine (75.0%) blood isolates (absorbance 0.389–1.063), and three (50.0%) strains from the vagina (absorbance 0.457–0.487). The most intense biofilm was created by strains derived from cerebrospinal fluid (mean absorbance value — 0.616 ± 0.341), which was significantly higher than the values established for vaginal strains (0.354 ± 0.147) and the reference strain (0.357 ± 0.076) (Fig. 1). The weakest biofilm-formers were found among strains isolated from the vagina (Fig. 2).

Drug resistance of *L. monocytogenes* strains tested

Among the 29 tested strains, resistance to cotrimoxazole was demonstrated in two (6.9%) strains, one from cerebrospinal fluid and one from vaginal smear. Erythromycin resistance was found in one (3.45%) isolate from cerebrospinal fluid.

Three drug resistance profiles were distinguished (Table 1). Profile I included 27 (93.1%) strains sensitive to all antibiotics tested, of which 12 (100.0%) were blood isolates, 10 (90.91%) were strains isolated from the cerebrospinal fluid, and five (83.33%) were isolates from the vaginal smear (Tab. 1). This profile was statistically rare among strains originating from vaginal smear. Profile II included one (3.45%) strain isolated from the cerebrospinal fluid, which was resistant to both erythromycin and cotrimoxazole. In turn, profile III comprised one (3.45%) strain isolated from a vaginal smear, in which resistance to cotrimoxazole was confirmed (Tab. 1).

There was no correlation (Guillford scale, correlation coefficient 0.211) between drug susceptibility of *L. monocytogenes* strains and the intensity of biofilm formation (Fig. 3).

DISCUSSION

It siniquitousness and the ability to form biofilms makes *L. monocytogenes* a serious pathogen, posing a threat to a wide range of patients. A particular risk group are catheterised patients. Due to its ability to produce biofilm, *L. monocytogenes* is more resistant to antibiotics, host immune system [7, 8], biocides, or stress [13, 14]. *L. monocytogenes* can produce biofilms on various surfaces used in medicine or industry, such as polystyrene, glass, and stainless steel [15, 16].

It was shown that all tested strains of *L. monocytogenes* formed a biofilm, but the intensity of its formation varied. As much as 66.67% (n = 20) of strains were characterised by a strong biofilm intensity, while 33.33% (n = 10) were characterised by medium intensity. Similar results were obtained by Cirkovic et al. (2016) [19], who showed that 66.67% (n = 8) of *L. monocytogenes* were strong biofilm formers, 25.0% (n = 3) medium biofilm formers, and 8.33% (n = 1) weak biofilm formers. In contrast, Raby et al. (2016) [17] demonstrated medium and strong biofilm formation ability in 60.0% (n = 18) and 40.0% (n = 12) of clinical strains, respectively. The strong and moderate ability of biofilm formation of clinical *L. monocytogenes* strains was also revealed by Borges et al. (2011) [18]. They confirmed that 68.0% of strains were characterised by medium intensity of biofilm formation whereas only 35.0% formed strong biofilm. In turn, Barbosa et al. (2013) [20] found that 70.3% of (n = 83) *L. monocytogenes* clinical strains formed a weak biofilm, and only 3.4% (n = 4) were strong biofilm formers [20]. Similar results were obtained by Doijad et al. (2015) [21], who showed that as much as 65.63% (n = 21) were characterised by low intensity of biofilm formation and 34.38% (n = 11) by medium biofilm intensity [21].

It was shown that strains isolated from cerebrospinal fluid formed biofilm the most intensively (average absorbance value — 0.616). On the other hand, the weakest biofilm formers were isolated from vaginal swabs (mean absorbance val-

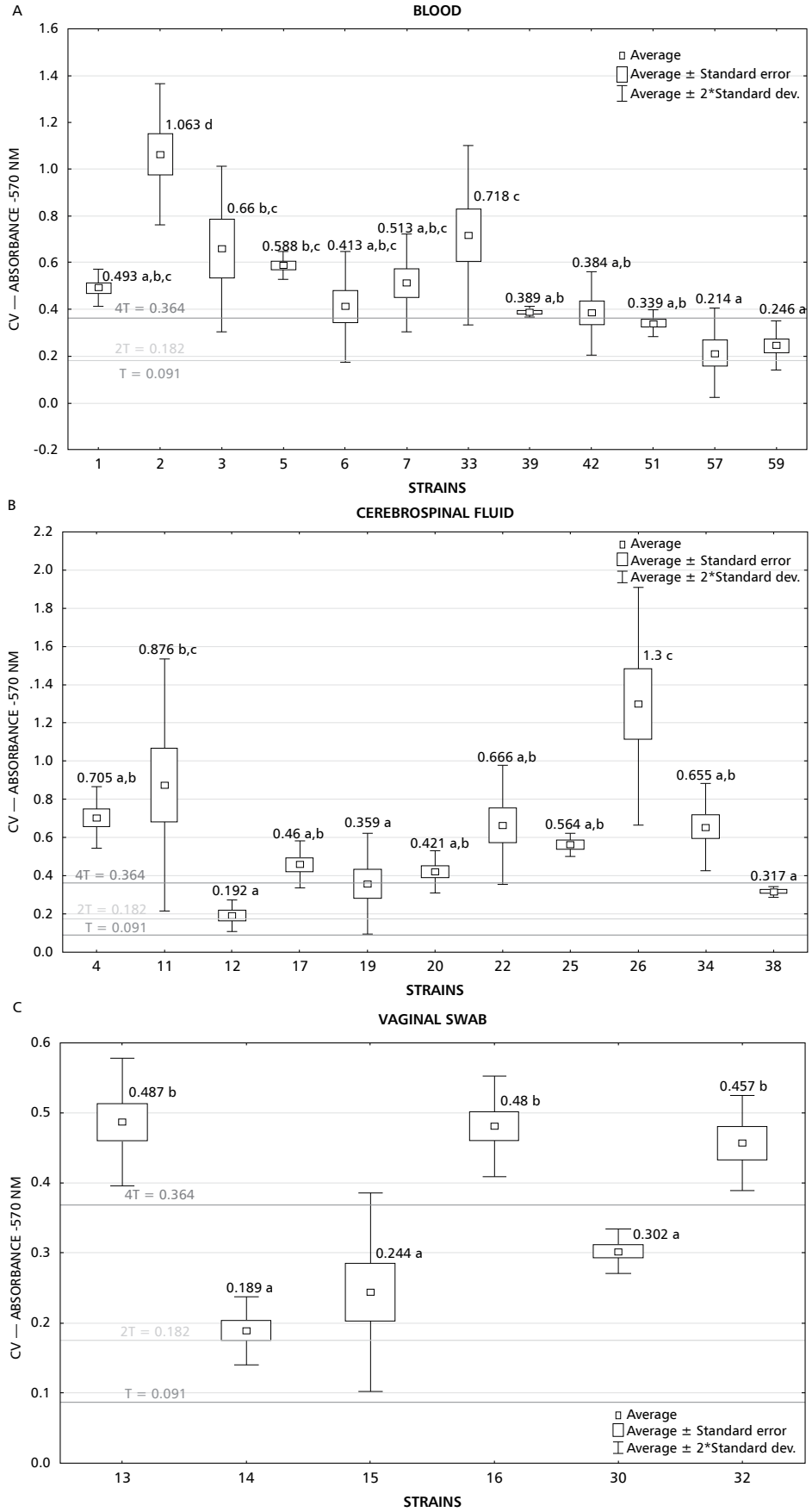

FIGURE 1. **A.** The intensity of biofilm formation among L. *monocytogenes* strains isolated from blood; **B.** Intensity of biofilm formation among *L. monocytogenes* strains isolated from cerebrospinal fluid; **C.** Intensity of biofilm formation among *L. monocytogenes* strains isolated from vaginal swabs; (a, b, c, d 3 values marked with different letters differ statistically significantly, $p \leq 0.05$)

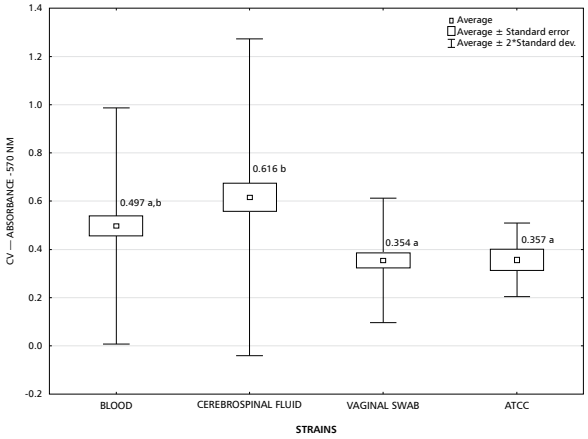

FIGURE 2. Comparison of mean values of absorbances obtained by CV between groups of *L. monocytogenes* strains (a, b — values marked with different letters differ significantly between each other, $p \leq 0.05$)

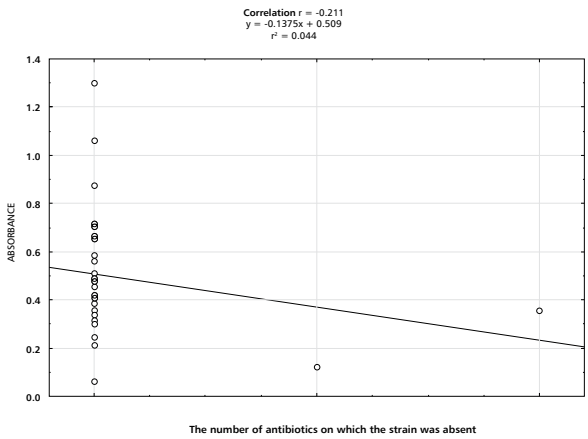

FIGURE 3. Correlation between the number of antibiotics to which the strain is resistant and the intensity of biofilm formation by *L. monocytogenes*

Table 1. Drug resistance profiles among the tested strains

The name of the drug resistance profile	Drug resistance profile	Number of strains depending on the origin [n, %]		
	BL	CSF	VS	
I	S: P, AM, E, MEM, SXT R: ---	12 (100.0%)[a]	10 (90.91%)[a]	5 (83.33%)[b]
II	S: P, AM, MEM, R: E, SXT	0[a]	1 (9.09%)[b]	0[a]
III	S: P, AM, E, MEM, R: SXT	0[a]	0[a]	1 (16.67%)[b]
Total [n, %]		12 (100.0%)	11 (100.0%)	6 (100.0%)

a, b — values marked with different letters differ significantly statistically ($p < 0.05$)

ue — 0.354). There are no data on the intensity of biofilm formation of *L. monocytogenes* isolated from different clinical materials. The study of Raby et al. (2016) [17] showed that strains of *L. monocytogenes* isolated from food produced a significantly stronger biofilm ($p \leq 0.05$) than the strains isolated from clinical material. Also, Doijad et al. (2015) [21] showed that the intensity of biofilm formation is correlated with the isolation source of the strain. Among animal isolates 34.38% (n = 11) were characterised by moderate biofilm formation intensity whereas 65.63% (n = 21) formed weak biofilm [21]. Among human isolates 55.56% (n = 10) showed a weak biofilm formation rate, and 44.44% (n = 8) were classified as biofilm-forming strains. In contrast, among strains isolated from meat, only 14.29% (n = 2) were classified into the strains with moderate biofilm intensity and as much as 85.71% (n = 12) were strains with low intensity. No strain isolated from these sources showed strong biofilm formation. On the other hand, among strains isolated from milk and milk products, 26.46% (n = 9) showed a strong biofilm formation ability, 17.65% (n = 6) of strains were moderate biofilm formers, and 55.88% (n = 19) were classified as strains of weak biofilm [21].

In our own study it was shown that 93.1% (n = 27) of strains were sensitive to all tested antibiotics. In two (6.9%) strains cotrimoxazole resistance was demonstrated, and in one (3.45%) strain – resistance to erythromycin. All isolates were sensitive to penicillin, ampicillin, and meropenem. Also, Raby et al. (2016) [17] showed that the majority of clinical strains 83.33% (n = 25) were susceptible to the tested antibiotics. However, they found three (10.0%) strains and two (6.67%) strains resistant to meropenem and penicillin, respectively [17]. The study of Winiarska (2017) [22] also revealed one strain resistant to meropenem and additionally three (30.0%) erythromycin-resistant strains, whereas

Borcan et al. (2014) [23] showed resistance to ampicillin (7.7%, n = 2), penicillin (7.7%, n = 2), and erythromycin (3.85%, n = 1). In turn, in the studies of Madeo et al. (2015) [24] and Caplan et al. (2014) [25] susceptibility to penicillin, meropenem, and erythromycin in all clinical isolates of *L. monocytogenes* was reported.

Conclusions

The results of our study show that *L. monocytogenes* strains isolated from clinical materials are characterised by a diverse ability to form biofilm. Therefore, it is recommended to control biofilm formation in catheterised patients.

Conflict of interest: The authors declare no conflict of interest.

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