ANTIMICROBIAL RESISTANCE AND BIOFILM FORMATION OF *YERSINIA PSEUDOTUBERCULOSIS* ISOLATED FROM PORK PRODUCTION CHAIN IN LITHUANIA

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Abstract. Limited information is available on antimicrobial resistance and biofilm formation of *Yersinia pseudotuberculosis*. Therefore, the goal of present study was to examine the antimicrobial resistance and biofilm formation of 27 *Y. pseudotuberculosis* 2/O:3 strains isolated from pork production chain. Antimicrobial resistance was performed with four antimicrobials by the detection of the minimum inhibitory concentrations (MIC).

All *Y. pseudotuberculosis* strains were resistant to erythromycin and sensitive to ciprofloxacin, meanwhile, 37% and 11% of tested bacteria were resistant to tetracycline and streptomycin, respectively. Obtained data on antimicrobial resistance revealed association between *Y. pseudotuberculosis* isolated from different pig farms and assigned to different genotypes ($p < 0.05$). All *Y. pseudotuberculosis* were able to form biofilms. However, no significant differences in biofilm formation of *Y. pseudotuberculosis* and antimicrobial resistance profile was observed. Additionally, no significant differences in biofilm formation and different bacteria sources, genotypes and farms were observed.

Considering the importance of this foodborne pathogen, the data presented is relevant for characterizing *Y. pseudotuberculosis* as one of human yersiniosis agents.

Keywords: *Yersinia pseudotuberculosis*, antimicrobial resistance, biofilm, pork, yersiniosis

Introduction. *Yersinia pseudotuberculosis* is one of two human-pathogenic *Yersinia* species that along with *Yersinia enterocolitica*, causes yersiniosis (Bottone, 1997). Yersiniosis was the third most commonly reported zoonosis in the EU in 2013 with the highest notification rates reported in North-Eastern European countries like Finland and Lithuania. Although *Y. enterocolitica* was the dominating species among confirmed cases, two fatal cases reported in 2013 were related to *Y. pseudotuberculosis* (EFSA and ECDC, 2015). *Y. pseudotuberculosis* is Gram-negative bacterium causing a variety of extra-intestinal and intestinal infections in humans and animals (Jalava, et al. 2006). This bacterium is widespread in nature and most often is found in the intestinal tract of pigs and various avian species (Niskanen, et al. 2003; Niskanen, et al. 2008). *Y. pseudotuberculosis* can be divided into four biotypes (1-4) and 15 serotypes (O:1-O:15) (Tsubokura and Aleksić, 1995; Bogdanovich, et al. 2003). Serotypes O:1-O:3 are mostly detected in Europe, while serotypes O:4-O:15 mainly occur in Asia (Niskanen, et al. 2009). According to European Food Safety Authority (EFSA), all *Y. pseudotuberculosis* strains and serotypes should be considered as human pathogenic (EFSA, 2007).

Y. pseudotuberculosis as well as other enteropathogenic *Yersinia* are known as cold tolerant bacteria and are able to survive and multiply at refrigeration temperatures (Palonen, et al. 2010). Additional factor, which increases survival properties of *Y. pseudotuberculosis* in the environment, is the ability to form bacterial populations known as biofilms. It has been observed that bacteria forming biofilms increase their resistance to a specific conditions like changes in temperature, pH and also to the resistance of antimicrobial agents (Mah and O'Toole, 2001; Gilbert, et al. 2002). The resistance levels to antimicrobial agents of *Y. enterocolitica* which is close related to *Y. pseudotuberculosis*, are on the rise (Fabrega and Vila, 2012). Meanwhile, data on the resistance of *Y. pseudotuberculosis* to antimicrobial agents is limited. Regular surveillance of *Y. pseudotuberculosis* antimicrobial resistance is necessary to identify the earliest possible changes in bacterial susceptibility to antimicrobials used for human yersiniosis treatment.

The aim of this work was to determine the resistance of *Y. pseudotuberculosis* 2/O:3 strains isolated from pig production chain to selected antimicrobials, as well to evaluate the ability of these bacteria to form biofilms. The data on antimicrobial resistance and ability to form biofilm of *Y. pseudotuberculosis* 2/O:3 strains isolated from pig production chain may be useful to better understand the survival of these pathogens in the pork production chain and their ability to cause human infection.

Materials and methods. In total 27 *Y. pseudotuberculosis* 2/O:3 strains were examined in this study. All *Y. pseudotuberculosis* isolates were obtained from pig production chain including 23 isolates from pig feces collected at slaughterhouses and farms (A, B, D, I and F), three isolates from pig carcasses and one isolate from pig farm B worker boots (Table 1). Tested bacteria strains were confirmed as biotype 2 using the methodology described by Tsubokura and Aleksić (1995) and as serotype O:3 based on slide agglutination test with commercial antisera O:3 for *Y. pseudotuberculosis* (Denka Seiken, Tokyo, Japan). *Y. pseudotuberculosis* 2/O:3 strains used in this study represent 2 different bacteria genotypes (Table 1). The typing was performed by Pulsed-field gel

electrophoresis method as described by Niskanen, et al. (2009). The detail methods of isolation, identification and characterization of these bacteria are described in our previous study (Novoslavskij, et al. 2013).

Detection of minimum inhibitory concentration (MIC). Antimicrobial susceptibility was tested by the agar dilution method according the CLSI guidelines (CLSI, 2012). The following four antimicrobial agents were tested: tetracycline (TET), erythromycin (ERY), streptomycin (S), and ciprofloxacin (CIP) (all Sigma-Aldrich, MO, USA). Antimicrobial susceptibility was tested on Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, UK) supplemented with antimicrobials with dilutions ranging from 0.25 to 128 mg/L for erythromycin, and from 0.0625 to 128 mg/L for ciprofloxacin, streptomycin, and tetracycline. For each isolate, 5 µl of approximately 1 x 10^7 CFU/ml bacterial suspension dissolved in PBS (phosphate-buffered saline, Oxoid, Basingstoke, Hampshire, UK) was spotted onto Mueller-Hinton agar containing antimicrobial agent and incubated at 30ºC for 24 h. The experiment for all isolates was performed in triplicate. The MIC was defined as the lowest concentration that produces complete inhibition of *Y. pseudotuberculosis* growth. The breakpoints for ciprofloxacin and tetracycline were determined according to CLSI recommendations for the family *Enterobacteriaceae* (CLSI, 2014), and breakpoint for erythromycin were determined according to CLSI recommendations for the *Campylobacter jejuni* (CLSI, 2006). The breakpoint for *Salmonella* from the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP, 2014) was applied for streptomycin, as it is not provided by the CLSI.

Table 1. **Antimicrobial resistance and biofilm formation of *Y. pseudotuberculosis* isolated from pork production chain**

Strain No	Strain data (genotype*/source/farm*)	Biofilm formation (optical density)	Antimicrobial resistance				
			Minimum inhibitory concentration (mg/L)				Resistance profile‡
			CIP†	TET†	S†	E†	
C	ATCC 29910	1.420±0.23	ns	ns	ns	ns	ns
N		0.21±0.08					
1	I/Carcass/D	3.165±0.27	<0.0625	2	2	64	E
2		2.612±0.19					E
3	I/Feaces/D	3.084±0.22					E
4		2.643±0.31					E
5		3.436±0.21					E
6		2.407±0.18					E
7		1.355±0.19					E
8		1.246±0.18					E
9		1.085±0.12					E
10		1.395±0.14					E
11	I/Feaces/B	3.653±0.24					E
12		0.919±0.13					E
13	I/Feaces/I	0.912±0.15					E
14	I/Feaces/A	0.709±0.11					E
15	I/Carcass/D	1.230±0.13			4		E
16	I/Feaces/D	1.575±0.15		4	2		E
17		1.336±0.16					E
18		0.788±0.11		64			E, TET
19		1.040±0.13	0.25	16	16		E, TET
20	I/Feaces/B	2.772±0.17	0.5	>128	8		E, TET
21		0.807±0.15					E, TET
22		1.106±0.21					E, TET
23		1.128±0.12					E, TET
24	I/Worker footwear/B	1.155±0.14		128			E, TET
25	II/Feaces/F	2.761±0.22	<0.0625	>128	>128		E, S, TET
26		0.726±0.17					E, S, TET
27		1.040±0.16		64			E, S, TET

C – control, *Y. pseudotuberculosis* ATCC 29910; N – negative control; ns – not studied; * Differences in antimicrobial resistance, $p < 0.05$; † E – erythromycin, TET – tetracycline, S – streptomycin, CIP – ciprofloxacin; ‡ Breakpoints: E – resistant if MIC ≥ 8 mg/L, TET - resistant if MIC ≥ 16 mg/L, S - resistant if MIC ≥ 32 mg/L, CIP - resistant if MIC ≥ 1 mg/L

Biofilm formation. Attached biofilms were assayed as described by Reeser, et al. (2007) with minor modifications. Twenty four well polystyrene plates (TPP® Tecno Plastic Products AG, Trasadingen, Switzerland) containing 1 ml of Mueller-Hinton broth (Oxoid, Basingstoke, Hampshire, UK) were inoculated with stationary phase bacterial cultures adjusted to OD600 = 0.25. The plates were incubated at 25°C for 24 h. After incubation the medium was removed and the wells were dried for 30 min at 55ºC. In total 1 ml of 0.1% crystal violet (CV) was added to each well for 30 min at room temperature. Unbound CV was removed and wells were rinsed two times with 1 ml of distilled water. The wells were dried at 55ºC for 15 min and bound CV was dissolved with 200 µl of 80% ethanol- 20% acetone mix. To determine biofilm formation 100 µl of dissolved CV was removed and placed into a 96-well microtiter plate and the absorbance at 540 nm (OD540) was determined using a microplate reader. The assay was repeated three times and the averages and standard deviations were calculated for each strain. In each assay, 1 ml of Mueller-Hinton broth without bacteria was included as a negative control. *Y. pseudotuberculosis* ATCC 29910 was used as positive control. The cut-off value for biofilm formation was OD540 = 0.21±0.08, which was the mean absorbance obtained from all negative controls.

Statistical analysis. The data were analyzed with SPSS 20.0 software with analysis of variance using the general linear model (GLM) procedure. Univariate analysis of variance was performed to determine the influence of the sources, farms and genotypes of *Y. pseudotuberculosis* on biofilms formation and antimicrobial resistance. Differences were considered statistically significant when $p \leq 0.05$.

Results. Performed analysis showed that *Y. pseudotuberculosis* 2/O:3 strains representing two different genotypes (I and II), different sources and farms were resistant to erythromycin with MIC of 64 mg/L and sensitive to ciprofloxacin with MIC varying from less than 0.0625 mg/L up to 0.5 mg/L (Table 1).

Altogether 37% of *Y. pseudotuberculosis* 2/O:3 strains were resistant to tetracycline with MIC varying from 16 mg/L up to more than 128 mg/L. Eleven percent of tested strains were resistant to streptomycin with MIC varying from 64 mg/L up to more than 128 mg/L. Additionally, these *Y. pseudotuberculosis* strains were confirmed as resistant to tetracycline. The data obtained on antimicrobial resistance revealed association between *Y. pseudotuberculosis* 2/O:3 strains found in different farms and belonging to different genotypes. We found that bacteria strains found in farm F and belonging to genotype II showed higher resistance level to tested antimicrobial agents ($p<0.05$). Meanwhile, no significant differences in antimicrobial resistance and different bacteria sources were observed.

All tested *Y. pseudotuberculosis* 2/O:3 strains were able to form biofilms. The optical density among biofilms forming strains varied among 0.709 and 3.4636, respectively (Table 1). However, no significant differences in biofilm formation and different bacteria sources, genotypes and farms were observed.

Discussion. The rise of antibiotic resistant bacteria including foodborne pathogens is an increasingly global threat to public health. Therefore the regular surveillance of antimicrobial resistance is necessary to identify the earliest possible changes in bacterial susceptibility to antimicrobials used for human treatment. Antimicrobial resistance of *Y. pseudotuberculosis* isolates is generally less studied compared to *Y. enterocolitica*. The lower interest in *Y. pseudotuberculosis* antimicrobial resistance studies may be consistent with the fact that this foodborne pathogen is not a main causative agent of human yersiniosis. However, it is worth noting that both fatal yersiniosis cases reported in EU in 2013 were related to *Y. pseudotuberculosis* (EFSA and ECDC, 2015), indicating relevance of this foodborne pathogen and necessity of antimicrobial resistance studies.

Consistent with previous studies (Martins, et al. 1998; Terentjeva and Berzins, 2010), our study detected a high level resistance of *Y. pseudotuberculosis* against agent of macrolide (erythromycin). The use of antibiotics as growth promoters was banned in EU since 2006; however erythromycin is still widely used as one of the pig gastrointestinal disease treatment modes in Lithuania. Thus, the resistance to erythromycin may be related to its use as a measure for preventing and treatment of infectious diseases in pigs (Teuber, 2001). Additionally, high resistance of examined *Y. pseudotuberculosis* to tetracycline and streptomycin (37% and 11%, respectively) found in our study may also be consistent with often use of these antimicrobials in veterinary practice in Lithuania. By contrast, studies from Poland, Latvia, Germany and Italy have reported that all tested *Y. pseudotuberculosis* strains were susceptible to streptomycin and tetracycline (Szych, et al. 2009; Terentjeva and Berzins, 2010; Bonke, et al. 2011; Bonardi, et al. 2016). Thus, our findings may indicate a possible increase in *Y. pseudotuberculosis* resistance to tetracycline and streptomycin. In agreement with mentioned studies, all *Y. pseudotuberculosis* strains tested in our study were susceptible to ciprofloxacin, supporting the general opinion that this antimicrobial agent is one of the most effective in human yersiniosis treatment. However, recent case report studies suggest that treatment by fluoroquinolone (ciprofloxacin) alone failed to cure the *Y. pseudotuberculosis* infection in human (Renvoisé, et al. 2015). It is noteworthy that an MIC of 0.5 mg/L was found in 19% of tested strains in our study, indicating that these strains are close to be confirmed as resistant to ciprofloxacin. In this study we attempted to identify differences in antimicrobial resistance either between *Y. pseudotuberculosis* strains collected from different pig farms, sources or between different genotypes. The data obtained on antimicrobial resistance revealed association between *Y. pseudotuberculosis* 2/O:3 strains found in different farms and belonging to different genotypes ($p<0.05$). These results suggest a limited geographical

distribution of resistant *Y. pseudotuberculosis* strains, which may be related to specific on farm practices including preventing and treatment of infectious diseases in pigs. On the other hand, such pig farm can be an important antimicrobial resistance reservoir (Williams, et al. 2016).

In our study a biofilm formation of *Y. pseudotuberculosis* 2/O:3 was performed together with antimicrobial resistance. Biofilms can be broadly defined as extracellular polymeric matrix-enclosed bacterial populations, adherent to each other and/or to surfaces or interfaces (Costerton, et al. 1995). Under the protection of biofilms, microorganisms become less sensitive to environmental changes and are more resistant to desiccation and treatment with antimicrobial and disinfection agents. Thus, bacteria forming biofilms are of great importance and challenge to medical science and food industry (Wu, et al. 2014; Kretli and Dietary, 2015). Historically, the formation of biofilms by *Y. pestis* has been better studied than biofilms of *Y. pseudotuberculosis* or *Y. enterocolitica*, thus limited data on biofilm formation of these bacteria species is available. The ability of *Y. pseudotuberculosis* to form biofilms on biotic as well as on abiotic surfaces was described in several studies (Joshua, et al. 2003; Terentieval, et al. 2015). According to studies performed with bacterial species other than *Yersinia* spp. significant differences on biofilm formation among the same bacterial species, serovars and genotypes can be detected (Borucki, et al. 2003; Deligianni, et al. 2010). Additionally, it has been described that biofilm formation significantly increases bacterial resistance to antimicrobial agents (Mah and O'Toole, 2001; Gilbert, et al. 2002).

The obtained results revealed that *Y. pseudotuberculosis* strains isolated from pig production chain were able to form biofilms, however, no significant difference in biofilm formation and different bacteria sources, genotypes and farms was observed. Despite the fact that, the difference in biofilm formation among tested bacteria strains varied up to 5 times, no correlation between biofilm formation and antimicrobial resistance of *Y. pseudotuberculosis* strains was found. Additionally, the lack of data on *Y. pseudotuberculosis* ability to form biofilms is limiting the possibility to compare our results with over reports. On the other hand, obtained results suggest that certain *Y. pseudotuberculosis* strains have a higher ability to form biofilms and can cause additional risk for humans (Wu, et al. 2014; Kretli and Dietary, 2015).

This is the first study to investigate antimicrobial resistance and biofilm formation of *Y. pseudotuberculosis* isolated from pork production chain in Lithuania. Considering the importance of this foodborne pathogen and lack of information about the ability to form biofilms and antimicrobial resistance of this bacterium, the data presented is relevant for characterizing *Y. pseudotuberculosis* as one of human yersiniosis agents.

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