COMPARISON OF ANTIMICROBIAL RESISTANCE AND BIOFILM FORMATION OF *YERSINIA ENTEROCOLITICA* 4/O:3 STRAINS ISOLATED FROM PIG PRODUCTION CHAIN AND PATIENTS CLINICAL SPECIMENS

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Abstract. The aim of the present work was to compare the biofilm formation and resistance of *Y. enterocolitica* 4/O:3 strains isolated from pig production chain and patients clinical specimens to five antimicrobials (minimum inhibitory concentration). Bacteria with different PFGE genotypes were selected for this study. Eleven *Y. enterocolitica* 4/O:3 strains from pig production chain and eight strains from patients stool clinical specimens were studied. The results of our study showed that *Y. enterocolitica* 4/O:3 strains isolated from pig production chain and patients stool clinical specimens shared similar antimicrobial resistance. All *Y. enterocolitica* strains tested were resistant to ampicillin and erythromycin, yet susceptible to ciprofloxacin. One and two of 19 tested strains were resistant to streptomycin and tetracycline, respectively. No statistically significant differences ($P<0.05$) between bacteria genotypes and antimicrobial minimum inhibitory concentration were observed. All tested *Y. enterocolitica* strains formed biofilms, however, no significant difference in biofilm formation was detected between different bacteria genotypes and no correlation between biofilm formation and antimicrobial resistance of different genotypes was observed.
Keywords: *Yersinia enterocolitica* 4/O:3, pig production chain, patients stool clinical specimens, MIC, biofilm.

YERSINIA ENTEROCOLITICA 4/O:3 PADERMIŲ, IŠSKIRTŲ IŠ KIAULIENOS, GAMYBOS GRANDINĖS IR ŽMONIŲ KLINIKINIŲ MĖGINIŲ PALYGINIMAS NUSTATANT ATSPARUMĄ ANTIMIKROBINĖMS MEDŽIAGOMS IR GEBĖJIMĄ SUDARYTI BIOPLĖVELES

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Santrauka. Tyrimai atlikti norint palyginti *Y. enterocolitica* 4/O:3 padermių, išskirtų iš kiaulienos gamybos grandinės ir sergančių žmonių išmatų mėginių, atsparumą penkioms skirtingoms antimikrobinėms medžiagoms ir gebėjimą sudaryti bioplėveles. Šioms bakterijų savybėms tirti atrinktos jersinijų padermės, priklausančios skirtingiems genotipams: vienuolika *Y. enterocolitica* 4/O:3 padermių, išskirtų iš kiaulienos gamybos grandinės, ir aštuonios padermės, išskirtos iš žmonių išmatų klinikinių mėginių. Tyrimais nustatyta, kad visos *Y. enterocolitica* 4/O:3 padermės buvo panašiai atsparios antimikrobinėms medžiagoms. Visos tirtos padermės buvo atsparios ampicilinui, eritromicinui, tačiau jautrios ciprofloksacinui. Viena *Y. enterocolitica* padermė buvo atspari streptomicinui, dvi padermės – tetraciklinui. Reikšmingų skirtumų ($p<0,05$) tarp skirtingo bakterijų genotipo ir minimalios antimikrobinės medžiagos slopinamosios koncentracijos nenustatyta. Visos devyniolika tirtų *Y. enterocolitica* padermių sudarė bioplėveles, bet reikšmingų skirtumų tarp atskirų jersinijų padermių gebėjimo sudaryti bioplėveles nenustatyta. Reikšmingų skirtumų nenustatyta ir tarp bakterijų genotipo bei gebėjimo sudaryti bioplėveles, jų atsparumo antimikrobinėms medžiagoms.
Raktažodžiai: *Yersinia enterocolitica* 4/O:3, kiaulienos gamybos grandinė, klinikiniai žmonių išmatų mėginiai, MSK, bioplėvelės.

Introduction. *Yersinia enterocolitica* is one of three human-pathogenic *Yersinia* species that along with *Y. pseudotuberculosis*, causes yersiniosis (Bottone, 1997). The third species, *Y. pestis* is the causative agent of plague and now is not detected in Europe (Anonymous, 2012). Yersiniosis is one of the three leading foodborne zoonoses in Lithuania, and an increase of human cases over the last decade has been observed (Anonymous, 2007, 2012). The incidence of 12.86 per 100 000 population was the highest among EU member states in 2010 (Anonymous, 2012). *Y. enterocolitica* is a gram-negative, oxidase-negative, facultatively anaerobic species and can be divided to several bioserotypes of witch bioserotype 4/O:3 is known as the main causative agent of human disease in continental Europe (Bottone, 1999; Anonymous, 2012). *Y. enterocolitica* is widespread in nature and is found in the intestinal tract of numerous mammals and avian species (Fredriksson-Ahomaa et al., 2006a). However, pigs are of particular importance in *Y. enterocolitica* epidemiology, as they are the main asymptomatic carriers and source of human enteropathogenic *Y. enterocolitica*, especially bioserotype 4/O:3 (Bottone, 1999; Fredriksson-Ahomaa et al., 2001, 2006b).

A majority of bacteria species increases their ability to survive in the environment or in specific conditions by

forming bacterial population referred to as biofilms. 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). Historically, the formation of biofilms by *Y. pestis* has been better studied than biofilms of *Y. enterocolitica*. Interestingly, it has been observed that the resistance of bacteria cells other than *Yersinia* spp. to antimicrobials in biofilm is significantly increased compared with what is normally seen with planktonic cells of the same bacteria. (Mah and O'Toole, 2001; Gilbert et al., 2002). Importantly, the levels of resistance of *Y. enterocolitica* strains to antimicrobial agents are on the rise (Fabrega and Vila, 2012). Regular surveillance of *Y. enterocolitica* antimicrobial resistance is necessary to identify the earliest possible changes in bacterial susceptibility to antimicrobials used for human yersiniosis treatment (Meyer et al., 2011). Thus, the data on antimicrobial resistance and ability to form biofilm of *Y. enterocolitica* 4/O:3 strains isolated from pig production chain and patients clinical stool specimens may be useful to better understand the survival of these pathogens in the pork production chain and their ability to cause human infection.

The aim of the present study was to compare the biofilm formation and resistance to selected antimicrobials (MIC) of different *Y. enterocolitica* 4/O:3 strains isolated from pig production chain and patients clinical stool specimens.

Materials and methods. *Y. enterocolitica 4/O:3 strains*. Nineteen *Y. enterocolitica* strains examined in this study were isolated in 2009–2010. Bacteria isolation and identification was performed as described in the previous study (Novoslavskij et al., 2010). Eleven *Y. enterocolitica* strains represent the pig production chain, and eight *Y. enterocolitica* strains isolated from patients with gastrointestinal symptoms were received from the Tuberculosis and Infectious Diseases Hospital, Vilnius University, Santariškių Klinikos (Vilnius, Lithuania). Pig production chain isolates were obtained from pig feces (6 isolates) and pig carcass swab samples (5 isolates). All *Y. enterocolitica* isolates were confirmed as biotype 4 using the methodology described previously (Wauters et al., 1987) and as serotype O:3 based on slide agglutination test with commercial antisera O:3 for *Y. enterocoltica* (Denka Seiken, Tokyo, Japan). The tested *Y. enterocolitica* 4/O:3 strains were previously characterised using pulsed-field gel electrophoresis (PFGE) method (unpublished data) and represent 12 different bacteria genotypes (seven *Y. enterocolitica* 4/O:3 genotypes from pig production chain and five genotypes from patients clinical stool specimens) (Table 1).

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 Mueller-Hinton broth (Oxoid, Basingstoke, Hampshire, UK) were inoculated with stationary phase bacterial cultures adjusted to OD_{600}= 0.25. Plates were incubated at 25°C for 24 h. After incubation the media was removed and the wells were dried for 30 min at 55ºC. 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 80% ethanol- 20% acetone. To determine biofilm formation 100 µl of this was removed and placed in 96-well microtitre plate and the absorbance at 540 nm (OD_{540}) 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. The cut-off value for biofilm formation was OD_{540} = 0.89, which was the mean absorbance obtained from all negative controls.

Table 1. **Tested *Y. enterocolitica* 4/O:3 strains**

Strain No	Source	Genotype
1	Pig carcass	I
2	Pig feces	
3	Human	
4	Pig carcass	II
5	Pig feces	
6	Human	
7	Pig feces	III
8	Pig carcass	
9	Human	
10	Pig carcass	IV
11	Pig feces	
12	Pig carcass	V
13	Pig feces	VI
14	Pig feces	VII
15	Human	VIII
16	Human	IX
17	Human	X
18	Human	XI
19	Human	XII

Detection of minimum inhibitory concentration (MIC). Antimicrobial susceptibility was tested by the agar dilution method according the CLSI guidelines (CLSI, 2006a). The following five antimicrobial agents were tested: tetracycline (TE), ampicillin (AM), erythromycin (ERY), streptomycin (S), and ciprofloxacin (CIP) (all Sigma-Aldrich, MO, USA). In total, 19 *Y. enterocolitica* strains, 11 isolated from the pig production chain and 8 from patients clinical stool specimens, were analyzed. Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, UK) with dilutions ranging from 0.25 to 128 mg/L for erythromycin and ampicillin, and 0.125 to 32 mg/L for ciprofloxacin, streptomycin, and tetracycline was prepared. 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 antimicrobial agent-containing Mueller-Hinton agar 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. enterocolitica* growth. For quality control, reference *Y. enterocolitica* DSM 13030 strain was used. The breakpoints for antimicrobial agents were determined according to CLSI recommendations for the family *Enterobacteriaceae* (CLSI, 2006b). The breakpoint for salmonellae from the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP, 2008) was applied for streptomycin, as it is not provided by the CLSI.

Statistical analysis. Statistical analyses of the quantitative data were performed using the SPSS version 9.0. One-way ANOVA was performed to determine the influence of the sources and genotypes of *Y. enterocolitica* 4/O:3 on biofilm formation and antimicrobial resistance. The differences among the factors were analyzed using LSD or Dunett (in control group) methods of comparisons. For all statistical analyses, $P<0.05$ was considered statistically significant.

Results. All investigated *Y. enterocolitica* 4/O:3 strains representing 12 different bacteria genotypes (I to XII) were resistant to ampicillin and erythromycin and sensitive to ciprofloxacin (Table 2).

Y. enterocolitica 4/O:3 originating from the pig production chain and clinical stool specimens shared similar antimicrobial resistance. No association between bacteria isolation source and antimicrobial resistance was detected. Two strains i.e. *Y. enterocolitica* genotype V isolated from pig carcass, genotype IX isolated from human specimen and one strain belonging to genotype XI isolated from human specimen were resistant to tetracycline. However, no statistically significant differences between bacteria genotypes and antimicrobial MIC were found.

All tested *Y. enterocolitica* 4/O:3 strains isolated from pig production chain and patients clinical stool specimens formed biofilms (Fig. 1). No statistically significant differences between different bacteria genotypes and antimicrobial resistance for biofilm formation were observed.

Discussion. In this study, we attempted to find possible differences in antimicrobial resistance either between *Y. enterocolitica* strains collected from patients clinical stool specimens and the pig production chain or between different bacteria genotypes. *Y. enterocolitica* strains isolated from humans and pigs shared very similar antimicrobial resistance patterns. In agreement to previous studies (Kwaga and Iversen, 1990; Prats e al., 2000; Baumgartner et al., 2007; von Altrock et al., 2010), the tested *Y. enterocolitica* strains showed high-level resistance against β-lactam (ampicillin) and macrolide (erythromycin). Importantly, *Y. enterocolitica* strains are known to be naturally resistant to ampicillin (Fabrega and Vila, 2012). The natural resistance to beta-lactam antibiotics is due to the production of two chromosomally encoded beta-lactamase genes *blaA* and *blaB* (Cornelis and Abraham, 1975). It is noteworthy, that use of antibiotics as growth stimulators was banned in the EU in 2005. However, these two antimicrobials are still widely used as one of the pig gastrointestinal disease treatment modes in Lithuania and Latvia (personal communication, Terentjeva and Bērziņš, 2010). Interestingly, even though streptomycin and tetracycline are also often used in veterinary practice in Lithuania, low resistance (only one and two tested strains, respectively) to these agents was determined among the *Y. enterocolitica* strains examined. In contrast, studies from Germany and Switzerland have reported a 12% resistance to streptomycin (Meyer et al., 2011; Baumgartner et al., 2007; von Altrock et al., 2010). It is difficult to compare the results on streptomycin resistance due to the different breakpoints and methods used in other studies. Therefore, standardized methodologies and MIC criteria for comparing results are needed (Meyer et al., 2011). The published data on the resistance of *Y. enterocolitica* to tetracycline differs among the countries. As example, 13% of tested strains were resistant to this antimicrobial agent in the Czech Republic (Simonova et al., 2008). However, only 1% of tested strains were resistant to tetracycline in Latvia (Terentjeva and Bērziņš, 2010). Moreover, the resistance of *Y. enterocolitica* strains to this antimicrobial was not found in Austria and Germany (Meyer et al., 2011; Mayrhofer et al., 2004). Our study results showed that all *Y. enterocolitica* strains were susceptible to ciprofloxacin. These findings are in agreement with previous studies (Meyer et al., 2011; Kwaga and Iversen, 1990; Baumgartner et al., 2007; Simonova et al., 2008) supporting the general opinion that this antimicrobial agent is the most effective one against *Y. enterocolitica* and should be considered as the first line agent in human yersiniosis treatment (Fabrega and Vila, 2012).

Interestingly, the MIC for ciprofloxacin of 0.25 mg/L was found in 42% of tested strains, whereas considerably lower MIC (≤0.06 mg/l) was detected for the majority of tested strains in the studies from the Czech Republic and Germany (Meyer et al., 2011; Simonova et al., 2008). These findings indicate that higher doses of ciprofloxacin may therefore be needed to treat some human yersiniosis cases. Despite the differences between bacteria genotypes and that antimicrobial MIC were detected these findings were not statistically significant.

Together with antimicrobial resistance, biofilm formation of selected *Y. enterocolitica* 4/O:3 strains was performed. Biofilm allow microorganisms to persist in environment, resist desiccation and treatment with antimicrobial and disinfection agents. It was shown that *Y. pestis* and *Y. pseudotuberculosis* strains form biofilm on glass or polystyrene (Joshua and others 2003; Patel and others 2006). However, limited data about biofilms of *Y. enterocolitica* exists. According to studies performed with other bacteria species significant differences on biofilm formation among same bacteria species, serovars and genotypes can be detected (Borucki et al., 2003, Deligianni et al., 2010). Moreover, biofilm formation significantly increases bacteria resistance to antimicrobial agents (Mah and O'Toole, 2001; Gilbert et al., 2002). The obtained results revealed that *Y. enterocolitica* strains isolated from pigs and humans formed biofilms, however, no significant difference in biofilm formation was found among different bacteria genotypes.

Table 2. **Antimicrobial resistance of 19 *Y. enterocolitica* 4/O:3 strains**

A*	Number (genotype/source) [a] of *Y. enterocolitica* O:3 strains with a minimum inhibitory concentration (MIC; mg/L) of:											
	<0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
ERY										3(I/F,C,H) 1(III/H) 2(IV/F,H) 1(V/C) 1(VI/F) 1(VII/F) 1(VIII/H) 1(X/H) 1(XII/H)	3(II/F,C,H) 2(III/ (F,C) 1(IX /H) 1(XI /H)	
CIP	3(I/F,C,H) 2(II/F,C) 2(III/F,C) 2(IV/F,C) 1(XII/H)	1(II/H) 1(III/H) 1(VI/F) 1(VII/F) 1(VIII/H) 1(IX/H) 1(X/H) 1(XI/H)	1(V/C)									
TE					3(I/F,C,H) 3(II/F,C,H) 3(III/F,C,H) 2(IV/F,C) 1(VI/F) 1(VII/F) 1(VIII/H) 1(X/H) 1(XI/H) 1(XII/H)				1(V/C) 1(IX H)			
AM												3(I/F,C,H) 3(II/F,C,H) 3(III/F,C,H) 2(IV/F,C) 1(VI/F) 1(V/C) 1(VII/F) 1(VIII/H) 1(IX H) 1(X/H) 1(XI/H) 1(XII/H)
S						1(I/H) 2(III/C,H) 1(XII/H)	2(I/F,C) 3(II/F,C,H) 1(III/F) 2(IV/F,C) 1(VI/F) 1(V/C) 1(VII/F) 1(VIII/H) 1(IX H) 1(X/H)		1(XI/H)			

Dark grey marked columns – Resistant; Light grey – Intermediate; Uncoloured columns – Susceptible;
* – Antimicrobial agent: TE- tetracycline, AM-ampicillin, ERY-erythromycin, S-streptomycin, CIP-ciprofloxacin;
a: F - pig feces, C – pig carcass, H – human clinical stool specimens

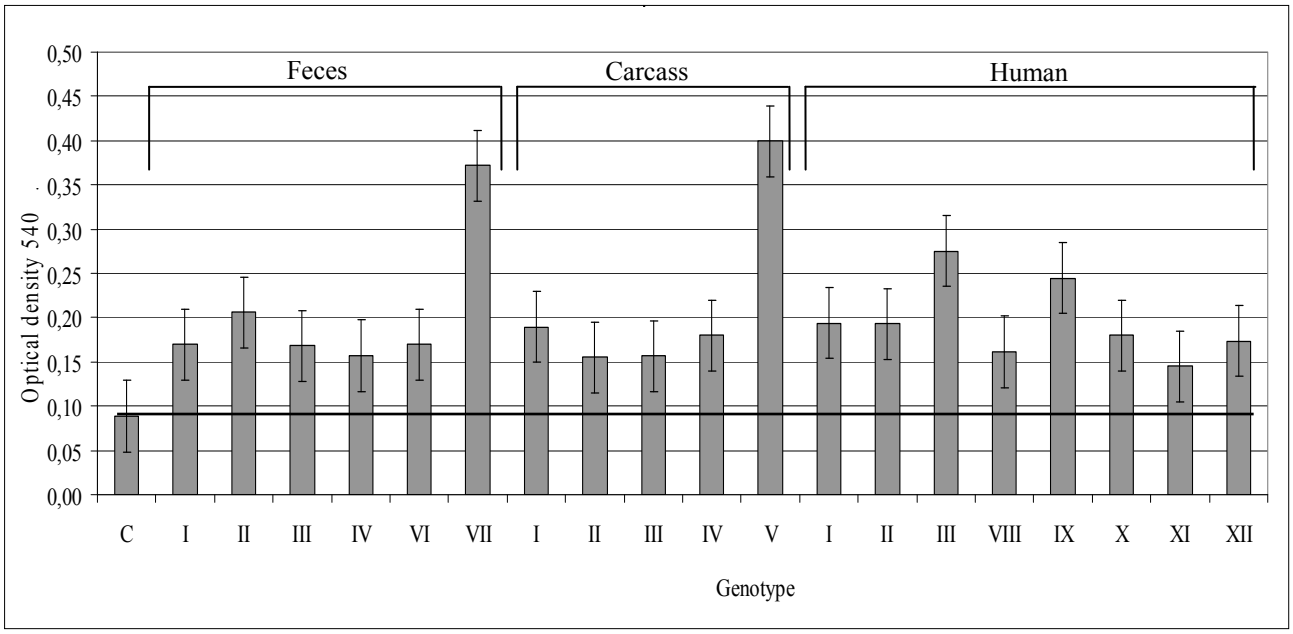

Figure 1. **Biofilm formation of 12 *Y. enterocolitica* 4/O:3 genotypes isolated from different sources**

C – negative control (Muller Hinton broth without bacteria)

Also no correlation between biofilm formation and antimicrobial resistance of different *Y. enterocolitica* 4/O:3 genotypes was observed. Interestingly, *Y. enterocolitica* genotype V and VII isolated from pig production chain and genotype III from human clinical samples showed better biofilm formation. Moreover, genotype V was found as resistant to tetracycline and it was the only genotype with MIC as high as 0.5 mg/L found for ciprofloxacin. These findings suggest that in certain cases biofilm formation might be related to antimicrobial resistance. We did not find any significant differences in biofilm formation of yersinia strains tested, presumably due to the limited number of strains used in the study. Moreover lack of the data on *Y. enterocolitica* biofilm formation is limiting the possibility to compare our results with other reports. Worth to mention, all tested *Y. enterocolitica* 4/O:3 strains shared high genetic similarity (>85%). This may probably explain that correlation among antimicrobial resistance and biofilm formation was not observed.

Conclusions.

The results of our study showed that the tested *Y. enterocolitica* 4/O:3 strains isolated from pig production chain and patients clinical stool specimens shared similar antimicrobial resistance. All bacteria strains were resistant to ampicillin and erythromycin and sensitive to ciprofloxacin. One and two out of 19 tested strains were resistant to streptomycin and tetracycline, respectively. No statistically significant differences between bacteria genotypes and antimicrobial MIC were observed. Also no significant difference in biofilm formation was found among different bacteria genotypes and no correlation between biofilm formation and antimicrobial resistance of different genotypes was observed.

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Received 11 May 2012
Accepted 20 March 2013