FENOTIPSKE I GENSKE ZNAČAJKE IZOLATA YERSINIA ENTEROCOLITICA IZDVOJENIH U LANCU PROIZVODNJE SVINJSKOGA MESA

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I Z J A V A

Ja, Valerij Pažin, potvrđujem da je moj doktorski rad izvorni rezultat mojega rada te da se u njegovoj izradi nisam koristio drugim izvorima do onih navedenih u radu.

_____

(potpis studenta)

Zagreb, 2021.

Doktorski rad izrađen je u Zavodu za higijenu, tehnologiju i sigurnost hrane Veterinarskog fakulteta Sveučilišta u Zagrebu.

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Predstojnik: izv. prof. dr. sc. Nevijo Zdolec

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SAŽETAK

Yersinia enterocolitica je među vodećim bakterijskim uzročnicima zoonoza u Europskoj uniji, a meso svinja smatra se najvažnijim izvorom infekcije za ljude. Ciljevi ovog istraživanja su: I) utvrditi prevalenciju patogene *Y. enterocolitica* u tonzilama svinja na liniji klanja i mesu iz maloprodaje, II) biokemijski i molekularno karakterizirati i usporediti izolate *Y. enterocolitica* s obzirom na podrijetlo sojeva tj. farmu, klaonicu, meso i godinu prikupljanja izolata.

Na liniji klanja uzorkovano je 234 tonzila svinja koje su potjecale s 26 farmi od čega su tri bile velike integrirane farme (N=69), deset srednjih farmi (N=130) i 13 obiteljskih poljoprivrednih gospodarstava (N=35). Uzorkovano je i 128 uzoraka porcioniranog i mljevenog svinjskog mesa u maloprodaji. Izolacija je provedena primjenom PSB i ITC bujona, CIN i CHROMagara™, a potvrda na MALDI-TOF MS i RT-PCR (*ail* gen). Sojevi (N=85) su biotipizirani, serotipizirani, podvrgnuti PFGE analizi i testiranju osjetljivosti na antibiotike. Biokemijske i molekularne značajke sojeva uspoređene su sa sojevima *Y. enterocolitica* iz 2014. godine (N=49).

Ukupna prevalencija patogene *Y. enterocolitica* u tonzilama svinja iznosila je 43,16 %. U tonzilama svinja s integriranih farmi utvrđena je prevalencija od 28,99 %, srednjih farmi 51,54 %, a obiteljskih gospodarstava 40,00 %. Kategorija biosigurnosti integriranih i srednjih farmi nije utjecala na razlike u prevalenciji patogene *Y. enterocolitica* ($P>0,05$), što nije bio slučaj za poljoprivredna gospodarstva ($P<0,05$). Sve presumptivne kolonije s CHROMagara™ (pYV+) su potvrđene na MALDI-TOF MS kao *Y. enterocolitica,* a RT-PCR-om je detektiran gen patogenosti *ail.* U tonzilama svinja neovisno o tipu farme, klaonici i godini izolacije (2014. naspram 2018.-2019.) perzistira patogena *Y. enterocolitica* biotipa 4 serotipa O:3. Također je PFGE tipizacijom utvrđena visoka genetska podudarnost (80,6 do 100 %) sojeva bioserotipa 4/O:3 što je pokazatelj njihove perzistentnosti u tonzilama svinja u Hrvatskoj. S druge strane, svi uzorci porcionirane i mljevene svinjetine bili su negativni na prisutnost patogene *Y. enterocolitica.* Statistički znakovito veći udio multirezistentnih sojeva patogene *Y. enterocolitica* zabilježen je u tonzilama svinja s integriranih farmi ($P<0,05$) uz dominantnu otpornost na cefalotin i ampicilin, ali i nalidiksičnu kiselinu, kloramfenikol i streptomicin.

Dobiveni rezultati ukazuju na perzistentnost patogene *Y. enterocolitica* bioserotipa 4/O:3 u tonzilama svinja iz različitih tipova farmi u Hrvatskoj, no rizik prijenosa kroz lanac svinjskog mesa je zanemariv. Po prvi puta je ovim radom potvrđena multirezistentnost *Y. enterocolitica* 4/O:3 u svinja koja je uvjetovana tehnologijom tova (velike integrirane farme) uz intrigantnu otpornost na pojedine klinički relevantne antibiotike u terapiji humane jersinioze. Ovo je prvo

istraživanje karakterizacije *Y. enterocolitica* u lancu proizvodnje svinjskog mesa u Hrvatskoj i temelj za razvoj kontrolnog programa ove zoonoze koji još nije uspostavljen u Europi.

Ključne riječi: *Yersinia enterocolitica*, tonzile svinja, farma, svinjsko meso, biokemijske i genetske značajke, veterinarsko javno zdravstvo

EXTENDED ABSTRACT

INTRODUCTION. The European Food Safety Authority (EFSA) has proposed the establishment of a comprehensive integrated meat inspection system to improve meat safety and meat inspection. The system is based on the identification of public health risks and the implementation of control measures throughout the production chain from farm to slaughterhouse. According to the risk analysis carried out, the main biological hazards in meat production at farm and slaughterhouse level are the bacteria *Salmonella* spp, *Campylobacter* spp, *Yersinia enterocolitica*, verotoxic *Escherichia coli* and the parasites *Trichinella* and *Toxoplasma gondii*. *Yersinia enterocolitica* is an important but very often neglected pathogenic bacterium in veterinary public health and meat hygiene. The main carriers of pathogenic *Y. enterocolitica* are pigs. Contamination of meat may occur most frequently during evisceration, tonsil removal and head splitting. Preliminary studies in Croatian slaughterhouses show that the prevalence of *Y. enterocolitica* in the tonsils of pigs ranges from 14 to 33%, and finding the bacteria in fresh pork on our market is occasional. In the European context, the prevalence in the tonsils of pigs varies between 1.8-93% and in the mandibular lymph nodes between 5-12.5%. Detection in lymphoid tissues at slaughter depends on several factors, such as the time of infection (on-farm, during transport, at the slaughterhouse lairage) or slaughter processing practices (cross-contamination). Consumption of raw and insufficiently heat-treated pork and untreated water is considered a risk factor for human infection. Although pork is considered the main source of human infection, numerous studies show that pathogenic *Y. enterocolitica* is rarely found in portioned pork on the market, but with the exception of carcass parts and organs that are more susceptible to contamination at slaughter (cheeks, head, tongue, throat). Risk factors for the occurrence and shedding of *Y. enterocolitica* at the farm level are considered to be the purchase of piglets from different sources, the supply of pig feed from different suppliers, lack of bedding, water supply from non-public water supply and direct contact of different groups of pigs. Existing housing systems on pig farms (integrated farms vs. family farms) differ significantly in the level of biosecurity and may therefore pose some risk. Risk factors at slaughter include failure to close the rectum, splitting the head along with the carcass, and measures to sanitize equipment during evisceration of thoracic organs. Measures that can reduce meat contamination are separation of the head along with the tongue and tonsils and rectum bunging/tying before evisceration. The high variability in results of prevalence studies, in addition to the risk factors mentioned above, is attributed to the complexity of isolation methods of pathogenic *Y. enterocolitica*, the accompanying microflora that interfere with

isolation of poorly contaminated samples, inconsistent sampling procedures, slaughter practices, and cross-contamination of carcasses. Conventional standard methods are supplemented by more reliable and rapid techniques such as RT-PCR, MALDI-TOF MS, PFGE and sequencing. Pathogenic biotypes and serotypes occur in both pigs and infected humans, with the most common biotype in most countries of the world being 4 serotype O:3. So far, *Y. enterocolitica* has rarely been considered as a significant entity in the context of meat safety in Croatia. Therefore, this study aims to determine the prevalence of *Y. enterocolitica* in pigs in the slaughter line and in different housing systems - integrated, medium and family farms, to characterize the strains biochemically and molecularly in terms of pathogenicity and to test their sensitivity to antibiotics. Genetic and phenotypic characteristics of the isolates will be compared in terms of time of isolate collection (years of research) and origin (farms and slaughterhouses) to determine the degree of persistence of pathogenic *Y. enterocolitica* strains in the pork production chain.

MATERIALS AND METHODS. In 2014, preliminary studies were conducted on the presence of *Y. enterocolitica* in the tonsils and lymph nodes of slaughtered pigs in Croatia. They were sampled in the slaughter lines of domestic pigs in four regional slaughterhouses in central, northern and eastern Croatia. These slaughterhouses process pigs from different husbandry systems (large integrated systems, medium farms and single households). Strains of pathogenic *Y. enterocolitica* from that study were also used in this study, with newly isolated isolates. In this study, samples were collected from the same slaughterhouses in 2018 and 2019 to gain insights into the persistence of pathogenic *Y. enterocolitica*, e.g., by comparing isolates in terms of biochemical characteristics, pathogenicity, and antimicrobial resistance profiles. In addition, the presence of *Y. enterocolitica* in portioned pork, minced meat and meat products from the same producers (slaughterhouses) in the market was investigated. Tonsils sampling was performed in selected slaughterhouses (slaughterhouses I, II, III, IV) by randomly selecting pigs, immediately after evisceration, using sterile equipment. A total of 12 sampling sessions were conducted, comprising pigs from 26 farms, and a total of 234 samples were collected. 10 grams of tonsils were homogenized in enrichment broth (Peptone, sorbitol, and bile salts, PSB, Sigma Aldrich, St. Louis, USA), of which 10 mL was transferred to 90 mL of selective enrichment broth (Irgasan™ ticarcillin and potassium chlorate, ITC, Sigma Aldrich, USA). Both solutions were incubated for 44 ± 4 h at 25°C ± 1°C. Then, 0.5 mL of the two enriched samples was treated with an alkaline solution (4.5 mL KOH) for 20 sec. Then, inoculation was performed on the selective agar Cefsulodin, Irgasan™ and novobiocin (CIN, Merck, Germany)

and the chromogenic agar CHROMagar™Y. enterocolitica, which differentiates pathogenic strains of *Y. enterocolitica* possessing the pYV plasmid. Incubation of the two selective agars was performed for 24 ± 2 h at 30 °C ± 1°C. Characteristic colonies on CIN agar (small, round, smooth, with dark red center and transparent margin) were examined microscopically for a morphology described as "bull's eye" and retained and transplanted for further identification and characterization. Colonies that were purple (pathogenic) on CHROMagar™ were also retained. Identification of selected isolates (N=84) was performed using MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight). Characteristic protein mass spectra were recorded for each isolate in the range m/z of 2,000-20,000 Da using a microflex LT mass spectrometer (Bruker Daltonik, Germany). The recorded mass spectra were processed using the computer program MALDI Biotyper 3.0 (Bruker Daltonik, Germany). The result of MALDI Biotyper is expressed as a logarithmic value in the range 0-3.0, which represents the probability of accurate identification of the isolates, which is then based on the comparison of the protein profiles of the unknown isolate with the reference spectrum in the database. A total of 100 isolates (from both studies) were selected for RT -PCR confirmation of the presence of the ail gene. The human isolate *Y. enterocolitica* biotype 4 serotype O:3 served as the positive control. DNA isolation was performed using Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, USA). PCR amplification and detection was performed according to the VIASURE *Yersinia enterocolitica* Real Time PCR detection kit protocol (Certest Biotec S.L., Zaragoza, Spain). Strain biotyping was performed according to HRN EN ISO 10273:2017 using reactions with esculin, xylose, pyrazinamidase, tweenesterase/lipase, trehalose and indole. Xylose and trehalose solutions, pyrazinamidase agar and Tween esterase/lipase plates were prepared at Croatian Veterinary Institute (12.2019). Esculin and indole reactions were tested on API 20E and Rapid 20E (bioMerieux, France). Serotyping was performed by agglutination of *Y. enterocolitica* O:3 antiserum (Statens Serum Institute, Denmark). The human isolate *Y. enterocolitica* biotype 4 serotype O:3 was used as a positive control. Comparison of molecular profiles of isolates from the pork production chain was performed using the Pulsed-field Gel Electrophoresis (PFGE) method. A total of 66 isolates (both studies) were tested. The PFGE method compared pulse types of pathogenic Y. enterocolitica from the pork production chain with a clinical isolate of human origin from the territory of the Republic of Croatia. PulseNet One-Day (24-28 h) Standardized Laboratory Protocol was used for Molecular Subtyping of *Yersinia pestis* (CDC, 2006). Selected strains of pathogenic *Y. enterocolitica* (n=85) were subjected to antimicrobial susceptibility testing using disk diffusion method. The susceptibility of pathogenic *Y. enterocolitica* strains from the first study conducted in 2014 was also

investigated (n=49). Eleven antibiotics were used: Levofloxacin (5 μg), Ciprofloxacin (5 μg), Ampicillin (10 μg), Cephalotin (30 μg), Cefotaxime (30 μg), Tetracycline (30 μg), Nalidixic acid (30 μg), Ceftazidime. 30 μg), Trimethoprim/sulfamethoxazole (25 μg), Chloramphenicol (30 μg), and Streptomycin (10 μg). Antibiotic susceptibility/resistance was interpreted according to the CLSI criteria for enterobacteria.

RESULTS. The study revealed a prevalence of *Y. enterocolitica* in pigs tonsils of 43.16% (N=234). The prevalence of *Y. enterocolitica* in pig tonsils was 28.99% in integrated farms, 51.54% in medium farms and 40% in family farms. The percentage of positive pigs from integrated farms ranged from 14.29% to 42.86%. Although the highest biosecurity category (3) was the same in all farms, a statistically significant difference was found in the percentages between farms IB and IC ($P<0.05$). The number of positive pigs slaughtered in the slaughterhouse II from six medium farms ranged from 15.38% to 66.67%. When comparing the differences found in the proportion within these six medium-sized farms, statistically significant differences were found between IIB (category 3) and IIE (category 3) and IIB and IIF (category 2). From five farms in biosecurity category 3, a total of 38.71% of pigs were positive. Comparing this result with the medium farms in the lower biosecurity category 2 (58.33% positive pigs), the difference was not statistically significant ($P=0.2104$, $\chi2=1.568$). Based on the analysis of these farms, the observed differences were not related to biosecurity rating. In addition, biosecurity category did not significantly affect the percentage of *Y. enterocolitica* findings in pigs from medium-sized farms slaughtered at the slaughterhouse III. Comparing the positive *Y. enterocolitica* findings taking into account the biosecurity of the farms but independent of the slaughterhouses, the following can be seen: Within biosecurity category 3, a total of 72 pigs were tested, of which 32 (or 44.44%) were positive. Within category 2, a total of 58 pigs were examined, of which 35 (or 60.34%) were positive, but this difference is not significant ($P=0.2482$; $\chi2 =1.333$), which also confirms the statement made above that in medium-sized farms, the detection of *Y. enterocolitica* in pig tonsils is not related to the biosecurity category of the farm. Comparing the results of *Y. enterocolitica* findings in the pig tonsils from medium size farms and depending on the place of slaughter (41.89%, 85.71% and 57.14%, slaughterhouses II, III and IV respectively), a significant difference was found between the slaughterhouse II and the slaughterhouse III ($P<0.05$). Within biosecurity category 3, no statistically significant differences were found between medium farms and integrated farms. Majority of family farms (76.92%) were in biosecurity category 2 and a total of 48.28% of positive pigs were identified (N=29). Compared to the family farms in category

3, without *Y. enterocolitica* findings, the difference was significant (P=0.0460, $\chi 2$ =1.333). Summarizing the results by slaughterhouse and farm type, the following emerges: The fewest *Y. enterocolitica*-positive porcine tonsils were found in integrated farms (28.99%; N=69), which belonged to the highest biosecurity category 3 and were processed in abattoir I. Most of the positive tonsils (85.71%) were from pigs from medium farms slaughtered at the abattoir III. The difference was statistically significant (P<0.01). A significantly lower percentage of positive pigs was found within the medium-sized farms associated with the slaughterhouse II (48.89%; N=74) compared to the medium-sized farms from the slaughterhouse III and a significantly higher percentage compared to the integrated farms from the slaughterhouse I (P< 0.05). When PSB broth was used exclusively and inoculated on CIN or CHROMagar™, the lowest number of positive samples (*Y. enterocolitica* pathogens in pig tonsils) was detected, and the agar used did not significantly affect the success of bacterial isolation (P=0.288). Neutralization of the culture from PSB broth with KOH showed a statistically significant increase in the frequency of isolation of pathogenic *Y. enterocolitica* by 5.3-fold on CIN agar and 3.7-fold on CHROMagar™, respectively (P=0.000; P=0.022). The frequency of isolation of *Y. enterocolitica* after neutralization of the culture from PSB broth was not statistically significantly different with respect to the selective agar used (P=0.05). Compared to PSB broth, enrichment in ITC broth showed a significantly higher number of Yersinia-positive tonsils after inoculation on CIN agar or CHROMagar™ (P<0.05). There were no differences in bacterial growth on the selective agars used (P=0.70). Neutralization of culture from ITC broth with KOH also showed an increase in the number of positive tonsil samples due to increased *Y. enterocolitica* on CIN agar, but without statistical significance compared to ITC broth without KOH (P=0.422). Similarly, the frequency of bacterial isolation on CHROMagar™ was not altered by neutralizing the culture in ITC broth (P>0.05). Thus, a statistically significant higher frequency of *Y. enterocolitica* was observed on CIN agar than on CHROMagar™ after neutralization of the culture from ITC broth (P=0.0002). Identification of morphologically suspect colonies with CIN and CHROMagar™ was performed by MALDI-TOF MS. Isolates (N=85) were confirmed with a very high probability to be *Y. enterocolitica* species, while atypical colonies (greenish on CHROMagar™) belonged to other species of *Enterobacteriaceae* (e.g. *Citrobacter, Serratia*). The presence of the ail gene was confirmed by RT -PCR. All suspected colonies of *Y. enterocolitica* belonged to biotype 4, characterized by a negative reaction of aesculin, xylose, pyrazinamidase, lipase and indole, with a positive reaction of trehalose. Serotyping confirmed that all strains of biotype 4 belonged to serotype O:3, regardless of the year of isolation from pig tonsils and the origin of the pigs, i.e. the type of

fattening farm. PFGE analysis showed low variability of pulse types within pathogenic *Y. enterocolitica* 4/O:3 strains. Considering the possibility of spread of the pathogen *Y. enterocolitica* through the pork chain, different cuts of portioned and minced meat were examined from the same producers in whose slaughterhouses tonsils were sampled. In addition, imported meat from retail outlets and meat from other FBOs was examined. 128 meat and minced meat samples were examined and the presence of *Y. enterocolitica* was not confirmed in any of them. In total (both 2014 and 2018/2019), 36 strains of *Y. enterocolitica* from integrated farms, 84 isolates from medium farms and 13 isolates from family farms were tested for susceptibility to 11 antibiotics. Comparing the widths of zones of inhibition when a single antibiotic was used in relation to the origin of the isolates, isolates of *Y. enterocolitica* from integrated farms were found to be more resistant to streptomycin, chloramphenicol and nalidixic acid than isolates from family farms and medium farms. In the group of pathogenic *Y. enterocolitica* isolates from integrated farms (N=36), dominant resistance to cephalothin (80.55%), ampicillin (77.77%), chloramphenicol (66.66%) and streptomycin (61.11%) was observed. The highest percentage of Y. enterocolitica isolates from medium-sized farms were resistant to ampicillin and cephalothin (85.71% and 78.57%, respectively), which was also confirmed in isolates from family farms (100% for both agents). In addition, correlations of sensitivity/resistance of *Y. enterocolitica* isolates to a single antibiotic were tested depending on the type of pig farming. There was a high correlation between the results of testing the susceptibility of *Y. enterocolitica* isolates to ampicillin and cephalothin between integrated farms and family farms. When the correlations of the results of susceptibility testing of *Y. enterocolitica* isolates within integrated farms were examined, a high correlation was found between all antibiotics tested ($r=0.93-0.99$) with a statistical significance of $P<0.05$. No high correlations were found among medium farms and correlation coefficients ranged from 0.21 to 0.49 ($P<0.05$). At the family farm level, significantly high correlation (0.99 and above) was found between most antibiotics in relation to qualitative indicators of susceptibility/resistance. High positive correlations occurred between 5 to 9 different antibiotics. No significant differences were found with respect to the susceptibility/resistance of *Y. enterocolitica* isolates from integrated farms and considering the year of isolation of the pathogen ($P>0.05$). Similarly, no statistically significant differences were found in the susceptibility/resistance of *Y. enterocolitica* isolates from medium and integrated farms considering the year of isolation of the pathogen ($P>0.05$). Examination of the relationship in the results (total) between years and farm types shows that in 2014, significantly more measurements showed susceptibility (S) of

isolates from medium farms and higher resistance in integrated farming. The same relationship was also found in the 2018/2019 survey.

CONCLUSION. The high prevalence of the pathogen *Y. enterocolitica* in pig tonsils indicates a significant risk of meat contamination. The highest prevalence of *Y. enterocolitica* was found in the tonsils of pigs from medium-sized farms, followed by pigs from family farms, and the lowest in pigs from large integrated farms. The prevalence of the pathogen was not related to the biosecurity category of the farms, except in the case of family farms. *Ail* positive pathogenic *Y. enterocolitica* biotype 4 serotype O:3 strains persist in the tonsils of pigs in Croatia. The same pathogenic bioserotype dominates, regardless of the year of isolation and the type of farm. The use of neutralization with KOH significantly affects the success of isolation of *Y. enterocolitica* from pig tonsils. PFGE is not a sufficiently discriminatory method for distinguishing pulse types of biotype 4 serotype O:3. Portioned and minced pork does not pose a public health risk with respect to transmission of pathogenic *Y. enterocolitica*. The prevalence of multidrug-resistant strains of *Y. enterocolitica* is significantly higher in pigs from integrated farms, which may be due to higher antibiotic use. This study is the first report on the characterization of *Y. enterocolitica* in the pork chain in Croatia and a baseline study for the development of a control program, which is not yet established in Europe.

Key words: *Yersinia enterocolitica*, pig tonsils, farm, pork, biochemical and molecular characteristics, veterinary public health

SADRŽAJ