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Original Scientific Article

ESTIMATION OF *Yersinia enterocolitica* PREVALENCE IN SLAUGHTERED PIG TONSILS IN SLOVENIA BY USING THREE CULTURAL ISOLATION PROCEDURES

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Summary: A survey was conducted to collect preliminary data concerning the prevalence of *Yersinia enterocolitica* in slaughtered pigs in Slovenia. At the same time, culture procedures for its isolation were compared. During the period between January and May 2008, 1490 tonsil swabs from 149 slaughtered herds were taken from slaughterhouses in Slovenia. One swab was used to sample the tonsils of one slaughtered pig and ten swabs were taken in each sampled herd. For each swab, three protocols were used to isolate *Y. enterocolitica*. Firstly, swabs were tested by direct cultivation on Cefsulodin-Irgasan-Novobiocin (CIN) agar. Secondly, the swabs were incubated in enriching Yersinia PSB broth (PSB) and then inoculated on CIN agar. In the third case of isolation, the PSB enrichment after incubation was treated with the potassium hydroxide solution (KOH) and then streaked on CIN agar. Out of 43 (29.3%) herds, *Y. enterocolitica* was isolated from at least one of the 10 tested swabs by any of the procedures used. In the case of isolation, the enrichment with KOH treatment, was the most efficient (74.4%). Isolation using the combination of direct plating and the procedure using enrichment with the KOH treatment was successful in all positive herds. *Y. enterocolitica* were biotyped and serotyped. Most isolated strains (80.4%) belonged to the pathogenic biotype 4 serotype O:3.

Key words: *Yersinia enterocolitica*; slaughter pigs; tonsils; isolation

Introduction

Yersinia enterocolitica is well established as a foodborne pathogen of human concern (10). It is primarily a gastrointestinal tract pathogen acquired through the oral route and epidemiologically linked to porcine source (18). Healthy pigs are often carriers of *Y. enterocolitica* strains pathogenic for humans, in particular strains of biotype 4 (serotype O:3) and biotype 2 (serotype O:9) (6, 16, 18, 22). The most common biotype associated with human disease in Europe is biotype 4 (1, 12, 19). *Y. enterocolitica* is commonly found in pig tonsils, intestine and faeces (4, 14). Although pork and pork products are considered major transmission sources of pathogenic *Y. enterocolitica* (13), it has only rarely been isolated from pork products with the exception of fresh tongues (2). Strains of the nonpathogenic biotype 1A are widely spread in the environment and often isolated from food. When *Y. enterocolitica* is present in meat or meat products, it has, due to its psychrophilic character, a potential to multiply during storage. Since the

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population of *Y. enterocolitica* in food samples is usually low, isolation procedures usually involve enrichment in liquid media followed by plating on selective media (3). When pigs are slaughtered at the age of 135 days or more, the estimated prevalence of pathogenic *Y. enterocolitica* on the tonsils is greater than the prevalence in faeces or on the carcasses (5, 15, 23, 24, 25). Due to a higher prevalence and a higher amount of *Y. enterocolitica* in tonsils than in faeces, the tonsils were chosen as the sampling site for this study. The aim was to find the most appropriate protocol for isolation of *Y. enterocolitica* on pig tonsils and to collect preliminary data of its prevalence in slaughtered pigs in Slovenia.

Material and methods

The sampling was carried out by inspectors of the Veterinary Administration of the Republic of Slovenia in all slaughterhouses in Slovenia that slaughter more than 1000 pigs per year. From 1st January to 14th May 2008, 149 herd samples were taken and 10 animals were randomly sampled per herd. Eleven slaughterhouses were involved, covering 97% of pig slaughtering in Slovenia. Only the animals raised in Slovenia were sampled. For each slaughterhouse, the number of sampled herds was calculated based on the number of slaughtered animals per year. According to the slaughtering capacity, 1 to 50 herds were sampled and ten pigs were sampled per herd randomly. For each pig carcass, one individual cotton swab was used. This resulted in a final sample size of 1490 tonsil swabs.

The surface of the tonsils was wiped with dry cotton on a stick (Dolgi bris, Plastika Kavčič, Ljubljana, Slovenia) and enclosed in a sterile tube. Samples were transported to the laboratory in portable coolers and analysed within 24 hours after collection. In the laboratory, each swab was tested following three isolation protocols, namely:

Procedure 1: Swab was streaked onto the surface of Cefsulodin-Irgasan-Novobiocin (CIN) agar (Biolife, Milano, Italy) and incubated at 30±1°C for 24 hours.

Procedure 2: Five ml of the Yersinia PSB broth (Biolife, Milano, Italy) enrichment media was added to each swab and incubated at 23±1 °C for 5 days. After incubation, 10µl of PSB was streaked onto CIN agar.

Procedure 3: A half of a millilitre of PSB after incubation, which resulted from the second protocol, was transferred to 4.5ml of the potassium hydroxide solution (0.5% KOH prepared with 0.5% saline solution) and mixed for approximately 20 seconds. Using a loop, 10µl of the treated PSB was streaked on CIN agar plate.

All of the inoculated agar plates were incubated at 30±1 °C for 24 hours. When growth was weak or there were no characteristic colonies, incubation was prolonged for another 24 hours. *Y. enterocolitica* formed small colonies with a deep red centre and sharp border surrounded by a clear colourless zone. Characteristic colonies from CIN plates were streaked to nutrient agar (Biolife, Milano, Italy) and incubated for 24h at 30± 1°C. After incubation, detection of oxidase (BD, Sparks, USA), urease (Merck, Darmstadt, Germany) and fermentation of sugars by TSI agar (Biokar Diagnostic, Beauvais Cedex, France) was performed. Oxidase negative, urease positive, glucose positive, lactose negative and H_2S negative cultures were further tested. Additional biochemical confirmation tests were performed: indol (Biolife, Milano, Italy), lysine decarboxylase and ornithin decarboxylase (Merck, Darmstadt, Germany), fermentation of sugars (sucrose (Kemika, Zagreb, Croatia), trehalose, rhamnose, xylose (Merck, Darmstadt, Germany)), and aesculin hydrolysis (Kemika, Zagreb, Croatia). Antiserums for O:3 and O:9 (Statens Serum Institute, *Copenhagen,* Denmark) were used for serotyping.

Agreements between the procedures used were tested by Kappa statistic test. Kappa values below 0 indicate no agreement, 0–0.20 slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1 almost perfect agreement (27).

Results

A herd was considered positive if at least one tonsil swab was positive for *Y. enterocolitica* by any of the procedures used. Forty-three (28.9%) out of 149 herds were positive. No one isolation procedure detected all of the positive herds. *Y. enterocolitica* was isolated from 16 herds (37.2%) using direct plating (procedure 1), from 16 using enrichment without treatment (procedure 2) and from 32 (74.4%) after enrichment using the treatment with KOH (procedure 3). Just one herd was found positive by all the procedures used. Nine

Table 1: Number of *Y. enterocolitica* positive herds and the rate (%) of successful isolations at positive herds by using each procedure or their combinations

Detection method	Number of positive results	% of positive results
Only procedure 1	9	20.9
Only procedure 2	0	0
Only procedure 3	14	32.6
Procedure 1 and procedure 2	2	4.7
Procedure 1 and procedure 3	4	9.3
Procedure 2 and procedure 3	13	30.2
All three procedures	1	2.3
All procedures and their combinations	43	100.0

Table 2: Coefficient of determination (R2) and p-values for model and effects of country (C), flock nested within country (H), litter size (L), parity (P), lambing season (S), interval between lambing and the first milk recording (I), and year of lambing (J)

Detection method	Number of positive results	% of positive results
Only procedure 1	22	25.6
Only procedure 2	4	4.7
Only procedure 3	36	41.9
Procedure 1 and procedure 2	1	1,1
Procedure 1 and procedure 3	3	3.5
Procedure 2 and procedure 3	20	23.2
All three procedures	0	0
All procedures and their combinations	86	100.0

herds were positive only by applying procedure 1 and 14 only by applying procedure 3. None of the herds was positive by using only procedure 2. In the case of 19 herds, isolation was successful using two of the procedures (Table 1).

Comparing procedures by Kappa test indicates statistical difference between procedures 1 and 2 ($x < 0$) and procedures 1 and 3 ($x < 0$), but just slight agreement between procedures 2 and 3 ($x = 0.16$). Between 32 herds detected positive by using procedure 3 were also 14 of 16 (87.5%) tested positive by procedure 2. This indicates better sensitivity of procedure 3, regarding procedure 2.

Within the positive herds, the number of positive swabs taken from individual animals varied from one to seven, with an average of 2 positives per herd. Out of 1490 individual swabs, 86 (6.0%) were positive by at least one of the methods used. Twenty-six swabs were positive after direct inoculation (procedure 1) and 64 using enrichment (procedures 2 and 3). By applying procedure 2, twenty-five swabs were positive and by applying procedure 3 fifty-nine. In the case of 20 swabs, *Y. enterocolitica* was isolated only by both enrichment procedures. In only four swabs *Y. enterocolitica* was isolated after direct plating (procedure 1) and after enrichment (procedure 2 or procedure 3). Numbers of positive isolations by one or more procedures used are presented in Table 2. *Y. enterocolitica* isolated from 37 herds belonged to the pathogenic biotype 4 serotype O:3 and from 9 herds to the non-pathogenic biotype 1A. In the case of 3 herds both biotypes were isolated.

Discussion

The results indicate that pigs are carriers of *Y. enterocolitica* in a significant proportion (28.9%) of herds in Slovenia. The prevalence reported in this study is comparable to the prevalence referred

by some other studies in which between 5% and 35% of positive animals, and from 20 to 80% of positive herds, have been reported (4, 5, 23, 24). Results of the studies, based on the prevalence of *Y. enterocolitica* in pig herds or slaughter batches from European countries reported by the European Food Safety Authority (EFSA) (9) varies greatly, from 1% reported in Germany to 48.4% in Spain. The highest prevalence of 52.0% indicated in pig tonsils was reported in Finland in 2007 (8). The reasons for such diverse data are probably due to thedifferent matrices and methods used. The results of this study are comparable to the results of Nesbakken et al. (23) from Norway, who found 5.2% positive animals and 25.0% positive herds. In many studies, the rate of contaminated animals and herds is higher (11, 14, 15). In the case of the French study (11) performed in one slaughterhouse by taking 20 swabs per batch, about 19.8% of positive tonsil swabs and 80% of positive pig herds, were reported. A higher number of tested swabs per herd could result in a higher number of positive herds due to the usually low number of infected animals per herd. They found 1 to 10 positive swabs out of 20 which agrees with this study's finding of 1 to 7 positives out of 10. Funk et al. (14) from the USA report that 92.2% lots and 23.9% of pigs, sampled by the swabbing of oral-pharyngeal surface, were infected with *Y. enterocolitica* and, 28.2% of lts and 13.2% of pigs with the pathogenic ones. In this study, high numbers of animals per lot were sampled (9 to 97 swabs at lots sized from 9 to 200 animals). They also found a low number (1 to 11) of animal carriers of pathogenic *Y. enterocolitica* per lot. Authors from Germany report that *Y. enterocolitica* persisted in tonsils in 38.4% (15). They also concluded, similarly to many of the previous studies, that tonsils are the most reliable tissue for *Y. enterocolitica* detection in slaughtered pigs (15, 20, 24) and that the relative difference of finding them in the tonsils and faeces is 6 to 1 (23). Due to this finding, sampling of tonsils by swabbing was chosen for this study. Protocols for enrichment procedures that were performed (procedures 2 and 3) are part of the International Standard Organization method (ISO 10273: 2003). In addition, direct plating on CIN (method 1) was implemented, due to the reports of good recovery rate (26), the ease of performing and a relatively fast result.

None of the herds were positive by only using procedure 2. With respect to the results of this study, and in agreement with Belgian findings (26), it can be concluded that the alkaline (KOH) treatment of enriched PSB cultures had a positive impact on *Y. enterocolitica* isolation. In this study, the procedure using the alkaline (KOH) treatment of enriched PSB was the most efficient procedure, with successful isolation at 74.4% of positive herds. *Y. enterocolitica* is able to resist weak alkaline treatment (3), and this property is used to select the organism while suppressing background flora. Successful isolation out of the treated enrichment broth (protocol 3) confirms the presence of *Y. enterocolitica* viable cells. Massive additional microflora which covered the target cells could be the reason for inability for isolation out of the enrichment broth without treatment (protocol 2).

In 9 out of 43 of the confirmed positive herds *Y. enterocolitica* were isolated only by applying direct plating (protocol 1). There is a theoretical possibility that all *Y. enterocolitica* cells were removed to the agar plates when direct plating or that the background microflora overgrew them during the following enrichment. By using both procedures in parallel both possibilities are overcome.

Other authors also indicate that the recovery rate at incubation in PSB for 5 days (procedures 2 and 3 of this study), is lower compared to the 2-day enrichment procedure, in particular as far as the method without KOH treatment (26) is concerned. Due to this finding, the 5-day incubation could be a minor reason for the relatively low isolation rate in this study.

Many surveys were performed using the PCR method and they reported a higher sensitivity of PCR compared with culture methods and in general they report a higher estimated prevalence (4, 12, 17, 21, 25).

The most frequently isolated serotype in the study was serotype 4 (O:3) which is the serotype most often recovered from pigs in Europe, Japan and Canada (2, 15, 18, 20, 23, 28) and the predominant serotype implicated in human illness (7, 18).

However, the study highlights the need for further development and improvement of the methods used for detection of pathogenic *Y. enterocolitica.* Among the procedures employed, a combination of the direct plating method (method 1) and enrichment using KOH treatment covered all positive isolations.

The number of animals sampled per herd could impact the estimation of herd contamination. It was confirmed that *Y. enterocolitica* is present in Slovenian pig herds, but an additional survey for its final estimation employing improved culture methods or PCR should be carried out.

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UGOTAVLJANJE PRISOTNOSTI BAKTERIJE *Yersinia enterocolitica* V TONZILAH KLAVNIH PRAŠIČEV V SLOVENIJI, Z UPORABO TREH POSTOPKOV IZOLACIJE

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Povzetek: Z raziskavo smo želeli pridobiti prve podatke o kontaminiranosti klavnih prašičev v Sloveniji z bakterijo *Yersinia enterocolitica*. Uporabili smo tri različne postopke izolacije ter njihovo uspešnost med seboj primerjali. V obdobju od januarja do maja 2008 je bilo v slovenskih klavnicah odvzetih 1490 brisov tonzil prašičev, ki so pripadali 149 klavnim serijam. Iz posamezne klavne serije je bilo vzorčenih po 10 živali. Postopki, ki smo jih uporabili za izolacijo *Yersinia enterocolitica*, so naslednji: 1. Vsebino brisa smo nanesli neposredno na gojišče agar Cefsulodin-Irgasan-Novobiocin (CIN); 2. Vsebino brisa smo inkubirali v PSB broth (PSB) Yersinia in jo nato po obogatitvi precepili na agar CIN; 3. Vsebini smo po obogatitvi v PSB dodali kalijev hidroksid (KOH) in jo nato precepili na agar CIN. V 43 (29,3 %) klavnih serijah smo bakterijo *Y. enterocolitica* izolirali iz brisa tonzil vsaj ene od 10 testiranih živali z vsaj enim od uporabljenih postopkov. Izolacija je bila največkrat uspešna če smo uporabili tretiranje s KOH (74,4 %). S kombinacijo neposrednega nasajanja na gojišče CIN in z uporabo obogatitve s tretiranjem s KOH, je bila izolacija uspešna pri vseh ugotovljeno pozitivnih serijah. Izolirani sevi bakterije *Y. enterocolitica* so bili biotipizirani in serotipizirani. Večina izoliranih sevov (80,4 %) je pripadala biotipu 4, serotipu O:3.

Ključne besede: *Yersinia enterocolitica*; klavni prašiči; tonzile; izolacija