APPLICATION OF MULTIPLEX PCR FOR THE EVALUATION OF THE OCCURRENCE OF \textit{ail}, \textit{ystA}, AND \textit{ystB} GENES IN \textit{YERSINIA ENTEROCOLITICA} STRAINS ISOLATED FROM WILD BOARS (\textit{SUS SCROFA})

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Abstract

The study aimed at the evaluation of the occurrence of genes directly connected with pathogenicity in \textit{Yersinia enterocolitica (Y. enterocolitica)} strains isolated from wild boars, obtained in the 2007/2008 hunting season in the North-East territory of Poland. Multiplex PCR, used for \textit{ail}, \textit{ystA}, and \textit{ystB} gene detection, was optimised in order to determine the existence of the genes in one reaction. Forty-six palatine tonsils, gained from 46 wild boars of various ages, were preliminary examined from the bacteriological, sero- and biotyping points of view; then bacterial DNA was isolated and multiplex PCR was performed. The presence of the product of volume corresponding to the \textit{ystB} gene fragment was found in two cases, which constituted 4.35% of the examined samples. The \textit{ail} and \textit{ystA} genes were not detected in any of the tested samples. The molecularly confirmed existence of \textit{Y. enterocolitica} in wild boar palatine tonsils, indicates the carrier state and possibly shedding of the microorganism into the environment, and suggests that wild boars, likewise pigs to a lesser extent, may constitute a reservoir of the bacteria and a potential source of infection for the man.

Key words: wild boars, \textit{Yersinia enterocolitica}, genes, multiplex PCR.

\textit{Yersinia enterocolitica (Y. enterocolitica)}, commonly in existence all over the world, is the Gram-negative bacterium causing yersinosis – zoonosis, with a growing epidemiologic significance, especially in Europe (8, 9, 13). The phenomenon of the significant correlation between the strains pathogenic for the man and the strains pathogenic for swine, as well as the fact that the main source of the microorganism for human beings is raw or half-raw pork meat, are the reason why pigs are the most frequent object of examinations concerning the \textit{Y. enterocolitica} strains which are potentially pathogenic for the man (4, 17). On the other hand, wild boars as free-living animals, closely related to swine, may also constitute a potential microbe reservoir and contribute to the occurrence of incidences of the disease among people. Due to the growing interest in venison as an alternative source of raw material with high nutrition values, it is necessary to microbiological inspect the meat gained in certain specific circumstances. The way wild boars are disembowelled in field conditions produces many possibilities for the contamination of the carcasses by microflora coming from various sources. \textit{Y. enterocolitica}’s ability to survive and, moreover, to multiply in low temperatures (the freezers in which the carcasses are stored), constitutes a potential threat for the consumer by means of contaminating the final product, \textit{i.e.} meat (11).

Taking into account \textit{Y. enterocolitica}’s characteristics and the necessity to carry on two kinds of cultures, the so called warm culture and cold culture, it has to be stated that the conventional methods of the isolation and identification of pathogenic strains of this microorganism are laborious and time-consuming. Therefore, it seems appropriate to apply the molecular diagnostics methods of \textit{Y. enterocolitica} infection, which not only shorten the waiting time for the test result and enable us to state precisely whether the \textit{Y. enterocolitica} strain exists in the examined sample, but also allows us to detect the presence of the so-called virulence markers, directly responsible for the pathogenic features of the tested strain. From among the six known biotypes, the strains belonging to biotypes 1B and 2-5, having the pYV virulence plasmid (plasmid \textit{Yersinia} virulence), the \textit{ail} and \textit{inv} chromosomal genes, and whose products are responsible for the processes of invasion and adhesion to the intestinal epithelial cells, as well as the \textit{ystA} gene coding of the production of the YstA enterotoxin, are considered as pathogenic for...
animals and people. The 1A biotype strains without the classical virulence markers were, until recently, regarded as non-pathogenic (14, 18, 20, 22). Lately however, there are more and more reports of clinical cases of yersiniosis caused exactly by the 1A biotype, which suggests that the strains of this particular biotype (5) should not be excluded in advance as being potentially pathogenic (5). The Y. enterocolitica belonging to the 1A biotype has, in the majority, the ystB gene responsible for the production of the YstB enterotoxin, being most likely the reason for diarrhoea in the course of the yersiniosis caused by this biotype.

The purpose of the study was to identify the ail, ystA and ystB genes in the Y. enterocolitica strains isolated from the wild boars gained during the 2007/2008 hunting season in the North-East of Poland, by means of multiplex PCR, optimised for the application of three pairs of starters in one reaction.

Material and Methods

Bacterial strains and culture conditions. The material for the study consisted of 46 palatine tonsils from 46 wild boars of various ages, 14 of which came from piglets and 32 from one to three-year-old animals. Two samples were taken from each tonsil, cultured simultaneously on ITC medium (broth with irgasan†, ticarcillin, and potassium chlorate) – warm culture, and PSB medium (broth with peptone, sorbitol, and bile salts) – cold culture. Further biochemical identification was carried out according to the PN-EN ISO 10273 standard, identically for both kinds of cultures, and this allowed for the preliminary selection of potentially pathogenic Y. enterocolitica strains, which were used for further examinations.

Serotype and biotype determination. The determination of the serologic group of the examined strains was performed using the slide agglutination test. Live bacterial cells from the 24-h-blood-agar-culture (Grasco) were used as an antigen, and the serum for the somatic antigens O:3, O:5, O:8, and O:9 came from ITEST company (Czech Republic). The cells of the tested strain were suspended in a drop of 0.85% NaCl placed on a glass slide, and then connected with a drop of serum placed nearby and mixed with the bacteriological oese. The occurrence of agglutination with one out of the four sera used after shaking for 1 min was considered to be a positive result. In the case of the lack of agglutination with any serum, the strain was regarded as being a non-typable one. The biotype determination of the examined strains was made in accordance with the PN-EN ISO 10273 standard.

DNA isolation. Genomic DNA isolation was performed with the “Genomic Mini” kit (A&A Biotechnology, Gdynia, Poland), destined for the isolation of DNA from bacteria, cell cultures, and solid tissues, making use of the ability of genomic DNA to bind to silica deposits in highly concentrated chaotropic salts. The isolation was made according to the manufacturer’s instruction. The purified DNA was stored in a test tube at -20°C for further analyses.

Primers and multiplex PCR conditions. Multiplex PCR included the amplification of three genes: ail, ystA, and ystB. The sequences of the primers, synthesised in the DNA Sequencing Laboratory of the Biochemistry and Biophysics Institute of the Polish Academy of Sciences, Oligo, Warsaw, were published by Harnett et al. (7) (ail) and Platt-Samaraj et al. (15) (ystA, ystB) (Table 1).

Multiplex PCR was carried out using HotStarTaq Plus DNA Polymerase (Qiagen) and HotStarTaq Plus Master Mix Kit (Qiagen). The reaction mixture of 20 µl volume contained about 120 ng of isolated DNA (from 1 to 3 µl), 10 µl of HotStarTaq Plus Master Mix 2x, 2 µl of CoralLoad Concentrate 10x, 0.1 µl of each of the primers (final concentration 0.5 µM), and the total was supplemented by up to 20 µl of RNase-free water. Three controls were applied to each reaction: two positive, with DNA isolated from the reference strains O:3 and O:5, and one negative, without DNA. The reaction was performed in a thermo-cycler (Mastercycler, Eppendorf). The applied reaction conditions included a preliminary 5-min denaturation at 95°C, and then 30 cycles with subsequent stages: denaturation at 94°C for 30 s, starter connection at 54°C for 30 s, and elongation at 72°C for 1 min. After the last reaction, the final chain synthesis took place at 72°C for 10 min. Once the reaction was over, electrophoresis separation was conducted in 2% agarosis gel containing 0.5 µg/mL of ethidium bromide, whose aim was to visualise the DNA fragments of the Y. enterocolitica strains obtained as a result of multiplex PCR. The volume of the obtained products was evaluated by means of a comparison with the standard mass of GeneRuler† 100 bp Ladder Plus (Fermentas). The electrophoresis results were recorded using the GelDoc gel evidence system (Bio-Rad).

Results

From among the 46 samples of wild boar palatine tonsils, the presence of genes directly related to Y. enterocolitica pathogenicity was detected only in two cases using multiplex PCR, which constituted 4.35% of the tested samples. In multiplex PCR, using three pairs of primers for the ail, ystA, and ystB genes, the following products of the reaction were searched for: fragments of the ail gene of 356 bp volume, the ystA gene of 134 bp volume, and the ystB gene of 180 bp volume, respectively. In none of the examined samples the presence of the ampicons of volume corresponding to the sought for fragments of the ail and ystA genes was demonstrated, whereas in two of the tested samples the presence of the product of the volume corresponding to ystB gene was found (Fig. 1).

The results of multiplex PCR, taking into consideration the age of the wild boar from which the sample was taken, and the kind of the culture from which the Y. enterocolitica strain was isolated for molecular tests, its biotype and serotype, are presented in Table 2.
Table 1  
Sequences of the primers used in multiplex PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of the primers</th>
<th>Product volume (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ail</td>
<td>$5'TGGTTATGCGAAAAGCATGT'3$ $5'TGGGAACTGGTTGAATTGCA'3$</td>
<td>356</td>
<td>Harnett et al. (7)</td>
</tr>
<tr>
<td>ystA</td>
<td>$5'GTCTCATTTGGGGATTGCCGAC'3'$ $5'AATCAGACTGACTTCCGCTTG'3'$</td>
<td>134</td>
<td>Platt-Samoraj et al. (15)</td>
</tr>
<tr>
<td>ystB</td>
<td>$5'TTCACTTATTCTCAACTTCCGCTTG'3'$ $5'GCCGATAATCATCATCAAG'3'$</td>
<td>180</td>
<td>Platt-Samoraj et al. (15)</td>
</tr>
</tbody>
</table>

bp – base pairs

Fig. 1. Electrophoresis separation of ail, ystA, and ystB gene amplification products from Y. enterocolitica strains in multiplex PCR. M - GeneRuler™ 100 bp DNA Ladder Plus volume marker. Path 1 – positive control, includes DNA isolated from the reference Y. enterocolitica strain O:3 (524/98+). Path 2 – positive control, includes DNA isolated from the reference Y. enterocolitica strain O:5. Path 3 – ‘zero’ control, does not include bacterial DNA, but includes primers for the ail, ystA and ystB genes. Paths 4, 5 – product corresponding to the fragment of ystB Y. enterocolitica gene in tested samples.

Table 2  
Culture and molecular test results and bio- and serotyping of Y. enterocolitica strains

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Wild boar age</th>
<th>Warm culture</th>
<th>Cold culture</th>
<th>Biotype</th>
<th>Serotype</th>
<th>ail</th>
<th>ystA</th>
<th>ystB</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Older</td>
<td>-</td>
<td>+</td>
<td>1A</td>
<td>O:5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>Older</td>
<td>-</td>
<td>+</td>
<td>1A</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

NT- serotype marked as non typable (lack of agglutination reaction with diagnostic sera for serotypes O:3, O:5, O:8, O:9)

Discussion

Y. enterocolitica presents itself as an interesting but diagnostically difficult pathogen. So far, 70 serotypes have been identified on the basis of differences in the structure of the O somatic antigen, as well as six biotypes: 1A, 1B, and 2-5, defined according to their biochemical features. Bacteriological studies, especially cold culture, require a long period of waiting for the inoculation result, the application of the biochemical row aims at demonstrating the bacteria characteristic features, as well as performing tests reveal the presence of plasmid, or the so called tests indirectly
PCR can be applied for the detection of was demonstrated, optimised in our research multiplex molecular biology seem to be the best solution. As it diagnostic difficulties, fast and reliable methods of Yersinia and the isolation of the bacterial strains from faeces, serological tests are carried out in many countries. Their results, however, may be difficult to trust, not only for the antigen affinity between the strains of the various serological groups of Y. enterocolitica and other Yersinia sp., but also due to the cross reactions with the microorganisms belonging to the different genera and families, for instance Brucella abortus, Salmonella sp., E. coli, or Vibrio sp. (8).

Taking into consideration the numerous diagnostic difficulties, fast and reliable methods of molecular biology seem to be the best solution. As it was demonstrated, optimised in our research multiplex PCR can be applied for the detection of ail, ystA, and ystB chromosomal genes not connected with plasmid, which the strain may lose, for instance by means of applying inadequate culture conditions (2). It is considered, that the Ail protein, coded by the ail gene, exists only in pathogenic strains of Y. enterocolitica and is responsible for the process of penetration into mammal cells, as well as for resistance to the bactericidal action of the complement system and of the human serum. On the other hand, Yst enterotoxins coded by yst genes are biologically analogical to thermo-stable St enterotoxin produced by E. coli. By means of cGMP activation, they cause the accumulation of liquids in the intestines, which in turn causes diarrhoea in the course of yersinosis (14). Thus, the indication of the presence of virulence markers in the form of ail and ystA genes confirms the pathogenic abilities of the examined strain, while the presence of the ystB gene confirms its affiliation to the 1A biotype, regarded previously as being non-pathogenic. Multiplex PCR applied in our research not only significantly shortens the time of waiting for the test result, but simultaneously confirms whether the examined sample contains Y. enterocolitica, and, what is important, does not ignore the 1A biotype strains. It has been indicated that not only do they produce enterotoxin with an intensity of action much stronger than YstA, but they also invade the epithelial cells much more effectively than classical pathogenic strains, based on the previously unknown pathomechanism, which suggests the existence of other different ways of cell penetration by 1A biotype strains. Numerous premises point out that the incidences of the disease caused by this biotype shall become more and more frequent, and that the currently used criteria for the Y. enterocolitica pathogenic evaluation, based mainly on the detection of plasmid virulence, may appear insufficient (2, 5, 6, 14, 16-19, 21). Therefore, in relation to the study by Woźniak-Kosek et al. (23), whose aim was the detection of the ail and ystA genes of Y. enterocolitica pathogenic strains, the range of the ystB genes detection was broadened in our research. Thus, the concentration was directed mainly at the detection of the genes responsible for enterotoxin production, as a predominant pathogenic factor, as opposed to the study by Kot et al. (12), which suggested the application of multiplex PCR (ail, ystB, inv, and yadA), including the detection of 1A biotype strains, but not indicating the presence of the ystA gene. With reference to the study by Platt-Samoraj et al. (15), the method was improved in such a way that ail, ystA, and ystB genes could be detected in one reaction – triplex PCR.

Interpreting the results obtained thanks to multiplex PCR, it has to be stated, that wild boars, just as pigs though to a lesser extent, may constitute a reservoir and potential source of Y. enterocolitica infection for the man. The presence of this microorganism in the palatine tonsils, though admittedly only 4.35% of the wild boars examined, points out the carrier state and possibly the bacterium being shedded into the environment. Papers regarding the presence of Y. enterocolitica in wild boars appear in Polish and foreign literature extremely rarely and bring diverse results. Koronkiewicz et al. (11) classified only one out of 45 strains isolated from wild boar faeces on the basis of biochemical tests regarding Yersinia sp. Unfortunately, serological and molecular identification was not performed, which might have allowed us to confirm its attachment to Y. enterocolitica. The analysis of the swabs from wild boar carcasses was negative (11). The same examinations, which also excluded the species’ attachment to the Yersinia sp. strains obtained from wild boars, were performed at the end of 1995 and at the beginning of 1996 in the North-East of Germany. Al Dahouk et al. (1) detected antibodies against Yop proteins, occurring in Yersinia sp. pathogenic strains, in 478 out of the 763 examined serum samples, which constituted 62.6%. The antibodies occurred independently of the sex and age of the animals from which the samples were taken. Koppel et al. (10), evaluating the occurrence of infectious diseases in wild boars in the territory of Switzerland, examined the reproductive organs from nine animals. The results of the bacteriological tests regarding Y. enterocolitica were negative (10). Therefore, the literature data confirm the growth of interest in the occurrence of pathogens in free-living animals, which may constitute a threat for the man. It is also confirmed by the review study of Gill (3), informing us about frequent Y. enterocolitica findings in wild boar faeces samples, as well as in venison offered in wholesale.

Thus, wild boars may constitute a reservoir of numerous pathogenic microorganisms for the man, being a potential source of infection in the case of many zoonoses, to which undoubtedly yersiniosis belongs. A particular risk of the infection concerns people being in a direct contact with wild boars, such as hunters or veterinary surgeons, as well as consumers, which makes it of significant importance for public health. Multiplex PCR, as applied in our research, is an excellent tool for the fast and reliable diagnosis of the presence of Y. enterocolitica in wild boar carcasses, and may also be successfully applied in other kinds of animals as well as in the man.
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References


