APPLICATION OF MULTIPLEX PCR FOR THE EVALUATION OF THE OCCURRENCE OF \( \text{ystA}, \text{ystB}, \text{ystC}, \) AND \( \text{ymoA} \) GENES IN \( \text{YERSINIA ENTEROCOLITICA} \) STRAINS ISOLATED FROM FATTENING PIGS

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Abstract

The purpose of the study was to evaluate the occurrence of genes directly connected with pathogenicity in \( \text{Yersinia enterocolitica} \) strains isolated from fattening pigs. Multiplex PCR, used for \( \text{ystA}, \text{ystB}, \text{ystC}, \) and \( \text{ymoA} \) gene detection, was optimised in order to determine the existence of the genes in one reaction. Material for the study consisted of 138 strains of \( \text{Y. enterocolitica} \), which were preliminary examined by the bacteriological, sero- and biotyping methods, then bacterial DNA was isolated and multiplex PCR was performed. The presence of the products of length corresponding to the \( \text{ystA} \) and \( \text{ymoA} \) gene fragments was found in each of the examined strains. The \( \text{ystB} \) and \( \text{ystC} \) genes were not detected in any of the tested samples. The molecularly confirmed existence of \( \text{Y. enterocolitica} \) in fattening pigs indicates the carrier state and a possibility of shedding the microorganism into the environment, and shows that pigs are an important reservoir of the bacteria and a potential source of infection for humans.

Key words: fattening pigs, \( \text{Yersinia enterocolitica} \), \( \text{ystA}, \text{ystB}, \text{ystC}, \) and \( \text{ymoA} \) genes, multiplex PCR.

\( \text{Yersinia enterocolitica} \) is the third aetiological factor in human diarrhoea in terms of the number of confirmed clinical cases, after \( \text{Salmonella} \) sp. and \( \text{Campylobacter} \) sp. (4, 6). Due to the symptomatic diversity of the disease, it is extremely difficult to recognise it by the clinical picture, both in humans and in animals. Laboratory tests seem necessary for proper diagnosis and selection of a treatment regimen. Bacteriological examinations used to isolate strains of \( \text{Y. enterocolitica} \) and those used to determine the bio- and serotype of the collected isolates are essential for further diagnostic procedures, but they are also time- and labour-consuming, and they do not provide a clear answer whether a strain is pathogenic (17). It has been repeatedly shown that strains classified to biotype 1A (3) – commonly regarded as non-pathogenic – may cause clinical form of yersiniosis (9, 15, 16). Classification to a specific serotype is not a clear reason for regarding a strain as pathogenic, because for example serotype O:8 includes both environmental, non-pathogenic strains (19), and such that cause the disease in humans (14). Therefore, molecular methods are more and more frequently applied in diagnostics and epidemiology. A number of genes specific for \( \text{Y. enterocolitica} \) are known, such as \text{ail}, \text{inv}, \text{yadA}, \text{yops}, \text{yst} \) and \text{virF}, referred to as markers of virulence, whose characteristic sequences are detected by PCR technique. The \( \text{yst} \) gene, which encodes production of \( \text{Yst} \) enterotoxins, is one of the important virulence markers typical of all \( \text{Y. enterocolitica} \) strains. Not all strains show the ability to produce the enterotoxin, but it has been shown to exist in pathogenic strains, isolated from clinical cases of yersiniosis, which indicates that they play an important role in aetiology of diarrhoea occurring during the disease. However, not all the strains with the \( \text{yst} \) genes produce the enterotoxins, which seems to be caused by the \( \text{ymoA} \) gene encoding production of \( \text{YmoA} \) protein, which inhibits expression of various genes (12, 20).

The purpose of the study was to identify the \( \text{ystA}, \text{ystB}, \text{ystC}, \) and \( \text{ymoA} \) genes in \( \text{Y. enterocolitica} \) strains isolated from the fattening pigs, by means of multiplex PCR, optimised for the application of four pairs of primers in one reaction.

Material and Methods

Material. The material for the study consisted of 138 strains of \( \text{Y. enterocolitica} \) isolated from fattening pigs, which were preliminary examined by the bacteriological, sero- and biotyping methods.

DNA isolation. Genomic DNA isolation was performed with the “Genomic Mini” kit (A&A Biotechnology, Poland), used 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
injection. The purified DNA was stored at -20°C prior to further analyses.

**Primers and multiplex PCR conditions.** The multiplex PCR method involved amplification of ystA, ystB, ystC, and ymoA genes. The primer sequences, synthesised at the DNA Sequencing Laboratory of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Oligo, Warsaw), were obtained from the papers by Platt-Samoraj et al. (13) (ystA, ystB) and Grant et al. (5) (ymoA). Primers for the ystC gene were designed based on the gene sequence from the GenBank (D63578), with the Primer-BLAST programme, available at the National Centre for Biotechnology Information website. Both designed primers had 20 nucleotides length; the ystC-1 primer (Forward), with the sequence: 5’TACGCAAGTGAGTGACGGAG3’, was complementary to the 63-82 position of the ystC gene, and the ystC-2 primer (Reverse): 5’CCTTTACTCGGCACGGAAT3’, annealed at the position of 346-327 nucleotides. Sequences of primers used in the reaction are shown in 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 volume was adjusted to 20 µl with 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 primers annealing at 46°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. Next, electrophoresis separation was conducted in 2% agarose gel containing 0.5 µg/mL of ethidium bromide, in order to visualise the DNA fragments of the *Y. enterocolitica* strains obtained in multiplex PCR. The length 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**

A multiplex PCR method has been developed for the purposes of molecular diagnostics, in which ystA, ystB, ystC, and ymoA genes could be detected in one reaction. Therefore, the optimum temperature-time profile of the reaction was established with a view to quick and specific diagnostics of infections with *Y. enterocolitica*, taking into account the ymoA gene.

A positive result of the reaction in the form of amplicons with the expected mass, obtained in the presence of positive controls and the absence of bands with the negative control indicates the correct course and specificity of the method.

Owing to application of a set of primers for regions encoding enterotoxins Yst in multiplex PCR, it was possible to detect proper amplicons by evaluation of the size of bands with reference to the mass standard. The use of multiplex PCR allowed detecting the 134 bp product of the ystA gene, 180 bp product characteristic for the ystB gene, and 330 bp product of ymoA gene. No 284 bp band was detected, indicating the absence of the ystC gene in analysed samples. In general all studied strains had the ystA gene, which indicates that they belong to the strains commonly regarded as pathogenic. Amplification of a fragment of ymoA gene was also detected in all examined strains. The results of multiplex PCR were documented by photographs of agarose gels with the presence of appropriate size bands clearly marked; an example of which is shown in Fig. 1.

**Discussion**

Bacteriological examinations and bio- and serotyping, commonly applied in diagnostics of infections with *Yersinia enterocolitica*, are time- and labour-consuming, at the same time they do not clearly identify pathogenic strains. Considering the above, molecular methods, such as hybridisation or PCR, including multiplex PCR – particularly useful in showing the presence of fragments of several genes, typical of *Y. enterocolitica* - and the qualitative real-time PCR method, seem to be the most effective, quickest and most reliable in diagnosing yersiniosis. Vazlerova and Steinhauserova (18) examined 2,982 samples from pigs and isolated 111 strains of *Y. enterocolitica* using classic biochemical methods, while the number of positive results in PCR was 120. This shows that not all pathogenic strains are isolated in bacteriological examinations. A similar study by Johannessen et al. (7) brought even more astonishing results, because for 50 positive results in PCR only six strains of *Y. enterocolitica* were detected with bacteriological methods. Bhaduri et al. (1) applied the PCR technique in molecular examinations and proved the presence of a sequence typical of the *ail* gene in 12.35% of faecal samples, while only 4.08% positive results were obtained in culturing studies. Kot et al. (8) conducted studies on 80 palatine tonsils obtained from pigs from three different farms and detected the presence of *Y. enterocolitica* by the multiplex PCR method in six cases, which accounts for 7.5% of the samples, while the bacteria were detected by culturing methods only in three cases (3.75%). These findings may provide grounds for the conclusion that molecular methods of *Y. enterocolitica* detection, especially PCR, are more precise and more specific in comparison with the bacteriological methods.
Table 1

<table>
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<th>Gene</th>
<th>Primer sequence</th>
<th>Product size (bp – base pairs)</th>
<th>Source</th>
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| ystA | 5’GTCTTCATTTGGAGGATTCCGGC3’
      | 5’AATCACTACTGACTTCGGCTGG3’    | 134    | Platt-Samoraj et al. (13) |
| ystB | 5’TGCACAGCATTTATTCTCAACT3’
      | 5’GCCGATAATGTATCATCAAG3’      | 180    | Platt-Samoraj et al. (13) |
| ystC | 5’TCGACAAGTGAGTGACGGAG3’
      | 5’CCCTTATCTCGGACGAATA3’       | 284    | D63578 GenBank            |
| ymoA | 5’GACTTTTCTCAGGGGATAAC3’
      | 5’GCTCAACGTTGTGTCTT3’         | 330    | Grant et al. (5)          |

Fig. 1. Electrophoretic separation of products of ystA, ystB, ystC, and ymoA genes’ amplification from strains of Y. enterocolitica in multiplex PCR. M – size marker GeneRuler™ 100bp DNA Ladder Plus (Fermentas). Lane 1 – positive control, contains DNA isolated from a reference strain O:8 (ACTT 23715). Lane 2 – positive control, contains DNA isolated from a reference strain O:5. Lane 3 – zero control, does not contain bacterial DNA, contains ystA, ystB, ystC and ymoA gene primers. Lanes 4 - 14 – products of amplification of ystA and ymoA gene fragments from strains of Y. enterocolitica isolated from the samples under study.

No connection has been found between traditional markers of pathogenicity and occurrence of diarrhoea. Since Pai and Mors (10), as well as Pai et al. (11) proved the ability of various bacterial strains to induce enterotoxin production on infant mice suckling test in 1978, Y. enterocolitica has been regarded as an enterotoxic pathogen. Currently, two main enterotoxins are identified: enterotoxin YstI, which includes variants YstA, YstB, and YstC, and enterotoxin YstII, whose mechanism of action probably differs from that of the known enterotoxins of Y. enterocolitica. As the nucleotide sequence for the gene encoding production of enterotoxin YstII has not been determined, a decision was taken to show by means of multiplex PCR characteristic sequences of known variants of the yst gene, which encode enterotoxin production. Attention was also paid to the ymoA gene, encoding a histone-like protein YmoA, which inhibits the expression of various genes.

The initial stage of the study involved optimisation of the temperature-time profile of multiplex PCR so that the presence of the ystA, ystB, ystC, and ymoA genes could be detected in one reaction. A proof of the presence of bands with the appropriate size for the applied positive controls, with the lack of a positive result for the negative control, confirmed the reaction specificity. Until now, experiments with such genetic configuration have not been carried out. In a study conducted by Grant et al. (5) concerning only strains of biotype 1A, fragments of genes ail, inv, myfA, ystA, ystB, and ystC were sought by the hybridisation method. The presence of a homologous sequence for the ystA gene was detected only in one of the studied strains, while hybridisation with the ystB gene was detected in 95 strains. However, no strain was found with a sequence homologous to the ystC gene. Additionally, the PCR method was used in search of a characteristic sequence of the ymoA gene in 20 investigated strains, including the 10 obtained from clinical cases and 10 of other origins. Amplification products of expected size were detected in all the examined strains. Similar studies on molecular characteristics of the strains of biotype 1A were carried out by Bhagat and Virdi (2) and involved determination of distribution of a number of genes related to virulence in the strains of this biotype. Seeking fragments of ystA, ystB, ystC, and ymoA genes in individual reactions yielded a positive reaction for the ystB and ymoA genes and a negative result for the ystA and ystC genes. Fragments of the other genes were found with variable frequency. These reports of the occurrence of the ymoA gene in the strains of Y. enterocolitica were also confirmed in our study, where the multiplex PCR method detected a 330 bp product specific for the ymoA gene in all 138 investigated strains of Y. enterocolitica, incidentally, it should be stressed...
that the examination was not restricted to the strains of biotype 1A. This implies that the ymoA gene occurs in all strains of \textit{Y. enterocolitica}, regardless of their potential pathogenicity.

A 134 bp band has been found in all the strains collected in our study, which indicates the presence of the \textit{ystA} gene. A similar result was reported by Woźniak-Kosek \textit{et al.} (21), who characterised 84 strains of \textit{Y. enterocolitica} isolated from pig oral cavity swabs, side surfaces, and faeces. The \textit{ystA} gene was found in all the strains under study based on the 134 bp product. However, higher variability was detected between the number of strains with the \textit{ystA} gene and the number of those with the \textit{ystB} gene. A study by Thoerner \textit{et al.} (17), in which genes connected with virulence of the studied strains, \textit{e.g.} \textit{ystA} and \textit{ystB}, were detected in 140 strains of \textit{Y. enterocolitica}, isolated from various sources, including 32.86% samples from pigs, which yielded a positive result for the \textit{ystA} gene in 92 (65.72%) cases. A 146 bp band, corresponding to the fragments of the \textit{ystB} gene, was obtained in 43 (30.71%) cases of the examined strains. No fragments of the \textit{yst} genes of interest were found in five strains of \textit{Y. enterocolitica}, including one from a pig, which accounts for 3.57% of all the samples. At the same time, a positive result for both \textit{ystA} and \textit{ystB} genes detected as two bands, 79 bp and 146 bp, respectively, with the weaker band for \textit{ystB}, was obtained for three strains of \textit{Y. enterocolitica}. Considering the PCR results for different sources of the strains of \textit{Y. enterocolitica} it can be concluded that the \textit{ystA} gene was present in most strains isolated from pigs. The 79 bp band, which corresponds to the \textit{ystA} gene, was found in 37 (80.44%) out of 46 samples. Of the remaining nine strains, fragments typical of the \textit{ystB} gene were found in eight (17.39%) strains, and one (2.17%) strain showed no the \textit{yst} genes. Therefore, a clear majority of the strains of \textit{Y. enterocolitica} isolated from pigs are those with the \textit{ystA} gene, responsible for the production of thermostable enterotoxin.

A study by Kot \textit{et al.} (8) involved the evaluation of 48 strains of \textit{Y. enterocolitica} and six strains of \textit{Y. pseudotuberculosis}, in terms of the presence of \textit{ail}, \textit{ystB}, \textit{inv}, and \textit{ysdA} genes. The multiplex PCR method, was developed by the authors, to serve as a quick assay for detection of \textit{Y. enterocolitica} strains, including the strains of biotype 1A, in which the \textit{ystB} gene, responsible for enterotoxin production, was usually present, as well as the strains of \textit{Y. pseudotuberculosis}. Most of the 48 (52.08%) strains of \textit{Y. enterocolitica} contained the \textit{ystB} gene, which was shown by the presence of a 69 bp band. The \textit{ail} gene was present in 47.92% of the strains, suggesting that they belong to the strains commonly regarded as pathogenic, in which the \textit{ystA} gene is also present. The newly-developed method was also used to test 80 samples from the palatine tonsils obtained from three different pig farms and three (3.75%) positive results were observed for the sequences of the \textit{ail} gene, together with three 69 bp bands, which indicates the presence of sequences typical for the \textit{ystB} gene of \textit{Y. enterocolitica}.

The molecularly proven existence of \textit{Y. enterocolitica} in fattening pigs indicates the carrier state and a possibility of shedding the microorganism into the environment, and shows that pigs are an important reservoir of the bacteria and a potential source of infection for humans.

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**References**


