OCCURRENCE OF YERSINIA ENTEROCOLITICA IN CANINE EXCREMENTS CONTAMINATING URBAN LAWNS

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Received for publication November 16, 2009

Abstract

The purpose of the studies was to determine, by means of bacteriological and molecular methods, the occurrence of Y. enterocolitica in the faeces of dogs, as well as to assess the degree of their spread in the urban environment. The faeces were collected from the lawns of six large districts of Olsztyn. In order to isolate Y. enterocolitica, “warm culture” (ITC/CIN) and “cold culture” (PSB/CIN) were used in parallel, together with biochemical tests. Next, genomic DNA of Y. enterocolitica was isolated and ail, ystA, and ystB genes were detected with the use of the multiplex PCR method. A relatively frequent occurrence of Y. enterocolitica strains in canine faeces contaminating the urban lawns of Olsztyn was demonstrated. The greatest percentage of contaminated faecal samples was found in the area of large housing estates. By means of molecular tests, the presence of ystB gene only, in the absence of ail and ystA genes, was demonstrated in the Y. enterocolitica genome, which could indicate a potentially pathogenic biotype 1A. Therefore, it seems important to keep monitoring the changes, which occur within this species of microorganisms, the epidemiological situation of yersiniosis in human and animal populations, as well as to continue the studies on the epidemiology of Y. enterocolitica infections also in the context of a reservoir of animals accompanying a man.

Key words: dogs, Yersinia enterocolitica, faeces, urban environment.

Within the last 30 years, Yersinia (Y.) enterocolitica has become a world-wide known human pathogen, constituting a cause of serious and miscellaneous health problems (27). The main signs of yersiniosis are gastrointestinal disorders in the form of diarrhoea with intensification at a varied degree, occurring especially in small children (16, 20). The disease may occur also in other forms – pseudoappendicular, septic, lymphonodular, and cutaneous, and in the form of erythema nodosum or Reiter’s syndrome (ocular-arthral-urethral). Moreover, reactive polyarthritis, abscesses of the liver, spleen, and colon, as well as bone inflammations, meningoencephalitis, pharyngitis, and panophthalmitis have been observed in its course (2, 4, 16, 17, 33).

Within the Y. enterocolitica species, there are many strains differing in antigen properties. Nowadays, six biotypes have been distinguished: 1A, 1B, 2, 3, 4, and 5, as well as 70 serotypes, isolated on the basis of the differences in the structure of the somatic O antigen. Biotypes 1B, 2, 3, and 4, and serotypes O:3, O:8, O:9, and O:5/27 are pathogenic for man and animals (9, 16). Biotype 1A is relatively frequently isolated from the environment (rivers, lakes, soil), food (vegetables, pork, poultry, pre-packed meat, seafood, raw milk and pasteurised dairy products), and healthy animals (birds, fish, insects, amphibians, various species of mammals, among others cattle, sheep, pigs, rats) (24, 34). Most animals, from which Y. enterocolitica of biotype 1A is isolated does not demonstrate the clinical signs of yersiniosis (36). In case of animals experimentally infected with biotype 1A strains, derived from the environment, the colonisation of the gastrointestinal tract has not been stated (24). So far, biotype 1A has been considered non-pathogenic, mainly because it does not have the virulence-associated plasmid pYV (plasmid Yersinia virulence) and ail (attachment-invasion locus) gene, which occur only in pathogenic strains. However, the latest studies provide the evidence that some serotypes belonging to biotype 1A may provoke the disease using factors and mechanisms independent of the virulence plasmid pYV (12, 24, 30, 36).

The pathogenicity of Y. enterocolitica is connected with its invasive and toxicogenic properties, which represent the ability of the microorganism to penetrate, proliferate, and produce toxins in the host cells. The pathogenic factors of Y. enterocolitica are encoded in the virulence plasmid pYV, as well as in chromosomal DNA (10, 11). The appropriate genes contain the information controlling the production of different proteins taking part in the process of adherence and penetration into the host cells, and conditioning the
defence mechanisms, which help to prevent the destruction by the immune system of the attacked organism (30). \( Ail \) gene, present only in pathogenic \( Y. \) enterocolitica strains, controls the production of the \( Ail \) protein regarded as one of the significant elements in the process of infection. This protein is localised on the external membrane of the bacterial cell and participates in adhesion and invasion, and also increases resistance to lysis, and protects against the activity of the complement system. \( Ail \) gene has been found only in biotypes \( 1B, 2, 3, 4, \) and \( 5 \). All these biotypes demonstrate features connected with \textit{in vitro} invasivenessness (10, 11, 18, 38).

A significant factor affecting the virulence of \( Y. \) enterocolitica strains is \( Yst \) enterotoxin (\textit{Yersinia stable toxin}), produced by all strains isolated from the clinical cases. \( Yst \) is a 71-amino-acid peptide consisted of three protein domains, which to a large extent show similarity to thermostable STI enterotoxin produced by \textit{Escherichia coli} strains (31, 35, 38). \( Yst \) is responsible for the increase in the concentration of cyclic guanosine monophosphate (cGMP) in enterocytes by means of the activation of guanylate cyclase, which leads to the passage of a large amount of liquid into the intestinal lumen, and consequently to diarrhoea. The \( Yst \) protein occurs in three variants - \( YstA \) (described above), \( YstB \), and \( YstC \). The \( YstB \) toxin is much more active than \( YstA \), its minimum effective dose amounts to 0.4 pmol/L and is much lower than the minimum effective dose of \( YstA \), equal 7.6 pmol/L (24, 29). Despite the fact that the studies have confirmed, that biotype \( 1A \) produces thermostable enterotoxin (\( YstB \)) at 28°C, its role in the pathogenesis of yersiniosis remains unexplained, due to the lack of evidence for the production of \( YstB \) \textit{in vitro} at 37°C, persisting in the host tissues (35). Thermostable \( Yst \) enterotoxin is chromosomally encoded. The possession of \( ystA \) gene, similarly to the \( ail \) gene, is attributed exclusively to pathogenic strains, whereas so far \( yst \ B \) gene has not been found in \( Y. \) enterocolitica strains belonging to the biotypes \( 1B, 2, 3, 4, \) and \( 5 \) (24, 29).

The main reservoir of \( Y. \) enterocolitica are pigs (3, 13); however, it is worth to emphasise the role of accompanying animals in the spreading of this microorganism. More and more often, they are considered to be the cause of family epidemics in humans (14, 27). It may be proved by the fact that \( Y. \) enterocolitica 4/0:3 was isolated from dogs and cats with diarrhoea, as well as from the faeces of healthy dogs. Clinical cases among dogs are observed most frequently in young individuals, while adult dogs are usually the asymptomatic carriers (21, 25). The studies of Fukushima et al. (8) indicated the presence of \( Y. \) enterocolitica 0:3 in 12.2% of pups under one year of age, and in 3.3% of older dogs. Serotype \( O:3 \) was isolated from the contents of the jejunum, whereas serotype \( O:5/27 \) occurred in dogs’ stomach, jejunum, and rectum. The outbreaks of yersiniosis concern dogs kept in animal shelters (5). Serotypes \( O:3, O:5/27, \) and \( O:9 \) were isolated from dogs; however, serotype \( O:8 \) was not isolated. It is worth noticing that the experimental infection of dogs with this serotype was successful (23).

Dogs excrete the microbe for several weeks after the infection, which makes them a potential source of infection for humans (7). The infections with pathogenic \( Y. \) enterocolitica are associated with the consumption of raw or undercooked pork, with which accompanying animals are often fed. After the consumption of raw pork meat containing the microbes, dogs excrete them together with faeces into the environment, where the bacteria survive about 23 days. Infections in man occur most probably via the alimentary route. The greatest risk of infection with \( Y. \) enterocolitica from accompanying animals relates to small children, due to their often close, direct contact with dogs, which may lead to the contamination of hands with excrements of infected animals (6, 7, 14, 25).

The purpose of this study was to determine, by means of bacteriological and molecular methods, the occurrence of \( Y. \) enterocolitica rods in the faeces of dogs, as well as to assess the degree of their spread in the urban environment.

**Material and Methods**

The samples of canine faeces, gathered from the urban lawns of Olsztyn, were examined. In general, 300 samples were collected in the period from November 2006 to April 2007 in six large districts of the city. Fifty faecal samples were gathered from each of the housing estate. The studies were divided into two stages: initial bacteriological identification and molecular tests. Bacteriological examinations were performed in accordance with the Polish PN-EN ISO 10273 standard (28).

**Bacteriological examinations.** Faecal samples were collected into the sterile faeces containers several hours before placing them in the proliferating medium. The examined material was proliferated in two selective liquid medium in parallel – ITC broth (irgasan, ticarcillin, and potassium chlorate) for 48 h at 22°C (warm culture) and PSB broth (peptone, sorbitol, and bile salts) for 21 d at 4°C (cold culture). The obtained cultures were then cultured on CIN agar (Difco USA) at 37°C, persisting in the host tissues (35). Thermostable \( Yst \) enterotoxin is chromosomally encoded. The possession of \( ystA \) gene, similarly to the \( ail \) gene, is attributed exclusively to pathogenic strains, whereas so far \( yst \ B \) gene has not been found in \( Y. \) enterocolitica strains belonging to the biotypes \( 1B, 2, 3, 4, \) and \( 5 \) (24, 29).

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**Molecular tests.** Bacterial DNA was isolated by means of a commercial Genomic Mini® isolation kit (A&A Biotechnology, Poland), according to the manufacturer’s instruction. The amount of isolated DNA was marked spectrophotometrically using BioPhotometer (Eppendorf, Germany). Isolated DNA was stored at -20°C for further analyses.

In order to indicate the presence of \( ail \), \( yst \ A \), and \( yst \ B \) amplicons, two multiplex PCR reactions were carried out. The aim of the first PCR was to amplify \( ail \) (531 bp) and \( yst \ B \) (180 bp) genes (26). The second PCR was executed in order to amplify \( ail \) (356 bp) and \( yst \ A \) (134 bp) genes (39). Oligonucleotide primers used for
the reactions were synthesised in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Biochemistry and Biophysics Institute of the Polish Academy of Sciences in Warsaw.

The reactions were performed in the presence of Tag DNA polymerase (Fermentas, Lithuania), in compliance with the manufacturer’s recommendations; the final MgCl₂ concentration amounted 1.5 mM. The amplification of ail and yst B genes was carried out according to the following schedule: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, primers’ annealing at 46°C for 1 min, elongation at 72°C for 2 min, and final elongation at 72°C for 5 min. The amplification of ail and yst A genes was carried out according to the following schedule: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, primers’ annealing at 56°C for 1 min, elongation at 72°C for 2 min, and final elongation at 72°C for 5 min. Electrophoretic separation of PCR products was conducted in 2% agarose gel, in a 1X concentrated TAE buffer in the presence of ethidium bromide (final concentration 0.5 µg/mL). The reaction was conducted for 30 min at a voltage of 5V per 1cm² of gel. For evaluation of the size of the products, GeneRuler™ 100 bp DNA Ladder Plus marker (Fermentas, Lithuania) was applied. The results were archived by means of GelDoc™ (Bio-Rad, USA) system for gel visualisation.

Results

The initial bacteriological identification allowed to qualify for further examinations 131 potential Y. enterocolitica strains, isolated from 300 collected faecal samples, including 28 from the ITC/CIN culture, which constitutes 9.3% of all the samples, as well as 103 from the PSB/CIN culture (34.3%), that is over three times more. It also allowed limiting the number of samples for molecular tests. Bacteriological examinations showed diversification in the percentage of isolated strains in particular housing estates: 2.0% - 16.0% in the warm culture, and 8.0% - 60.0% in the cold culture, which demonstrates considerable differences of a potential threat to humans from the population of dogs living in these city areas. The greatest amount of Y. enterocolitica strains in both types of culture, (38 out of 50 collected faecal samples) were isolated within the largest district of Olsztyn (L), in which tower-blocks and high-density housing are predominant. It has been found that Y. enterocolitica strains coming from one sample were not always isolated from both: the warm culture and the cold culture. In 14 cases Y. enterocolitica was isolated from the warm culture, however, it was not isolated from the cold one. In 89 cases the strains isolated from the cold culture were not detected in the warm culture. In 14 cases, Y. enterocolitica was isolated from both the cold and the warm culture. From 131 bacterial strains qualified for molecular tests, yst B gene was detected in 104 (79.4%), including 85 strains out of 103 examined in the cold PSB/CIN culture (81.7%), and 19 strains out of 28 examined in the warm ITC/CIN culture (67.3%). The percentage of strains having yst B gene was high in strains isolated in two types of culture, with greater diversity in case of the warm culture (25.0% - 100.0%), rather than the cold one (50.0% - 95.0%). No ail and yst A genes were found in the genetic material of the examined Y. enterocolitica strain. Table 1 and Fig. 1 present the obtained results.

Fig. 1. Electrophoretic separation in 2% agarose gel of ail (531 bp) and ystB (180 bp) gene amplification products from Y. enterocolitica strains in multiplex PCR. M – molecular weight marker (GeneRuler™ 100 bp DNA Ladder Plus). Lane 1 – positive control, includes DNA isolated from the reference Y. enterocolitica strain O:5 and primers for ystB gene. Lane 2 – positive control, includes DNA isolated from the reference Y. enterocolitica strain O:3 and primers for ail gene. Lane 3 - “zero” control without bacterial DNA, but with primers for yst B and ail genes. Lanes 4-9 – examined samples.
Table 1
The results of bacteriological and molecular examination of dogs’ faecal samples for *Yersinia enterocolitica*

<table>
<thead>
<tr>
<th>District</th>
<th>Number of samples</th>
<th>Bacteriological examination</th>
<th>Molecular examination</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Warm culture</td>
<td>Cold culture</td>
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<tr>
<td></td>
<td></td>
<td>Number of strains</td>
<td>%</td>
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<tr>
<td>G.</td>
<td>50</td>
<td>4</td>
<td>8.0</td>
</tr>
<tr>
<td>J.</td>
<td>50</td>
<td>8</td>
<td>16.0</td>
</tr>
<tr>
<td>L.</td>
<td>50</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>D.</td>
<td>50</td>
<td>8</td>
<td>16.0</td>
</tr>
<tr>
<td>M.</td>
<td>50</td>
<td>5</td>
<td>10.0</td>
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<tr>
<td>Z.</td>
<td>50</td>
<td>2</td>
<td>4.0</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>28</td>
<td>9.3</td>
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Discussion

For the purpose of *Y. enterocolitica* identification, traditional bacteriological examinations are usually conducted, in accordance with the Polish PN-EN-ISO 10273 standard (28). In this method the CIN medium, used as a differing medium, is considered to be the most effective for this pathogen. However, *Y. enterocolitica* grows well also on the majority of media designed for the culture of intestinal microorganisms. The horizontal method of detection of *Y. enterocolitica* rods is time-consuming, because it requires several days to obtain the result, and allows determining only potentially pathogenic strains. For the diversification of pathogenic strains, their ability to absorb Congo red can be defined. The strains, which absorb the virulence plasmid pYV (9). However, according to the latest knowledge, the presence of the pYV is not tantamount to their pathogenicity. It is also known that the strains belonging to biotype 1A, without the plasmid, may sometimes cause the clinical signs (36). Therefore, there is a need to develop new standards for identification of this pathogen. In order to increase the efficiency, and to accelerate the identification process, more and more frequently the PCR method is applied, allowing a fast detection of the virulence markers. This method is much quicker and of greater sensitivity than the traditional microbiological methods. Plasmid genes, as well as genes localised in chromosomes may be used as pathogenic markers. Because of the fact that *Y. enterocolitica* easily loses its plasmid during the passages, it seems more justified to search for chromosomal virulence markers (11). Recognised chromosomal genes responsible for the pathogenicity of the microorganism are *ail* and *ystA* genes. The studies of Woźniak-Kosek et al. (39) concerned serotypes O:3, O:5/27, and O:8, in which *ail* and *ystA* genes were searched. These genes were detected in all pathogenic *Y. enterocolitica* strains. That was the reason why in case of our own studies it was of great significance to precede the molecular analysis after the initial bacteriological identification, which enabled to reduce the number of samples destined for the isolation of DNA.

The comparison of differences between the same samples, which occurred after the culture with the warm and cold methods, indicated that from the cold PSB/CIN culture much more *Y. enterocolitica* strains were obtained including also more strains containing *ystB* gene. The obtained results confirm, however, the necessity of conducting two types of culture for the correct evaluation of the epizootic situation in the given population of animals.

Pigs are considered to be the main reservoir of *Y. enterocolitica* strains, therefore, the greatest number of published studies concerning this microorganism was conducted using faecal samples, as well as internal organs of pigs (3, 13, 16). However, there is growing evidence that an important reservoir of the microbe, constituting a potential threat to humans, are accompanying animals. *Y. enterocolitica* is isolated not only from sick animals, but also from the faeces of healthy dogs (5, 8, 22). These animals become infected mainly as a consequence of consuming raw pork meat, permitted for retail sales. It has been demonstrated that this meat can be contaminated by more than one of *Y. enterocolitica* serotypes (7). Freezing of the meat does not constitute an appropriate protection against this pathogen. This explains why prior heat treatment of the meat destined for dogs is so important. *Y. enterocolitica* is sensitive to high temperature, and 72°C destroys it within 18 s (37).

In the examined samples, gathered from the urban lawns of Olsztyn from November 2006 to April 2007, no *Y. enterocolitica* strain of serotype O:3 was found. All the isolates contained *ystB* gene only, which may prove that they belong to the environmental strains. Similar results were obtained by Pedersen and Winblad (22), who isolated only two strains of *Y. enterocolitica* O:3 from the samples coming from 115 dogs. In many remaining samples the presence of *Y. enterocolitica* belonging to biotype 1A, not constituting a potential threat to human health and life, was detected. In the studies performed several years earlier in Denmark, Pedersen (23) demonstrated that from 40 samples taken from dogs, *Y. enterocolitica* was isolated from three samples, but only one isolate was qualified to serotype O:3. By contrast, on the territory of Italy, among 63 faecal samples collected from clinically healthy dogs, coming from an animal shelter, *Y. enterocolitica* biotype 4 serotype O:3 was found in 17. Much more frequently, however, other species of *Yersinia* were isolated, such as *Y. frederiksenii* or *Y. intermedia* (5). The situation was different in New York, where mainly *Y. enterocolitica* strains belonging to serotypes O:3, O:5/27, and O:8 (1) were isolated. Hayashidani et al. (15) carried out the experimental infection of the Beagle dog breed with serotype O:8 of *Y. enterocolitica*. The animals were infected per os, and the microorganism was excreted for 7 to 21 d post infection (dpi). A part of the animals were subjected to euthanasia on 3rd and 7th dpi, and the microorganisms were detected in the contents of jejunum and in Peyer’s nodes, and bile, as well as the samples from the contents of jejunum were taken. *Y. enterocolitica* organisms were detected in the contents of jejunum and in Peyer’s patches; however, they did not colonise other examined organs at that time. Moreover, after reinfection, dogs did not demonstrate the clinical signs in the gastrointestinal tract. However, according to these researchers, sufficient evidence that *Y. enterocolitica* O:8 isolated from dogs plays a significant role in causing infections in humans, especially as the direct transmission from animal to man, has not been definitively proven (32).

Studies of Shayegani et al. (34), concerning the human population in New York showed that in 7.62% of the samples taken from people, the microorganisms classified to *Yersinia* were found, among which as much as 80.5% were found to be *Y. enterocolitica* species. However, among the samples taken from the environment and from animals, the presence of *Y. enterocolitica* was detected in 28.4%. Analysing the above results, one may hazard a hypothesis that dogs, although they are the carriers of this microorganism, do
not constitute a serious threat to man, because serotype O:3 is rarely found in them. Special attention should be paid, however, to children, who have close contact with the infected dogs during play, since direct contact may sometimes promote the transmission of this microorganism (6). It was confirmed in Bottone’s studies (1), who showed that as much as 59% of the examined samples, in which he found Y. enterocolitica, were taken from the patients, who were under one year of age. Different results were obtained in the studies conducted in 1974–1976 in Washington, in which only five cases of diarrhoea in children were caused by Y. enterocolitica infection (20). Faecal samples were also taken from children with diarrhoea in the province of Siena in Italy. From 2,500 faecal samples examined, only 1.4% were containing Y. enterocolitica, but 94.2% of the isolated Y. enterocolitica strains were classified to biotype 4, serotype O:3 (19). The same serotype was isolated also from animals, including dogs (5). Studies of Frederiksson-Ahomaa et al. (7) conducted in years 1998–1999 in Finland, on dogs under one year of age, fed on raw pork, demonstrated that raw pork is an important source of Y. enterocolitica biotype 4, serotype O:3 in the infections of animals accompanying man. The authors stated also that dogs could constitute a link transmitting the pathogen between pigs and small children.

In our studies, Y. enterocolitica was isolated from the dog faecal samples collected from large housing estates, which are characterised by a high density of humans and animals, making use of the same living spaces (lawns, playgrounds etc.). The high density as well as the lack of awareness of dogs’ owners, connected with threats caused by the excrements left by their animals, can be a cause of spreading this microbe in the dog population of a given estate. Contaminated with Y. enterocolitica canine excrements in playgrounds constitute a direct threat to children. Bottone’s studies (1) conducted in USA in 1974-1982 indicate that in the high-density areas, such as New York, the morbidity caused by Y. enterocolitica serotype O:3 keeps growing. In 1978-1979, an average of three cases of the disease caused by Y. enterocolitica O:3 had been reported a per year. Then, the number of cases increased and only two years later Y. enterocolitica was isolated already from an average of 10 patients per year, manifesting the clinical signs of yersiniosis. A significant increase in the number of isolated Y. enterocolitica strains, particularly those belonging to serotype O:3 in the urban areas, raises the risk of the epidemic. The same studies indicate also a significant decrease in the number of Y. enterocolitica O:8 and O:5/27 infections. After 1980, the number of detected infections with these serotypes fell to 1–2 on an annual basis (1).

To sum up, our research demonstrates that the populations of dogs in the urban environments could be an important reservoir of Y. enterocolitica, constituting a potential threat to public health. A relatively frequent occurrence of Y. enterocolitica strains in the canine faeces contaminating the urban lawns of Olsztyn was stated. The greatest percentage of faecal samples containing these microorganisms was reported on the territory of large housing estates. Molecular tests showed the presence of ystB gene only, in the absence of ail and ystA genes, in the Y. enterocolitica genome, which might indicate the potentially pathogenic biotype 1A, and a little threat from classic pathogenic strains. However, despite the evidence supporting the fact that accompanying animals are mainly the carriers of the environmental strains, the risk related to biotype 1A should not be underestimated. The strains belonging to this biotype are more frequently isolated from the clinical cases of gastritis and enteritis in humans, characterised by alternatively occurring periods of acute disease signs and remissions, lasting weeks or even months. Therefore, it is important to keep monitoring the changes, which occur within this species of microorganisms, the epidemiological situation of yersiniosis in the population of humans and animals, as well as to continue the studies on the epidemiology of Y. enterocolitica infections, also in the context of a reservoir, which animals accompanying man can constitute.

**References**
