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Research Article

Virulence genes and antibiotic resistance of *Yersinia enterocolitica* strains isolated from children

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ABSTRACT

Yersinia enterocolitica is a foodborne pathogen which is primarily responsible for gastrointestinal infections. The presence of the virulence genes in *Y. enterocolitica* strains isolated from children and antimicrobial resistance was studied in this work. The PCR, biotyping and disc diffusion method were used for analysis of *Y. enterocolitica* strains. Most of *Y. enterocolitica* strains belonged to biotype 4 and all carried *ail*, *myfA* and *ystaA* genes. Most of them also had the plasmid *yadA* gene. These genes were also detected in the strains of biotype 2, while in the two strains of biotype 1A only *myfA* gene was found. The *blaA* gene was present in all the strains of biotype 4 and 2, while *blaB* in the strains of biotype 2 and in some of biotype 4 strains. The presence of β-lactamase genes in *Y. enterocolitica* was not detected in biotype 1A. All strains were resistant to ampicillin, 76.2% and 47.6% were resistant to ticarcillin and piperacillin, respectively. Two strains (9.5%) were resistant to amoxicillin/ clavulanic acid and aztreonam, three (14.3%) to chloramphenicol, four (19%) to amikacin and trimethoprim/sulfamethoxazole, six (28.6%) to gentamicin. A few strains of *Y. enterocolitica* were multidrug resistant. The *Y. enterocolitica* strains isolated from the faeces of children suffering from diarrhea carried virulence genes and some of them were resistant to antibiotics used in extra-intestinal yersiniosis treatment.

Keywords: *Yersinia enterocolitica*; Virulence genes; Antibiotic resistance; PCR; Yersiniosis.

1. INTRODUCTION

Yersinia enterocolitica is an important human pathogen with the global distribution and a variety of clinical disorders such as enteritidis, enterocolitis, gastroeneritidis, mesenteric lymphadenitis and others [1]. Yersiniosis is a zoonotic foodborne bacterial disease with high public health relevance. In Europe it is the third most common bacterial enteric disease after campylobacteriosis and salmonellosis [2]. Animals such as pigs, rodents, sheep, goats, cattle, horses are reservoirs of *Y. enterocolitica*. Pigs are a major reservoir for human pathogenic strains, especially for bioserotype 4/O3 [3]. This microorganism is considered an important foodborne pathogen including strains of diverse pathogenicity. Infections are most often acquired through ingestion of contaminated pork, milk, dairy foods, vegetables and contaminated drinking water or pet animal contact [4, 5]. The pathogenic *Y. enterocolitica* strains were also isolated from waste water samples in Turkey [6] or from river water in Poland [7]. *Y. enterocolitica* is rarely transmitted

through contaminated blood during transfusion [8]. The species *Y. enterocolitica* is divided into six biotypes. Strains of biotype 1A are generally regarded as nonpathogenic, whereas strains of biotypes 1B, 2, 3, 4, and 5 carry a virulence plasmid pYV. This plasmid encodes type III secretion system and the outer membrane protein YadA (*Yersinia* adhesin A). YadA was found to play multiple functions in pathogenesis because it protects bacterial cells against antibacterial activity of complement and mediates specific binding of *Y. enterocolitica* to laminin, collagen and cellular fibronectin [9]. The chromosomal *Y. enterocolitica* virulence markers are *ail*, *ystA* and *myfA* genes. The *ail* gene encodes a small outer membrane protein (Ail adhesin), which promotes adhesion of *Y. enterocolitica* and invasion of epithelial cells. The *ystA* gene encodes enterotoxin YstA, which activates the guanylate cyclase that leads to the increased cGMP level. High level of cGMP causes fluid accumulation in the intestine [10]. The major subunit of antigen Myf is encoded by the *myfA* gene. This fibrillar structure promotes the colonization of the intestine by yersiniae [11]. Biotyping is used for clinical and epidemiological classification of *Y. enterocolitica*, but the heterogenous nature of *Y. enterocolitica*, including differences in virulence, requires genotyping methods and this may be a novel way of pathogenic characterization of this microorganism.

The aim of this study was the description of *Y. enterocolitica* strains isolated from the faeces of children suffering from diarrhea by using PCR assays for the detection of some virulence genes and *in vitro* evaluation of antibiotic sensitivity of this pathogen. The presence of genes coding β-lactamases was also detected in the genome of *Y. enterocolitica* strains.

2. MATERIALS AND METHODS

2.1. Strains

Twenty one *Y. enterocolitica* strains were isolated from the faeces of children suffering from diarrhea. The strains were isolated from children treated in different hospitals and outpatients in Warsaw (Poland) over the period 2009-2015. The identification of the strains was performed with the VITEK GNI card system (VITEK 2 instrument, version 4.01, bioMérieux). Biotyping of *Y. enterocolitica* strains was performed according to Wauters et al. [12]. The strains were stored at -70°C in Brain Heart Infusion (BHI) Broth (BHI; BBL, Becton Dickinson) containing 15% glycerol.

2.2. Antibiotic susceptibility testing

The susceptibility of the strains was tested with a disc diffusion method using the following antibiotic discs (Oxoid, Basingstoke, UK): ampicillin (25 µg), amoxicillin/clavulanic acid (20/10 µg), cefepime (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), imipenem (10 µg), norfloxacin (10 µg), piperacillin (100 µg), ticarcillin (75 µg), tobramycin (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), chloramphenicol (30 µg) and trimethoprim/sulfamethoxazole (1.25/ 23.75 µg). The results were recorded by measuring the inhibition zones and scored as susceptible, intermediately susceptible, and resistant, according to the Clinical and Laboratory Standards Institute [13].

2.3. DNA isolation

Genomic DNA was isolated from *Y. enterocolitica* strains by using the Genomic DNA PrepPlus (A&A Biotechnology, Poland), according to the manufacturer's protocol. 2.5 µl of the total extracted material from each test sample was used as a template DNA for PCR application.

2.4. Primers and PCR conditions

The primers specific for the *ail*, *ystA*, *myfA*, *yadA*, *blaA*, *blaB* and 16S rRNA genes of *Y. enterocolitica*, synthesized at DNA-Gdańsk (Gdańsk, Poland), are listed in Table 1. The duplex PCR for *ail* and *ystaA* genes was performed in a 25-µl volume containing 2.5 µl of DNA template, 1×PCR buffer, 0.2 mM each dATP, dCTP, dGTP, and dTTP (Fermentas, Lithuania), the *ail*-specific primers and *ystA*-specific primers at 50 nM, with 1 U of RedTag Genomic DNA polymerase (Sigma-Aldrich, Germany). The amplification was carried out under the following conditions: initial denaturation (94°C, 3 min), followed by 30 subsequent cycles consisting

of denaturation (94°C, 1 min), primer annealing (52°C, 1.5 min), extension (72°C, 1.5 min), and final extension (72°C, 10 min).

The duplex PCR for *blaA* and *blaB* genes was also performed in a 25 µl volume containing 2.5 µl of DNA template, 1 x PCR buffer, 200 µM of each: dATP, dCTP, dGTP, and dTTP (Fermentas, Lithuania), 100 nM of the *blaA* and the *blaB* pair of specific primers, and 1U of RedTag Genomic DNA polymerase (Sigma-Aldrich, Germany). The amplification was carried out under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 0.5 min, primer annealing at 50°C for 0.5 min and extension at 72°C for 1 min. A 5 min extension at 72°C was performed at the end of the final cycle. The monoplex PCR for *myfA* gene and *yadA* gene as described earlier [19] and monoplex PCR for the 16S rRNA gene for species identification as described by Wannet et al. [18] were also performed.

The amplifications were carried out in the Multi Gene II thermal cycler (Labnet International, Inc., USA). The PCR products were analysed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. Molecular size markers (Sigma-Aldrich) were also run for product size verification. The gel was electrophoresed in 2 × Tris-borate buffer at 70 V for 1.5 h.

Table 1. Oligonucleotide primers used in the study.

Primers	Sequence (5' → 3')	Amplicon length (bp)	References
ail-a (F)	TGGTTATGCGCAAAGCCATGT	356	[14]
ail-b (R)	TGGAAGTGGGTTGAATTGCA		
ystA-a (F)	GTCTTCATTTGGAGGATTCGGC	134	[14]
ystA-b (R)	AATCACTACTGACTTCGGCTGG		
myfA-1 (F)	CAGATA CAC CTG CCT TCC ATCT	272	[15]
myfA-2 (R)	CTCGACATATTCCTCAACACGC		
yadA-1 (F)	TAAGATCAGTGTCTCTGCGGCA	747	[16]
yadA-2 (R)	TAGTTATTTGCGATCCCTAGCAC		
blaA-1 (F)	AAATGCGCTACCGGCTTCAG	439	[17]
blaA-2 (R)	AGTGGTGGTATCACGTGGGT		
blaB-1 (F)	CCCACTTTATACCTTGGCACAAA	781	[17]
blaB-2 (R)	GAACATATCTCCTGCCTGGAAAT		
16S rRNA-Y1 (F)	AATACCGCATAACGTCTTCG	330	[18]
16S rRNA-Y2 (R)	CTTCTTCTGCGAGTAACGTC		

3. RESULTS

Biotype 4 was most numerously represented by 71.4% of *Y. enterocolitica* strains. A small group included strains of biotype 2 and biotype 1A (Table 2).

The 330 bp fragment, specific amplification product for the *Y. enterocolitica* 16S rRNA gene, was obtained in case of all the strains (Fig. 1A). A duplex PCR was used for the detection of the *ystA*-specific PCR product of 134 bp and the *ail*-specific product of 356 bp (Fig. 1B). These genes were present in all the strains of 4 and 2 biotype (Table 2). The *yadA*-specific amplification product of 747 bp was detected in all the strains of biotype 2 and the majority of strains belonging to biotype 4 (86.6%) (Fig. 1C). The *myfA*-specific PCR product of 272 bp (Fig. 1D) was detected in all the strains which belonged to different biotypes. Using multiplex PCR, 439 bp fragment for *blaA* gene in all the strains of biotype 4 and 2 was obtained (Fig. 1E). The amplification products for *blaB* (827 bp) were detected in all strains of biotype 2, and only in eight strains of biotype 4. The presence of β-lactamase genes in *Y. enterocolitica* was not detected in biotype 1A.

Table 2. Virulence genes and resistance profiles of *Y. enterocolitica* strains from the faeces of children with intestinal yersiniosis**.** Bt - biotype, AMP - ampicillin, TIC - ticarcillin, AMC - amoxicillin plus clavulanic acid, PIP - piperacillin, GM - gentamicin, AN - amicacin, C - chloramphenicol, SXT - trimethoprim/sulfamethoxazole, ATM - aztreonam, * - multidrug resistance strains, „-„ no amplification.

Strains	**Year**	**Bt**	**Results of PCR for:**						**Resistance profile**
			ail	***yadA***	***myfA***	***ystA***	***blaA***	***blaB***	
9996	2009	2	+	+	+	+	+	+	AMP/TIC/AMC
6068	2010	1A	-	-	+	-	-	-	AMP/PIP
10743	2010	4	+	+	+	+	+	+	AMP/TIC/GM
15869	2010	4	+	+	+	+	+	+	AMP/PIP/TIC
6528	2010	4	+	-	+	+	+	+	AMP/AN/C/SXT*
6701	2010	4	+	-	+	+	+	+	AMP/TIC
7217	2012	2	+	+	+	+	+	+	AMP/PIP/TIC/SXT
20179	2013	2	+	+	+	+	+	+	AMP/TIC/C/SXT*
10510	2013	1A	-	-	+	-	-	-	AMP/PIP/TIC/SXT
15395	2013	4	+	+	+	+	+	+	AMP/GM
26530	2014	4	+	+	+	+	+	+	AMP/PIP/AN/GN
13004	2015	4	+	+	+	+	+	-	AMP/PIP/TIC
2	2015	4	+	+	+	+	+	-	AMP/TIC
13571	2015	4	+	+	+	+	+	-	AMP/TIC/ATM/AMC
601	2015	4	+	+	+	+	+	-	AMP/TIC
1	2015	4	+	+	+	+	+	-	AMP, TIC
158	2015	4	+	+	+	+	+	-	AMP/TIC
450/6	2015	2	+	+	+	+	+	+	AMP/PIP/TIC/GM
448/7	2015	4	+	+	+	+	+	+	AMP/PIP/ATM/AN/GN/C*
511/8	2015	4	+	+	+	+	+	+	AMP/PIP/TIC/AN/GM
301/3	2015	4	+	+	+	+	+	-	AMP/TIC

The *Y. enterocolitica* strains showed high resistance to antibiotics belonging to penicillin group because all the strains were resistant to ampicillin, above 76% of the strains were resistant to ticarcillin and about 48% were resistant to piperacillin. Additionally, two strains (9.5%) were resistant to amoxicillin/clavulanic acid. About 29% and 19% of the strains were resistant to gentamicin and amikacin, respectively. Moreover, two strains (9.5%) were resistant to aztreonam. In case of chloramphenicol, 14.3% of the strains showed resistance and 19% of the strains were resistant to trimethoprim/sulfamethoxazole. All the strains were sensitive to cephalosporins, fluoroquinolones, imipenem and tobramycin (Fig. 2). Among the tested *Y. enterocolitica*, three strains were multidrug resistant. Two strains of biotype 4 showed resistance to antimicrobial agents from four various chemical groups and one strain of biotype 2 was resistant to antimicrobial agents belonging to three different chemical groups (Table 2).

4. DISCUSSION

Y. enterocolitica is an important foodborne pathogen which is primarily responsible for gastrointestinal infections in young children. The incidence of *Y. enterocolitica* infection is highest among children under 5 years of age [20].

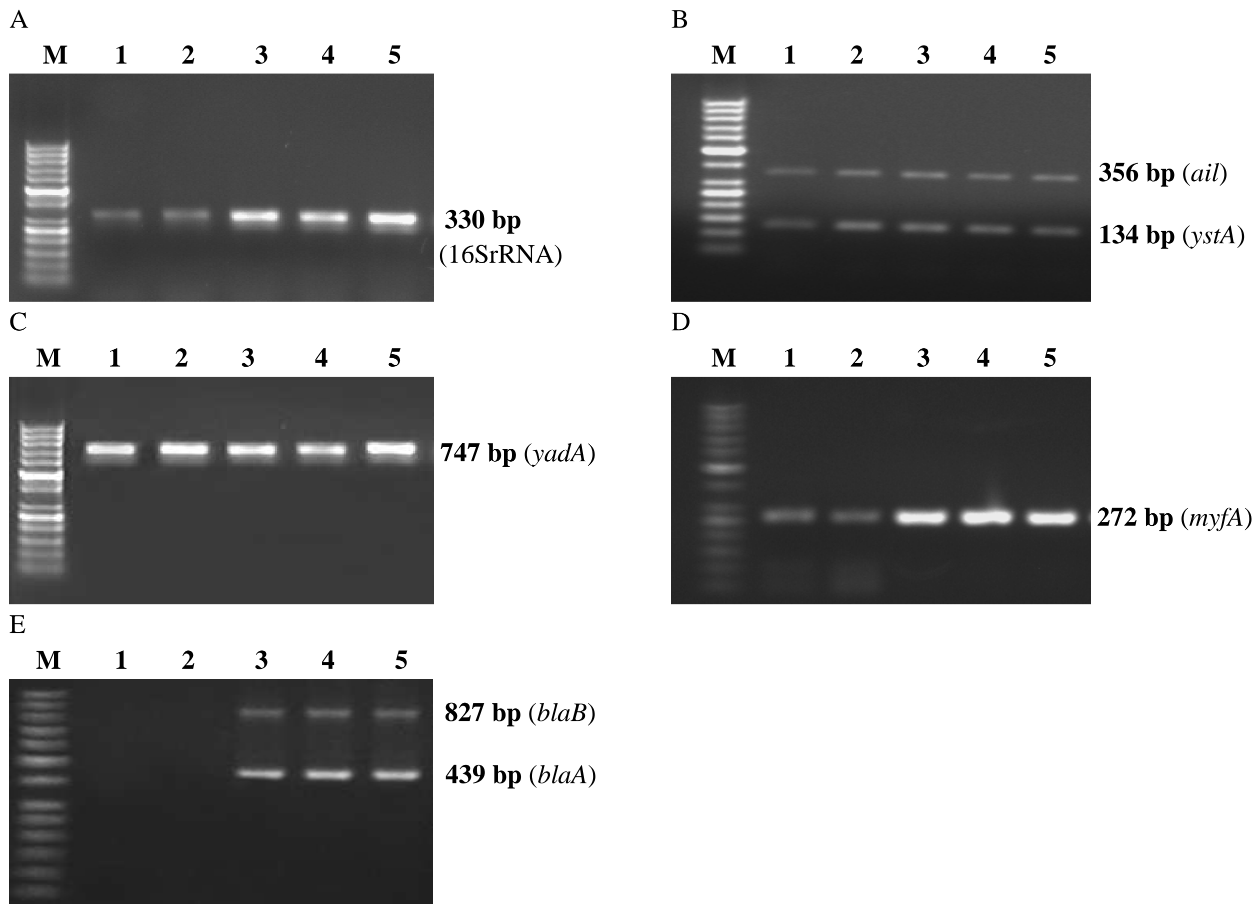

Figure 1. Electrophoresis in 1.5% agarose gel PCR products obtained by using specific primers for 16S rRNA gene (A), *ail* and *ystA* genes (B), *yadA* gene (C), *myfA* (D) and *blaA* and *blaB* genes (E).

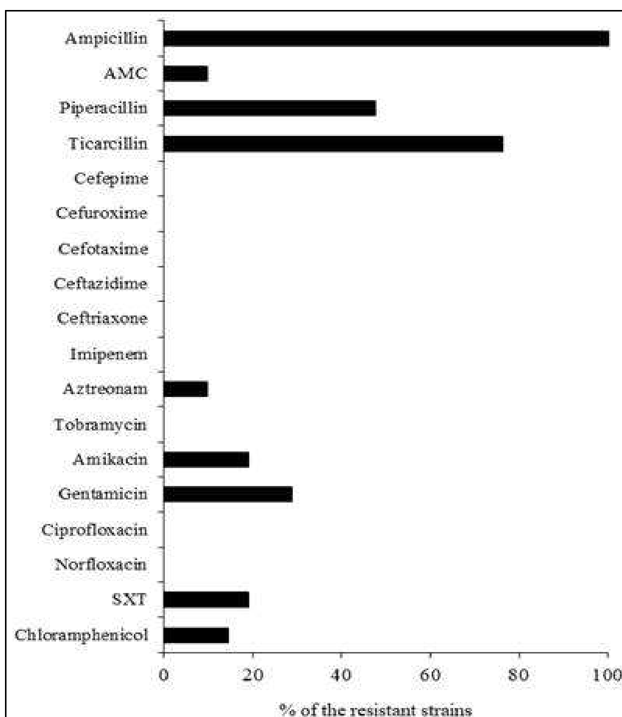

Figure 2. Antimicrobial resistance of *Y. enterocolitica* strains isolated from the faeces of humans with intestinal yersiniosis. AMC - amoxicillin/clavulanic acid, SXT - trimethoprim/sulfamethoxazole.

The high incidence of *Y. enterocolitica* infections in this age group, compared with other gastrointestinal infections, such as salmonellosis and campylobacteriosis, may result from eating food prepared from raw pork products, use of baby's dummy or contact with domestic animals, such as dogs and cats [21]. In addition, factors that may contribute to the high incidence of *Y. enterocolitica* infection in young children include an increased rate of exposure to this pathogen as a result of fecal-oral contamination, predisposition to infection due to immature immune system [22] and higher frequency of testing stool samples in case of children when affected [23]. In our research we investigated *Y. enterocolitica* strains isolated from the faeces of children suffering from diarrhea. Among them, strains belonging to biotype 4 carrying the *ail*, *myfA* and *ystaA* genes predominated. Most of them had also the plasmid gene *yadA*, confirming the presence of the plasmid pYV. These results demonstrated the pathogenic potential of the investigated strains to susceptible hosts. Our results are similar to those

obtained by other authors that also showed that strains belonging to biotype 4 are responsible for most infections caused by *Y. enterocolitca* in Europe [4, 20]. The strains of biotype 2 are rarely isolated from humans. The pathogenic potential of the biotype 2 strains examined in this study was highlighted by the occurrence of the virulence markers investigated. Similar results were obtained by Frazão and Falcão [24], who also studied strains of *Y. enterocolitica* biotype 2.

Uncomplicated course of yersiniosis usually does not require the use of antibiotics. However, some cases of yersiniosis, such as sepsis, focal extra-intestinal infection or infection in immune-compromised patients require antimicrobial treatment. *Y. enterocolitica* strains are β-lactamase producers. Most *Y. enterocolitica* strains harbored chromosomal genes *blaA* and *blaB* encoding BlaA (a non-inducible broad-spectrum carbenicillinase) and BlaB (an AmpC-type inducible cephalosporinase) [25].

In our study, the presence of *blaA* gene in all the strains of biotype 4 and 2 was detected, while *blaB* gene was carried by biotype 2 strains and over 50% of the biotype 4 strains. These genes were not detected in the strains of biotype 1A, although in previous studies, in which were used additional primers designed using the conserved regions of the *blaA* genes of *Y. enterocolitica* 8,081, biotype 1B, has been shown the presence of this gene in the majority of *Y. enterocolitica* strains of biotype 1A [26]. Heterogeneity in *blaA* gene of *Y. enterocolitica* of biotype 1A was confirmed by Sharma et al. [27]. Inability to detect *blaA* gene in these strains may result from a genetic variability in *blaA* preventing the binding of primers. The antimicrobial susceptibility test revealed high resistance of *Y. enterocolitica* to antibiotics belonging to penicillin group such as ampicillin, ticarcillin and piperacillin. This was in accordance with the results obtained by other authors [28]. Two strains (9.5%) belonging to 2 and 4 biotype were also resistant to amoxicillin with clavulanic acid, while Frazão et al. [29] showed that 19/34 of *Y. enterocolitica* strains isolated from different sources in Brazil were resistant to this combination. In our study, all the strains were sensitive to the second (cefuroxime), third (cefotaxime, ceftazidime, ceftriaxone) and fourth generation cephalosporins (cefepime), fluoroquinolones and imipenem. Fluoroquinolones and the third generation cephalosporins are the best therapeutic options to treat enterocolitis in compromised hosts and in patients with septicemia or invasive infection [30]. In case of extra-intestinal yersiniosis, also aminoglycosides in combination with other antibiotics are used for treatment. In our research, four (19%) and six (28.6%) strains were resistant to amikacin and gentamicin, respectively. Rusak et al. [28] obtained one strain (2%) resistant to amikacin, while all the strains were sensitive to gentamicin. In Switzerland during 2001-2010 also no gentamicin-resistant strains were found [4]. Trimethoprim/sulfamethoxazole are also used to treat yersiniosis. In this study, four strains (19%) were resistant to this sulfonamide. Sporadic resistance to trimethoprim/sulfamethoxazole occurred in Switzerland [4], while in Brazil trimethoprim/sulfamethoxazole resistance was found in 8.8% to 10% of the strains [28, 29]. In our study, three strains were multidrug resistant. Two strains belonging to biotype 4 showed resistance to four different classes of antimicrobial agents (penicillins, aminoglycosides, chloramphenicol, sulfonamides and penicillins, aminoglycosides, chloramphenicol, monobactams) and one strain of biotype 2 was resistant to antimicrobial agents belonging to three groups (penicillins, chloramphenicol, sulfonamides). Multiple resistance phenotypes were rarely reported in *Y. enterocolitica*. Only one out of from 60 *Y. enterocolitica* strains investigated by Rusak et al. [28] showed resistance to the three classes of antimicrobial agents (cephalosporin, sulfonamide, and tetracycline). Fredriksson-Ahomaa et al. [4] also reported that only one out of 128 *Y. enterocolitica* strains isolated from human clinical samples in Switzerland showed resistance to multiple antimicrobial agents. The multiresistance of *Y. enterocolitica* strains (19%) was found in Finland, and these strains were significantly associated with traveling abroad [31].

Our study showed that *Y. enterocolitica* strains from children in Poland belonging to biotype 4 and 2 had all investigated virulence genes, including the plasmid gene *yadA*, except the two strains of biotype 4 in which this gene was not detected. These strains showed high resistance to penicillin, although they remain susceptible to drugs used for treating gastroenteritidis, as well as extra-intestinal infections. However, it should be stressed

that some strains were resistant to antibiotics used in extra-intestinal yersiniosis treatment and few strains were multidrug resistant.

AUTHORS' CONTRIBUTION

BK: study design, laboratory investigation, data interpretation, preparation of manuscript; MP and KJ: laboratory investigation, literature analysis. The final manuscript has been approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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