

Expression of *blaA* and *blaB* and Susceptibility to Penicillins and Cephalosporins in *Yersinia enterocolitica* from Different Foods

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ABSTRACT

Objective: *Yersinia enterocolitica* which is an important foodborne pathogen causing illness in humans is an extremely heterogeneous species consisting of different subtypes. It has the intrinsic resistance to β -lactam antibiotics because of the production of β -lactamases, BlaA and BlaB. *Y. enterocolitica* exhibits variable susceptibilities to β -lactams.

Materials and Methods: The expression of the *blaA* and *blaB* genes by polymerase chain reaction, and the susceptibility to some β -lactams including penicillins and cephalosporins using the broth microdilution and disk diffusion methods were determined. A total of 18 *Y. enterocolitica* isolates were examined.

Results: Overall, 33.3% of these isolates carried the *blaA* and *blaB* genes, all of which were recovered from chicken meat. The wide range of MIC for ampicillin (≤ 2 -128 $\mu\text{g/mL}$) and ceftazidime (≤ 0.0625 -2 $\mu\text{g/mL}$) was also observed. Of the *Y. enterocolitica* isolates, 55.6% were resistant to ampicillin (≥ 32 $\mu\text{g/mL}$) while the remaining isolates (44.4%) were susceptible to ampicillin (≤ 8 $\mu\text{g/mL}$). All isolates were susceptible to ceftazidime at the concentration tested. According to the disk diffusion test, 55.6% and 33.3% of the isolates were resistant to ticarcillin and cefoxitin, respectively. No resistance to piperacillin and ceftriaxone was found.

Conclusion: The results showed that the presence of the *blaA* and *blaB* genes and intrinsic resistance against penicillins and cephalosporins were variable among *Y. enterocolitica* food isolates. Furthermore, the *blaA* and *blaB* genes were expressed in most of the resistant isolates to β -lactams, which may indicate the contribution of the genes to the drug resistance.

Keywords: *Yersinia enterocolitica*, *blaA*, *blaB*, β -lactams, antimicrobial susceptibility, food

INTRODUCTION

Yersinia enterocolitica is a Gram-negative, rod-shaped, nonlactose-fermenting and facultative anaerobic bacterium from the *Enterobacteriaceae*. This organism is an extremely heterogeneous species consisting of different subtypes that the subtypes have differences in geographic distribution, habitat and virulence characteristics (1-3).

Y. enterocolitica has been widely found in various sources; water, food, sewage, animal and human (1). Many previous studies reported that foods including

ground beef, chicken meat, cheese, and raw milk were frequently contaminated with *Y. enterocolitica* (4-8). *Y. enterocolitica* as a foodborne pathogen is transmitted to human via consumption of contaminated food and leads to yersiniosis, the most common form of gastroenteritis (1,9). It also causes some extraintestinal diseases such as urinary tract and respiratory tract infection, erythema nodosum, osteoarticular infection, and axillary abscesses. Severe infections including septicemia, pneumonia, meningitis, and endocarditis can often result in death in immunocompromised patients (1,3).



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Resistance to β -lactams is the most common and important mechanism in the *Enterobacteriaceae*. *Y. enterocolitica* is generally resistant to β -lactam antimicrobials such as penicillin, ampicillin and first generation cephalosporins (10-13). β -lactam resistance in *Y. enterocolitica*, which was first reported in 1975, is due to two chromosomally encoded β -lactamases, BlaA and BlaB (14). BlaA is a penicillinase-type β -lactamase which is produced constitutively while BlaB is an inducible class C cephalosporinase (15,16). The expression of these β -lactamases is quite variable among different species and the strain (17). Previous studies indicated that in *Y. enterocolitica* strains which are extremely heterogeneous, differences in the susceptibility to β -lactams are mainly due to differences in the expression of β -lactamases. Different *Y. enterocolitica* subtypes exhibit variability in the level and spectrum of resistance (18-21).

Therefore, this study aims to investigate the expression of the genes *blaA* and *blaB* and determine the susceptibility to β -lactam antimicrobials including penicillins and cephalosporins using the disk diffusion and broth microdilution methods in the *Y. enterocolitica* isolates from different foods.

MATERIALS AND METHODS

Bacterial Isolates

The 18 *Y. enterocolitica* isolates obtained from various foods were used. The isolates were identified using phenotypic methods and polymerase chain reaction (PCR) amplification of the *Y. enterocolitica* 16S rRNA-specific gene (22,23). These isolates belonging to biovar 1A were recovered from chicken meat containing breast and leg parts (n=10), open white cheeses (n=3), ground beef (n=3) and raw milk (n=2). All *Y. enterocolitica* isolates were kept individually at -20°C in Brain Heart Infusion Broth (BHI, Merck, Darmstadt, Germany) containing 20% glycerol. To activate the cultures, a 400 μ l of glycerol stock was inoculated into 5 mL BHI Broth and incubated for 18-24 h at 28°C. The resulting culture was streaked on Nutrient Agar (Merck) to obtain individual colonies.

DNA Extraction

Genomic DNA of the *Y. enterocolitica* isolates was extracted using hexadecyltrimethylammonium bromide-method (CTAB) according to Ausubel et al. (24). The isolates in 5 mL BHI Broth (Merck) were incubated at 28°C for 18 h. Then 1.5 mL of the culture was transferred to an Eppendorf tube and centrifuged. Then pellet was suspended in 567 μ l TE buffer (10mM Tris, 1mM EDTA), 30 μ l SDS (10%) and 3 μ l proteinase K (20 mg/mL) (1h at 37°C). After addition of 100 μ l NaCl (5M), 80 μ l cetyltrimethyl ammonium bromide was added and incubated (65°C/10 min). Equal volume chloroform/isoamyl alcohol (24:1) solution was added and centrifuged. The supernatant was transferred to a new centrifuge tube and equal volume phenol/chloroform/isoamyl alcohol (25:24:1) solution was added. After centrifugation, the DNA pellet was precipitated with isopropanol and washed with 70% ethanol. Finally, the DNA was dissolved in 100 μ l TE buffer.

Determination of the *blaA* and *blaB* Genes

The *blaA* and *blaB* genes were investigated by PCR in all 18 *Y. enterocolitica* biovar 1A isolates from food. The *blaA* primer se-

quences were A9-f (5'-GAG ATT CAG GAA TGA AGC ACT CTT CG-3') and A10-r (5'-TCA GGA TAT TTG CGA CAA AAT TAT-3'), which were predicted to yield an 896 bp product (2). The *blaB* primer sequences were blaB5 (5'-CCC ACT TTA TAC CTT GGC ACA AA-3') and blaB3 (5'-GAA CAT ATC TCC TGC CTG GAA AT-3'), which were predicted to yield an 827 bp product (18). All reactions were carried out with the T100 thermal cycler (Bio-Rad, Hercules, USA). PCR mixture (50 μ L) contained 5 μ l of 10 \times PCR buffer (Vivantis Technologies, Selangor DE, Malaysia), 4 mM MgCl₂ (Vivantis), 0.2 mM dNTP mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.4 μ M (each) primers (Biomers, Ulm, Germany), 1.5 U of Taq DNA polymerase (Vivantis), 5 μ l of 50 ng of template DNA and 35.7 μ l nuclease free water (AppliChem, Darmstadt, Germany). PCR reaction conditions included an initial denaturation (94°C, 5 min), 30 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), extension (72°C, 1 min) followed by final extension (72°C, 8 min). The products were analyzed by electrophoresis (Bio-Rad) in a 1% agarose gel and photographed using a UV transilluminator (DNR Minilumi Bio-imaging Systems Ltd., Jerusalem, Israel). *Y. enterocolitica* ATCC 23715 was used as a control in this study.

Minimum Inhibitory Concentration (MIC) by Broth Microdilution

Broth microdilution method which is used to determine the minimum inhibitory concentration (MIC) offers the possibility to quantify resistance by actual concentrations of a particular agent and might yield reliable results (25,26). In this study, susceptibility to ampicillin and ceftazidime against *Y. enterocolitica* isolates was evaluated by the broth microdilution MIC according to the CLSI guidelines (27). Antimicrobial powder of ampicillin and ceftazidime (Sigma, St. Louis, Missouri, USA) was stored as recommended in the manufacturer's instructions. Stock solutions of ampicillin and ceftazidime were prepared considering the potency of each antimicrobial agent. All stock solutions of antimicrobials were dispensed into aliquots and kept frozen at -20°C. The stock solutions of ampicillin and ceftazidime were diluted in sterile distilled water to obtain 512 μ g/mL and 32 μ g/mL concentrations, respectively. At the end of each day, stock solutions used were discarded and never refrozen. The MICs were determined by the microdilution method in 96-well plates (TPP, Switzerland). Firstly, *Y. enterocolitica* isolates were cultured overnight on Nutrient Agar plates. Several colonies were taken and suspended into Mueller Hinton broth (MH, Merck) to adjust the bacterial turbidity to 0.5 McFarland standard (about 10⁸ CFU/mL). Serial dilutions of antimicrobials were prepared in sterile MH broth in 96-well plates. The adjusted inoculum suspension was first diluted 1:150 in MH broth, and 50 μ l was transferred to each well (5 \times 10⁴ CFU/well) of the microplate. For each test, a sterility control well (only MH broth) and a growth control well (containing MH broth with bacterial culture, no antimicrobial) were included. The microtiter plates were incubated for 18 h at 35°C. MIC experiments were carried out in triplicates. Bacterial growth was measured by optical density (ELISA reader, Thermo Electron Corporation, Vantaa, Finland). The MIC value for each *Y. enterocolitica* isolate was defined as the lowest concentration of antimicrobial which completely prevents the growth of a microorganism (27). According to CLSI, ampicillin MIC breakpoints for *Y. enterocolitica* are \leq 8 μ g/mL for

susceptible, 16 $\mu\text{g/mL}$ for intermediate, and $\geq 32 \mu\text{g/mL}$ for resistant. The breakpoints of ceftazidime are $\leq 4 \mu\text{g/mL}$, (susceptible), 8 $\mu\text{g/mL}$ (intermediate), and $\geq 16 \mu\text{g/mL}$ (resistant). Reference strains for the antimicrobial susceptibility test were *Escherichia coli* ATCC 25922 and *Y. enterocolitica* ATCC 23715.

Standard Disk Diffusion Method

All *Y. enterocolitica* isolates were examined for susceptibility to some antimicrobials belonging to the penicillin group and the cephalosporin group by the disk diffusion method (27). The antimicrobial agents tested were ticarcillin (75 μg), piperacillin (100 μg), cefoxitin (30 μg) and ceftriaxone (30 μg) (Oxoid, Basingstoke, UK). Cell suspensions adjusted to 0.5 McFarland standards were spread on Mueller Hinton Agar (Merck). The antimicrobial disks were applied on the agar surface. All plates were incubated for 24 h at 30°C. The zones of growth inhibition were evaluated as susceptible, intermediate or resistant (27).

RESULTS

In the present study, the existence of the *blaA* and *blaB* genes was examined in the 18 *Y. enterocolitica* isolates belonging to biovar 1A using PCR. Out of the 18 *Y. enterocolitica* isolates, 33.3% were found to be positive for both the *blaA* and *blaB* genes (Table 1). These positive isolates were only isolated from chicken meat. However, 66.7% of the isolates from different foods including open white cheese, ground beef, and raw milk were not found to be positive for both *blaA* and *blaB* gene (Table 1). Furthermore, Figure 1 shows the agarose gel electrophoresis image of the *blaA* and *blaB* genes in the *Y. enterocolitica* isolates.

In this study, the MIC values of the ampicillin and ceftazidime by the broth microdilution method were determined in all *Y. enterocolitica* isolates (Table 1). Ampicillin resistance was detected in 55.6% of the isolates. Antimicrobial susceptibility to ampicillin varied ranged from 2-128 $\mu\text{g/mL}$. The MIC₅₀ and MIC₉₀ were 32 $\mu\text{g/mL}$ and 128 $\mu\text{g/mL}$, respectively. Ampicillin resistance was observed in 80% of the chicken isolates. None of the isolates originated from ground beef were resistant to ampicillin. All isolates

were sensitive to ceftazidime (MIC $\leq 4 \mu\text{g/mL}$). For ceftazidime, the MIC₅₀ and MIC₉₀ were 0.25 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$, respectively.

Susceptibilities of the isolates to some β -lactam antimicrobials belonging to the penicillin and cephalosporin group by the disk diffusion method are given in Table 1. Resistance to ticarcillin was detected in 55.6% of the isolates. On the contrary, none of the isolates were resistant to piperacillin. All isolates (100%) were found to be sensitive to ceftriaxone while 33.3% were found to be resistant to cefoxitin. All cefoxitin resistant isolates carried both *blaA* and *blaB* genes. In addition, ampicillin resistant isolates were also resistant to ticarcillin.

DISCUSSION

Y. enterocolitica is transmitted through consumption of contaminated food or water and mainly cause of gastrointestinal infections. Most *Y. enterocolitica* strains are β -lactamase producers which harbor chromosomal genes *blaA* and *blaB* encoding BlaA and BlaB, respectively (1). In the present study, these genes were detected in 33.3% of the *Y. enterocolitica* biovar 1A isolates. Numerous studies reported the presence of the genes associated with β -lactamase production of *Y. enterocolitica* (19,28,29). In India, all biovar 1A strains of *Y. enterocolitica* carried genes *blaA* and *blaB* (30). In a study, *Y. enterocolitica* biovar 1A strains isolated from different countries were found to be positive for both *blaA* and *blaB* genes (2). Sharma et al. (16) reported that all *Y. enterocolitica* biovar 1A strains from different sources also harbored both *blaA* and *blaB* genes. In Poland, in a study conducted by Kot et al. (29), the presence of the *blaA* and *blaB* genes of *Y. enterocolitica*, isolated from the feces of children suffering from diarrhea, was 90.5% and 57.1%, respectively. They indicated that the *blaA* gene was detected in all strains of biovar 4 and 2, while found the *blaB* gene in some strains of biovar 2 and biovar 4. On the other hand, in their study, biovar 1A strains did not carry both the genes. It was documented in the study of Ye et al. (28) that the *blaA* and *blaB* were found to be positive of 97% and 100% of the *Y. enterocolitica* biotype 1A isolates from retail foods in China, respectively. In Germany, all *Y. enterocolitica* biovars 2, 4 and 5 strains had the *blaA* and *blaB* genes (18). Stock et al. (19) observed that all biovar 1A, 1B and 3 strains carried the *blaB*. The *blaA* was found in some biovar 1B and 3 strains, but not in biovar 1A strains. These studies of Stock et al. (18,19) suggested that expression of β -lactamase genes, *blaA* and *blaB*, varied with the biovar. Furthermore, variable expression and activities of *blaA* and *blaB* genes might have an effect on the level and spectrum of β -lactam resistance (18,30). Strain variations have been shown to be depending on the different subtypes and geographical origin of the isolates (2,18,31-33).

In this study, more than half of the isolates (55.6%) were positive for ampicillin resistance, but all isolates were sensitive to ceftazidime. Many studies have been performed to determine the levels of resistance to ampicillin and ceftazidime in *Y. enterocolitica* strains from different sources (8,34). *Y. enterocolitica* strains from retail poultry and swine feces presented 100% resistance to ampicillin and 100% susceptibility to ceftazidime

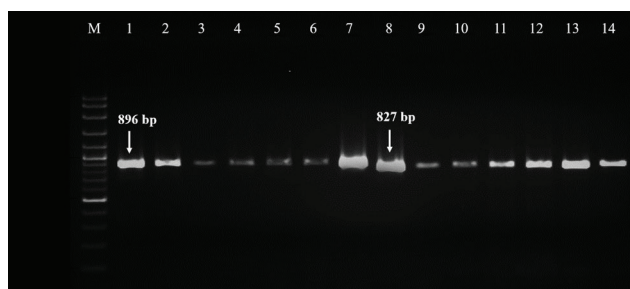


Figure 1. Agarose gel electrophoresis of PCR-amplified *blaA* and *blaB* gene products from the *Y. enterocolitica* isolates. Lane M: 100 bp DNA Ladder. Lane 1: *Y. enterocolitica* ATCC 23715 positive control for *blaA* (896 bp). Lanes 2-7: *blaA* of the chicken meat isolates in this study. Lane 8: *Y. enterocolitica* ATCC 23715 positive control for *blaB* (827 bp). Lanes 9-14: *blaB* of the chicken meat isolates in this study.

Table 1. Presence of β -lactamase genes and susceptibility profiles to penicillins and cephalosporins of 18 *Y. enterocolitica* isolates from food.

Isolate	Origin	PCR analysis			Ampicillin MIC			Ceftazidime MIC			Disk Diffusion		
		<i>blaA</i>	<i>blaB</i>	MIC ^c /Interpretation	MIC ₅₀	MIC ₉₀	MIC ^d /Interpretation	MIC ₅₀	MIC ₉₀	TIC	PRL	FOX	CRO
T1	Chicken meat	+ ^a	+	32/R			0.5/S			R	S	R	S
T2	Chicken meat	+	+	32/R			1/S			R	S	R	S
T3	Chicken meat	+	+	64/R			2/S			R	I	R	S
T4	Chicken meat	- ^b	-	64/R			0.25/S			R	S	S	S
T5	Chicken meat	+	+	32/R			0.25/S			R	S	R	S
T6	Chicken meat	+	+	128/R			1/S			R	I	R	S
T7	Chicken meat	-	-	128/R			0.25/S			R	S	S	S
T8	Chicken meat	-	-	8/S			0.25/S			S	S	S	S
T9	Chicken meat	+	+	32/R	32	128	0.25/S	0.25	1	R	I	R	S
T10	Chicken meat	-	-	2/S			0.0625/S			S	S	S	S
K1	Ground beef	-	-	4/S			0.25/S			S	S	S	S
K2	Ground beef	-	-	4/S			0.25/S			S	S	S	S
K3	Ground beef	-	-	4/S			0.25/S			S	S	S	S
M1	Open white cheese	-	-	128/R			1/S			R	I	S	S
M2	Open white cheese	-	-	2/S			0.5/S			S	S	S	S
M3	Open white cheese	-	-	4/S			0.0625/S			S	S	S	S
S1	Raw milk	-	-	8/S			0.25/S			S	S	S	S
S2	Raw milk	-	-	128/R			0.25/S			R	S	S	S

MIC: Minimum inhibitory concentrations were recorded in the unit of $\mu\text{g/mL}$; R: Resistant, I: Intermediate, Susceptible: S, TIC: Ticarcillin, PRL: Piperacillin, FOX: Cefoxitin, CRO: Ceftriaxone; ^aPositive for the gene; ^bNegative for the gene; ^cMIC breakpoints for ampicillin; Susceptible, ≤ 8 ; Intermediate, 16; Resistant, $\geq 32 \mu\text{g/mL}$; ^dMIC breakpoints for ceftazidime; Susceptible, ≤ 4 ; Intermediate, 8; Resistant, $\geq 16 \mu\text{g/mL}$.

by the broth microdilution method (34). Similarly, MIC results for ceftazidime in *Y. enterocolitica* from swine and pork products showed that all strains were categorized as susceptible (35). All *Y. enterocolitica* from the pork production chain (8) and from fruits and vegetables (36) were resistant to ampicillin. In some studies, the resistance to ampicillin was detected in 92% of the isolates from different samples including human and nonhuman and 87.2% of *Y. enterocolitica* clinical isolates (37,38). In a study conducted by Weiner (39), ampicillin MICs were ≥ 32 $\mu\text{g/mL}$ (resistant) in all *Y. enterocolitica* isolates from animals.

In this study, *Y. enterocolitica* isolates were resistant to ticarcillin (55.6%) and cefoxitin (33.3%) whereas resistance to piperacillin and ceftriaxone was not observed. Resistance to ticarcillin in *Y. enterocolitica* strains was detected in Brazil at the level of 94%, in Canada at the level of 90% and in Poland at the level of 76.2% (10,29,40). However, Ye et al. (41) reported that all *Y. enterocolitica* strains from retail frozen foods were sensitive to ticarcillin. In previous studies, the rates of susceptibility to piperacillin were 100% (10,35). Susceptibility against cefoxitin (62%) and ceftriaxone (100%) was found by Kwaga et al. (35), similar to our ceftriaxone results. Similarly, Baumgartner et al. (42) in Switzerland indicated that no strains of *Y. enterocolitica* from human patients, pigs and retail pork were resistant to ceftriaxone. In contrast to our results, the *Y. enterocolitica* strains isolated from clinical and non-clinical sources in Brazil were not positive for cefoxitin resistance (40).

β -lactamases, BlaA and BlaB that offer ampicillin and first-generation cephalosporin resistance in *Y. enterocolitica* have been reported in previous studies (13,36,37,43). In this study, the positive isolates for *blaA* and *blaB* genes were ampicillin resistant and ceftazidime susceptible according to MIC values. However, some ampicillin resistant isolates on the basis of MIC break-points did not carry the β -lactamase genes. In contrast to our study, Peng et al. (34) reported that all *Y. enterocolitica* isolates carried the *blaA* and *blaB* were also resistant to ampicillin and susceptible to ceftazidime by the broth microdilution method. On the other hand, some studies showed that some ampicillin sensitive *Y. enterocolitica* strains carried the *blaA* and *blaB* genes. Most ceftazidime sensitive strains also were positive for these genes. Although there is some correlation between MIC results and β -lactamase induction, it may not possible to predict the expression of BlaA and BlaB enzymes from MIC results (18,28).

CONCLUSION

In this study, both *blaA* and *blaB* genes which confer resistance to β -lactam antimicrobials were detected in *Y. enterocolitica* biovar 1A isolates from foods, particularly in chicken meat isolates. More than half of the isolates were resistant to ampicillin whereas all isolates were sensitive to ceftazidime. The isolates carried both the *blaA* and *blaB* genes were resistant to ampicillin, ticarcillin and cefoxitin. Consequently, the results of this study demonstrated that the presence of the *blaA* and *blaB* genes and intrinsic resistance against penicillins and cephalosporins were variable among *Y. enterocolitica* food isolates. Furthermore, the *blaA* and *blaB* genes were expressed in most of the resistant iso-

lates to β -lactams, which may indicate the contribution of the genes to the drug resistance.

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