



The Relationship of *Yersinia* Isolates Bioserotypes with Minimum Inhibitory Concentration of Ampicillin, Cefazolin, and Cefotaxime

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Abstract

Background: *Yersinia* is a genus in the family Enterobacteriaceae that its species include *Yersinia enterocolitica*, *Yersinia intermedia*, *Yersinia frederiksenii*, and *Yersinia kristensenii*. Among these species, *Yersinia enterocolitica* is the most important one that causes various diseases such as gastroenteritis, mesenteric lymphadenitis, and erythema nodosum. Since antibiotic-resistance makes some problems in the treatment of diseases caused by these groups of bacteria, this study was designed to determine the relationship between serotypes and minimum inhibitory concentration (MIC) of Ampicillin, Cefazolin, and Cefotaxime in *Yersinia* isolates and also the sensitivity of *Yersinia* to these antibiotics.

Methods: In this descriptive study, 50 different strains of *Yersinia* (39 ones isolated from human, environmental, and food resources of Iran, and 11 ones purchased from Pasteur Institute of Paris) were used to determine MIC of three selected antibiotics using broth macrodilution test.

Results: Cefotaxim showed the lowest MIC (≤ 0.125 $\mu\text{g/ml}$) indicating that it can be used as the most effective antibiotic against *Yersinia*-related infections. All the species showed 100% resistance to Ampicillin and very low sensitivity to Cefazolin.

Conclusion: Among four studied species, *Yersinia kristensenii* and *Yersinia frederiksenii* were respectively the most sensitive and the most resistant species to Beta-lactam antibiotics. *Yersinia enterocolitica* and *Yersinia intermedia* compared to the others showed intermediate sensitivity.

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Introduction

Diarrhea is caused by various parasites and microorganisms, and *Yersinia enterocolitica* similar to the other dangerous pathogens like *Shigella*, *Salmonella*, and *E. coli* plays a key role in bacterial gastroenteritis. Although these organisms cause similar disease, they infect host cells through several different mechanisms (1-3). *Yersinia enterocolitica* is motile and grows well at 25° C, but it is immotile at 37°C. More than 70 serotypes of *Yersinia enterocolitica* have been recognized, that among them, only 11 serotypes are significant in foodborne diseases (4, 5). This bacterium has 5 biotypes classified into 5 categories (1-5). Bio type 1 is divided into two biotypes 1A and 1B. Biotypes 2, 4, and 1B are human pathogens. Among them, bio serotypes 1B/O: 8, 2/O: 5, 27, 2/O: 9, 3/O: 3, and 4/O: 3 are often associated with human Yersiniosis and the others are related to biotype 1A and are environmental (6,7).

The role of *Yersinia enterocolitica* as an intestinal pathogen has been proved in the population of 30 countries. This organism frequently grows in cold regions like Western Europe and Scandinavia. However, high frequencies of this organism have been reported in Japan and America too (7-9). In a study performed in Tehran (2004) on 300 diarrhea samples obtained from children (0-12 years), *Yersinia* (2.7% including 5 *Yersinia enterocolitica* and 3 *Yersinia inermidia*), *Escherichia coli* Enteropathogenic

(5.7%), *Shigella* (3%), and *Salmonella* strains (2%) have been reported as responsible pathogens (10).

Some researchers believe that contaminated water is the main source of *Yersinia* infections (11, 12), although most of the recognized Human-pathogenic strains have not been isolated from water sources. Considering the role of *Yersinia enterocolitica* in poisoning food and its fecal-oral transmission, and since it is cold-resistant and can grow in low temperatures, its detection in the process of evaluating infection sources and transmission routes is very important (13,14).

Most *Yersinia* infections are associated with Self-limiting diarrhea and no beneficial effect of antibiotics on these infections have been reported. In acute cases of diarrhea, intravenous (IV) gentamicin (5 mg/kg/day) or oral chloramphenicol (50 mg/kg/day) is recommended. Beta-lactam antibiotics have been recommended as alternatives in *Yersinia* infections among which almost all the samples were resistant to Ampicillin and the first generation Cephalosporins but sensitive to Co-amoxiclav and the third generation Cephalosporins such as Cefotaxime, Cefprozime, and Ceftriaxone (15-16).

Different biotypes have shown different sensitivity patterns to 4 different beta-lactam antibiotics. All microorganisms of biotype 4 were resistant to Carbenicillin and Ticarcillin, and sensitive to Amoxicillin, Clavulanic acid, and Cefoxitin. In contrast, all samples of biotype 3

showed sensitivity to Carbenicillin and Ticarcillin and resistance to Cefoxitin and Co-amoxiclav. Biotype 1 showed resistance to all 4 antibiotics (17).

Considering the effect of *Yersinia* different biotypes and species on the sensitivity or resistance of this bacterium to the antibiotics, knowing how local isolates obtained in Iran respond to Beta-lactam antibiotics is very important. Therefore, the aim of this study was to find out the relationship of biotypes and serotypes of *Yersinia* isolates with MIC of three Beta-lactam antibiotics and the sensitivity of *Yersinia* to these antibiotics.

Methods

This descriptive study was performed in 2014 on 50 strains of *Yersinia* including 39 strains from various sources in Iran (8 strains from stool of children with diarrhea, 26 ones from water, 3 ones from raw milk, and 2 ones from vegetables) that were previously isolated at 27° C and 11 strains purchased from Pasteur Institute of Paris to determine the MIC of three antibiotics (Ampicillin, Cefazolin, and Cefotaxime).

First, all strains were kept in the brain heart infusion (BHI) broth in an incubator at 25°C for 18 hours, then one loop of cloudy liquid medium was transferred to CIN (Cefsulodin, Irgasan, Novobiocin) agar.

All mannitol positive colonies were selected as suspicious colonies and their motility was determined using differential tests like oxidase,

ortho-Nitrophenyl- β -galactoside (ONPG), urease, ornithine decarboxylase (ODC), arginine dihydrolase, and lysine decarboxylase tests at 25 and 37°C. Colonies of mannitol, urease, ortho-Nitrophenyl- β -galactoside, and ornithine decarboxylase that were positive and motile at 25°C and colonies of lactose and lysine decarboxylase that were negative and immotile at 37°C were classified as *Yersinia* species. Then, the species of *Yersinia* colonies were determined using fermentation test of sugars such as sucrose, rhamnose, melibiose, and raffinose, and the results were confirmed by plaque API-20E.

Biotype and Serotype Determination

The biotype of strains confirmed as *Yersinia enterocolitica*, was determined and classified according to the table of Wauters et al (1987) (18) using tests of acid production from xylose, salicin and trehalose, indole production, pyrazinamidase activity, lipase activity, esculin hydrolysis, and production of acetoin. According to this table, biotype 1A is an environmental biotype and the others (biotypes 1B, 2, 3, 4, and 5) are pathogenic.

Serotype was determined using slide agglutination test at Pasteur Institute of Paris.

In order to evaluate the sensitivity of Iranian isolates and to compare with that of 11 standard strains of Pasteur Institute, as controls, minimum inhibitory concentration was determined using broth macrodilution test as follows.

Procedure

The MIC of Ampicillin, Cefazolin, and Cefotaxime (Mast Group LTD) was determined using Macrodilution broth test (19).

To prepare the stock solution, standard antibiotic powders with exactly defined potency are required. In this study, due to the lack of standard powders, 1g vials of Ampicillin, Cefazolin, and Cefotaxime (purchased from Exir Pharmaceutical Co. Iran) were used.

Different concentrations of antibiotics were prepared based on the break point and at a concentration as twice as the desired final concentration. Since in this technique, 1 ml of inoculum is combined with 1ml of antibiotic and consequently the final concentration of each will be reduced by one-half, in order to obtain the desired final concentration, the prepared concentration in the first phase should be twice.

After weighing the powder, it was dissolved in 20 ml of sterile distilled water or any other solvent that can dissolve powder well, and using a sterile syringe, it was filtered on 0.22 μm pore size membrane filters.

Then, 10 ml sterile distilled water (or other solvents) was added to the each of the 6 small sterile tubes. One-half of the 20 ml filtered solution was added to the first small tube and shaken well. Again, 10 ml of the solution of the first tube was added to the second one and this process was continued to the last tube. At last, 10 ml of the solution of the last tube was poured out.

Afterwards, the contents of these tubes based on the concentration of each, were transferred into Erlenmeyer flasks containing 90 ml of sterile broth. Thus, 7 different concentrations of one antibiotic were prepared and arranged from maximum to minimum concentration. As mentioned before, different concentrations are prepared based on twice of the three concentrations before and after the break point. For instance, for Ampicillin with break point of 16, serial dilutions of 2-128 $\mu\text{g/ml}$ were prepared. These solutions can be kept in refrigerator for 5-7 days.

To prepare a microbial suspension, first, the fresh cultures of microorganism were prepared near the flame and placed in an incubator at 27°C. After 18-24 hours, some freshly grown colonies were removed aseptically and transferred into 2-3 ml sterile broth. The obtained suspensions were incubated until opacity was obtained. The opacity should be equivalent to a McFarland tube 1. Then, 0.02 ml of the suspension was diluted with 10 ml sterile nutrient broth. Therefore, there are 5×10^6 cfu/ml of microorganisms in the suspension.

In order to determine the MIC of antibiotics for each strain, a series of 9 small sterile tubes (7 test tubes and 2 positive and negative controls) were placed in a tube racks. Then, 1 ml of bacterial suspension and 1 ml of target antibiotic dilutions were added to each tube so that the first tube had the minimum antibiotics concentration and the last one had the maximum concentration. In the

positive control tube, 1ml of microbial suspension was added to 1ml of sterile broth, and in the negative control tube, 1 ml of antibiotic with maximum concentration was added to 1ml of sterile broth. Since *Yersinia* is a psychrophilic (cold loving) microorganism, all tubes were incubated at 25°C for 18-24 h.

After incubation, all tubes were investigated in terms of the opacity. In the negative control tube, no growth should be observed. The tubes were compared with positive and negative control tubes. Thus, the first tube with no opacity was considered as the MIC of the antibiotic for that strain.

Results

The aim of this study was to determine the MIC of Ampicillin, Cefazolin, and Cefotaxime for 50 strains of different species of *Yersinia* at 27°C. The results are as following:

Table 1 shows the MIC of antibiotics for strains obtained from Pasteur Institute of France (*Yersinia enterocolitica*, *Yersinia intermedia*, *Yersinia frederiksenii*, and *Yersinia kristensenii*). As it is seen, the MICs of Ampicillin, Cefazolin, and Cefotaxime were respectively $\leq 2-128$, $\leq 4-256$ and $\leq 0.125-0.25\mu\text{g/ml}$ for *Yersinia enterocolitica*,

16, 32 and $\leq 0.125\mu\text{g/ml}$ for *Yersinia intermedia*, 32, 256 and $\leq 0.125\mu\text{g/ml}$ for *Yersinia frederiksenii*, and 16, ≤ 4 and $\leq 0.125\mu\text{g/ml}$ for *Yersinia kristensenii*

Table 2 shows the MIC of antibiotics for isolates of Iran. As it is seen, the MIC of Ampicillin, Cefazolin, and Cefotaxime were respectively $>16-128$, 8- 64 and 0.125- 0.5 $\mu\text{g/ml}$ for *Yersinia enterocolitica*, $\leq 4-64$, 4-32 and $\leq 0.125\mu\text{g/ml}$ for *Yersinia intermedia*, $> 32-128$, $> 64-256$ and $\leq 0.125-0.5\mu\text{g/ml}$ for *Yersinia frederiksenii* and $\leq 8-32$ and $\leq 0.25\mu\text{g/ml}$ for *Yersinia kristensenii*.

Comparison of two tables show that the strains obtained in Iran compared to those obtained in France, except *Yersinia kristensenii*, were more resistant.

In this study, the serotype distribution of strains obtained from France and isolated in Iran was limited to only 5 serotypes. Although there are similar serotypes in both groups, there are also some serotypes in Europe that have not been identified in Iran before this study (Tables 1 and 2). In this study, the predominant biotypes of *Yersinia enterocolitica* were 1, 1A, 1B, 2, and 4.

Table 1. The MIC of Ampicillin, Cefazolin, and Cefotaxime for strains obtained from Pasteur Institute of France

Species	Code of strain	Serotype	Cefotaxime	MIC ($\mu\text{g/ml}$) Cefazolin	Ampicillin
<i>Y. enterocolitica</i>	22079	O: 3	0.25	128	> 128
	22080	O: 3	0.25	256	> 128
	96	O: 4,32	0.25	16	32
	124	O: 5	≤ 0.125	32	16
	161	O: 8	≤ 0.125	≤ 4	64
	22069	O: 8	0.25	16	16
	9	N.AG	≤ 0.125	≤ 4	≤ 2
	21	O: 6	≤ 0.125	≤ 4	≤ 2
<i>Y. intermedia</i>	3953	N.AG *	≤ 0.125	32	16
<i>Y. frederiksenii</i>	867	N.AG	≤ 0.125	256	32
<i>Y. kristensenii</i>	105	N.AG	0.125	≤ 4	16

*: Non agglutinable

Table 2. MIC of Ampicillin, Cefazolin, and cefotaxime for strains obtained in Iran

Species	Code of Strain Sent to Pasteur Institute	Serotype	Cefotaxime	MIC ($\mu\text{g/ml}$) Cefazolin	Ampicillin
<i>Y. enterocolitica</i> resource: stool of children with diarrhea	1	O:7,8,19	0.5	32	64
	3	O:7,8,19	0.25	32	64
	7	N.AG *	≤ 0.125	32	64
	8	N.AG	≤ 0.125	16	64
	52	N.AG	≤ 0.125	32	64
resource: water	12	N.AG	≤ 0.125	32	64
	16	O:7,8,19	0.5	32	32
	17	O: 7,13	0.25	16	32
	18	O: 7,13	0.5	16	32
	21	O: 7,13	≤ 0.125	8	32
	24	O: 7,13	≤ 0.125	16	128
	30	O:7,8,19	≤ 0.125	16	64
	31	O:7,8,19	≤ 0.125	16	64
	32	O:7,8,19	≤ 0.125	16	64
	33	O:7,8,19	≤ 0.125	16	64
	34	O:7,8,19	0.25	32	64
	41	O: 5	≤ 0.125	32	16
	42	O: 5	≤ 0.125	16	32
	44	O: 5	≤ 0.125	32	16
	45	O: 5	≤ 0.125	16	16
resource: milk	26	N.AG	≤ 0.125	16	32
	47	O: 5	≤ 0.125	32	64
	50	O: 7,13	≤ 0.125	16	32
resource: Vegetable	55	-	≤ 0.125	64	32
<i>Y.intermedia</i> resource: stool of children with diarrhea	5	O: 17	≤ 0.125	≤ 4	128
	6	O: 17	≤ 0.125	16	128
resource: water	9	N.AG	≤ 0.125	32	32
	10	O: 37	≤ 0.125	16	64
	14	O: 32/4	≤ 0.125	32	32
	15	O: 40	≤ 0.125	32	16
	20	O: 14	≤ 0.125	≤ 4	16
	22	N.AG	≤ 0.125	≤ 4	16
	23	O: 32/4	≤ 0.125	16	16
40	O: 14	≤ 0.125	$4 \geq$	16	

<i>Y. frederiksenii</i>					
resource: stool of children with diarrhea	2	O: 39	0.5	128	32
	11	N.AG	≤0.125	128	128
resource: Water	13	N.AG	0.25	64	128
	19	N.AG	≤0.125	256	> 128
resource: vegetable	56	-	4	> 256	>128
<i>Y. keristensenii</i>					
resource Water	65	N.AG	≤0.25	8 ≥	32

Discussion

In this study, it was tried to investigate the sensitivity of *Yersinia* to Beta-lactam antibiotics with respect to factors like *Yersinia* serotypes, biotypes and strains.

Almost all samples, which were selected from different serotype and biotypes, showed complete sensitivity to Cefotaxime compared to Cefazolin and Ampicillin. The results show that the strains with different biotypes and serotypes show different pattern of sensitivity, and difference in sensitivity or resistance depends on these two factors.

It was revealed that all O:3 serotypes of French strains were resistant to Ampicillin while most of the O:8 strains were sensitive to Ampicillin. However, sometimes in spite of similarity in serotypes and biotypes, there are some differences in the MIC that can be interpreted by taking into account the other factors such as temperature.

Sensitivity to Ampicillin and Cefazolin showed slight differences based on the type of biotype and serotype; for example, *Yersinia enterocolitica* biotype 4 compared to the biotypes 2, 1A, and 1 showed the highest resistance to Ampicillin and

Cefazolin so that the MIC of Ampicillin for enterocolitica biotype 2 was 16 mg/L while the MIC of Ampicillin for biotype 4 was 64 mg/L. It was reported that in terms of sensitivity, biotype 2 was followed by biotypes 1 and 1A, and biotype 1B was more resistant than biotype 1A.

Therefore, among biotypes 1, 1A, 1B, 2, and 4 of *Yersinia enterocolitica* investigated in this study, biotypes 1A and 2 showed sensitivity to ampicillin while biotype 4 was the most resistant biotype to Ampicillin and Cefazolin.

Among *Yersinia intermedia* biotypes 1, 2, and 3, biotype 3 was the most sensitive and biotype 1 was the most resistant one to Cefazolin. In regard to Ampicillin, too, biotype 3 showed less resistance.

Since Cefotaxime is a third generation cephalosporin and has significant inhibitory and bactericidal effects on gram-negative bacteria, it can be considered as the most effective antibiotic against *Yersinia* species among beta-lactam antibiotics. However, its sensitivity compared to Cefazolin is less and depends on the type of biotype, serotype, and other factors. Since different strains of *Yersinia* rarely were sensitive to

Ampicillin, it can be considered as an ineffective antibiotic against *Yersinia*.

In the similar studies in France, Hornstein et al in their study on the sensitivity of 126 *Yersinia enterocolitica* strains to 21 beta-lactam antibiotics, have reported that the most effective anti-microbial agents were Ceftriaxone, Cefotaxime, and Ceftizoxime (MIC: 0.06-0.08 mg/l). And, Mezlocillin (MIC: 1.36 mg/L) and Piperacillin (MIC: 1.57 mg/L) were also recognized as the effective Penicillins. However, all strains showed resistance to Ampicillin (MIC > 4 mg/L) and Cephalothin (MIC > 8 mg/L) (20).

Melchior & Keiding (21) also reported that all *Yersinia* strains that were selected from different biotypes and serotypes, showed resistance to Ampicillin (MIC: 32 – 256 mg/L), but sensitivity to Amoxicillin-clavulanic acid, Cefoxitin, Carbenicillin, and Ticarcillin differed based on the biotype; that is, all strains of biotype 4 were resistant to Carbenicillin and Ticarcillin but sensitive to Amoxicillin-clavulanic and Cefoxitin. However, strains of biotype 3, showed completely reverse results. In addition, all *Yersinia* strains belonged to biotype 1A were sensitive to all four antibiotics. Therefore, this study also confirms that different biotypes show different sensitivity patterns (19). Serotypes O:3 and O:8 showed sensitivity and resistance to Ampicillin respectively (21). In the study of Bent and Young, *Yersinia enterocolitica* biotype 1B was pathogenic

and had two resistant genes (BlaA and BlaB) to β -lactamase (22).

In another study performed by Preston et al in Canada on the sensitivity of *Yersinia enterocolitica* to antimicrobial agents, no sensitivity to Ampicillin, Carbenicillin, Ticarcillin, and Cephalotin was observed in the majority of cases (over 90%), and strains of serotypes O:3, 27, O:5, and O:8 showed different sensitivity patterns to these antibiotics. Strains isolated in Canada showed resistance to Ampicillin, Amoxicillin, Carbenicillin, Ticarcillin, and Cephalotin, whereas *Yersinia* serotypes O:3, O:5, O:8, O:9, and 27 isolated in France, showed sensitivity to Ampicillin, Cephalexin, Cephalotin, and Carbenicillin (23).

Comparison of Tables 1 and 2 shows that *Yersinia enterocolitica* strains obtained from Pastor Institute of France were more sensitive to Ampicillin and Cefazolin than those isolated from human and environment sources in Iran. As shown in Table 1 and 2, 25% of *Yersinia enterocolitica* strains obtained from France showed sensitivity to Ampicillin (MIC \leq 2 mg/L), while those obtained in Iran, showed 100% resistance to Ampicillin in this concentration and even higher ones.

Generally, the results show that the strains obtained from France were more sensitive than the clinical and environmental strains obtained in Iran. But despite of this high sensitivity, the range of MIC of French strains was much wider than those obtained in Iran and this can be due to the presence

of different serotypes and biotypes in 9 strains. As mentioned before, different serotype and biotypes of a microorganism usually show different sensitivity patterns to antimicrobial agents. In the present study, among four species of *Yersinia* (*enterocolitica*, *intermedia*, *frederiksenii*, and *kristensenii*.), *Yersinia kristensenii* was reported as the most sensitive one, and *Yersinia frederiksenii*, *Yersinia enterocolitica*, and *Yersinia intermedia* were reported as the resistant species, respectively.

Soltan Dallal et al showed that most of the *Yersinia* species were resistant to Ampicillin and Cephalothin, and sensitive to Ciprofloxacin. They also reported that some of the *Yersinia enterocolitica* strains isolated from poultry and meat were resistant to several drugs (Tetracycline, Streptomycin, Cephalothin, Nalidixic acid, Amoxicillin) (24).

Ahmedy in his studies in France, has shown that 98.9% of *Yersinia enterocolitica*, *Yersinia intermedia*, *Yersinia frederiksenii*, and *Yersinia kristensenii* strains isolated from food were sensitive to non-beta-lactam antibiotics and 87% of the strains were resistant to Ampicillin and Carbenicillin. Using serial dilution-agar plating method, the MIC of Ampicillin and Carbenicillin for 125 *Yersinia kristensenii* strains and *Yersinia enterocolitica* biotype 3 was determined, and Carbenicillin (MIC<8 mg/l) was considered as the sensitive antibiotic to these strains, but all *Yersinia enterocolitica* biotype 1, *Yersinia intermedia*, and *Yersinia frederiksenii* with MIC >90-256 mg/l

were considered as the resistant antibiotics to Carbenicillin (25).

Conclusion

Among the three Beta-lactam antibiotics (Aminopenicillin and the first and third generation Cephalosporins), the third generation Cephalosporins like Cefotaxime, Ceftriaxone, Ceftazoxime, and Ceftazidime are considered as the preferred treatment for *Yersinia*-related infections. The first generation Cephalosporins like Cefazolin have low effect on gram-negative bacteria compared with the third generations; therefore, a high concentration of these antibiotics is required to inhibit the growth of microorganisms or to destroy them.

It is worth mentioning that Ampicillin which belongs to Aminopenicillin antibiotics has almost no inhibitory effect on *Yersinia*. Therefore, it can be concluded that among Beta-lactam antibiotics, the third generation Cephalosporins in very low doses can be used as effective antibiotics in the treatment of *Yersinia*-related infections.

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