Title: Isolation and Antibiotic Resistance Evaluation of *Yersinia enterocolitica* from Raw Milk of Ruminants in Sistan and Baluchestan, Iran

Running title: Isolation of *Yersinia enterocolitica* from Raw Milk of Ruminants

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Abstract

There is a great importance of *Yersinia enterocolitica* (*Y. enterocolitica*) and increasing concerns about antibiotic resistance. Hence, objective of this study was to investigate the level of contamination of ruminants' raw milk with *Y. enterocolitica* and determine its antibiotic resistance pattern. A total of 200 raw milk samples (100 from cows and 100 from sheep) were collected over five months (July 2023 to December 2023) from bulk tank supply centers in the Sistan region, Iran. CIN agar was used accurately as a selective medium for *Y. enterocolitica*. The isolated colonies were transferred to Simon Citrate, Lysine Iron Agar, and Urea medium for final confirmation. Moreover, the susceptibility of the obtained isolates to ampicillin (AM), amikacin (AN), imipenem (IMP), nalidixic acid (NA), ciprofloxacin (CP), amoxiclav (AMC), gentamicin (GM), ceftriaxone (CRO), tetracycline (TE), and sulfamethoxazole (SXT) antibiotics was assessed. Of the 200 raw milk samples, 8 (4%) were contaminated with *Yersinia* (95% CI: 1.5%-7%). The highest contamination with *Y. enterocolitica* was associated with raw cow's milk, and the lowest contamination with *Y. enterocolitica* was associated with raw sheep's milk; however, these differences were not statistically significant ($P>0.05$). The highest resistance for *Y. enterocolitica* in raw cow's milk was related to antibiotic AM, and antibiotic SXT had the highest sensitivity. Additionally, the highest resistance to *Y. enterocolitica* in raw sheep's milk was associated with AN, SXT, CRO, NA, GM, and AMC, but CP and TE exhibited the highest sensitivity. This study confirms the presence of multidrug-resistant Y. enterocolitica in raw milk, highlighting a potential public health risk and the need for improved antimicrobial stewardship in veterinary and agricultural practices. To prevent the transmission of foodborne diseases such as milk-borne diseases, it is recommended that raw cow's and sheep's milk be minimized and consumed in pasteurized or sterilized conditions.

Keywords: Antibiogram test; Antibiotics; Bacterial infection; Cattle; Prevalence

1. Introduction

A serious threat to human health is posed by *Yersinia enterocolitica*, a zoonotic pathogen recognized worldwide as a high-priority pathogen (1). At low temperatures, this gram-negative bacterium can be motile and propagate under various conditions, including raw and unprocessed foods such as raw milk, meat, and animal products such as cheese, yogurt, and other dairy products (2). *Y. enterocolitica* usually causes infections through the consumption of contaminated food products and can cause a wide range of illnesses, including enteritis, yersiniosis, and septicemia in some cases, which are life-threatening. There has been an increase in the incidence of antibiotic resistance due to the emergence of resistant strains of *Y. enterocolitica* species in recent years, resulting in a decline in the effectiveness of standard treatments and a rise in mortality rates (3).

Due to the nutritious environment in which raw milk is produced, it is potentially a vector for various pathogens, including *Y. enterocolitica* (4). The bacterium is capable of growing under refrigeration conditions, reaching infectious levels even at low refrigerator temperatures. It has been shown that raw milk and unpasteurized dairy products, such as yogurt and butter, are among the most significant routes of transmission of Y. enterocolitica to humans through consumption (5). The importance of this becomes more apparent in areas where pasteurization is not fully practiced or in areas where dairy products from local producers are commonly consumed. Several factors must be considered in these circumstances (e.g., raw milk contamination with *Y. enterocolitica*, its antibiotic resistance pattern, etc.) to minimize health risks and control the contamination of raw milk and even meats (6).

It has become increasingly apparent over recent decades that antimicrobial resistance among pathogens is a growing concern on a global scale (7). Due to the overuse of antibiotics in animal husbandry and the food industry, strains of *Y. enterocolitica* have developed that are resistant to many antibiotics, making the treatment of infections caused by *Y. enterocolitica* more difficult (8). As a result, it is not only a threat to public health, but it also increases healthcare costs, extends the time for patients to recover, and increases the risk of death due to drug-resistant infections as well.

Various methods, including microbial culture, Polymerase Chain Reaction (PCR), and serological tests, are used to detect *Y. enterocolitica* in raw milk. Molecular techniques have been widely used in recent years for the rapid identification of this pathogen due to their higher sensitivity and specificity (9). However, identifying antibiotic-resistant strains remains a serious challenge and requires further research to improve screening and control methods.

It is essential to adopt preventive strategies to reduce the prevalence of *Y. enterocolitica* and control antibiotic resistance. Implementing hygiene standards in livestock farms, maintaining food quality, accurately implementing the pasteurization process, and reducing the excessive use of antibiotics in the livestock industry are practical measures to mitigate the risk of transmission of this bacterium (10). Moreover, developing and implementing strict monitoring policies by health institutions at the international level can play a significant role in reducing the threats posed by *Y. enterocolitica*. Given the global importance of *Y. enterocolitica* and increasing concerns about antibiotic resistance, this study aims to investigate the level of contamination of raw milk with this bacterium and determine its antibiotic resistance pattern.

2. Methods and materials

2.1.Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed, and we confirm that the study was conducted in compliance with the ARRIVE guidelines. The Animal Welfare Committee's ethical committee at Zabol University has approved the study procedure. All authors have reviewed the moral issues, including plagiarism, consent to publish, misconduct, data fabrication and falsification, double publication and submission, and redundancy.

2.2.Study design

The rationale for selecting Sistan and Baluchestan province can indeed be strengthened by mentioning the high rate of milk consumption in the local diet and the significant presence of animal farms in the region. Hence, in the current study, 200 raw milk samples (100 from cows and 100 from sheep) were collected over five months (July 2023 to December 2023) from bulk tank supply centers in the Sistan region, Iran. Under sterile conditions and next to the ice, the samples were transferred to the food quality control laboratory of the Veterinary School at Zabol University.

2.3.Study area

Zabol is located in the Sistan and Baluchestan province in southeastern Iran. The climate is arid during the summers and mild during the winters (11). Due to its barren surroundings and desert landscapes, the city experiences hot, dry weather throughout the year.

2.4.Sampling

Two hundred raw milk samples (100 from cows and 100 from sheep) from the bulk tank were placed directly into special, sterile containers, and the sampling process was carried out according to standard principles. For each sample, 25 cc of raw cow's milk and 25 cc of raw sheep's milk were poured into sterile, leak-proof containers, and the product specifications were recorded.

2.5.Bacterial isolation

Lactose broth culture media, McConkie agar, *Yersinia* CIN agar, Simon citrate culture medium, and Lysine iron agar were used to examine the samples (12). 25 cc of the samples were added to 225 ml of broth, lactose, and then transferred to McConkey agar medium, and incubated at 37°C for 24 hours. CIN agar was used as a selective medium to identify and isolate *Y. enterocolitica* accurately. The incubation was carried out at 37°C for 24 hours. The presence of pink colonies confirmed the presence of *Y. enterocolitica*. The isolated colonies were transferred to Simon Citrate, Lysine Iron Agar, and Urea medium for final confirmation.

2.6.Antibiogram test

Mueller-Hinton culture medium was used to determine the sensitivity of bacteria to antibiotics (13). The susceptibility of the obtained isolates to ampicillin (AM), Amikacin (AN), Imipenem (IMP), nalidixic acid (NA), Ciprofloxacin (CP), Amoxiclav (AMC), gentamicin (GM), ceftriaxone (CRO), tetracycline (TE), and sulfamethoxazole (SXT) antibiotics were assessed. After 24 hours, plates were removed from the incubator, and the diameter of the growth inhibition zone was measured using a caliper ruler.

2.7.Statistical analysis

SPSS version 23 statistical software and the Chi-square test were used to analyze the data. The prevalence of *Yersinia* infection was calculated with a 95% CI. The level of significance was considered $P<0.05$.

3. Results

Of the 200 raw milk samples examined in this study, 8 (4%) were contaminated with Yersinia (95% CI: 1.5%-7%). The highest contamination with *Y. enterocolitica* was associated with raw cow's milk, and the lowest contamination with *Y. enterocolitica* was associated with raw sheep's milk; however, these differences were not statistically significant ($P>0.05$). The sensitivity of isolates from sheep's milk to those from cow's milk and different antibiotics did not differ

significantly from each other (*P>0.05*) (Table 1). The highest resistance for *Y. enterocolitica* in raw cow's milk was related to antibiotic AM, and antibiotic SXT had the highest sensitivity (Figure 1). Additionally, the highest resistance to *Y. enterocolitica* in raw sheep's milk was associated with AN, SXT, CRO, NA, GM, and AMC; but CP and TE exhibited the highest sensitivity (Table 2).

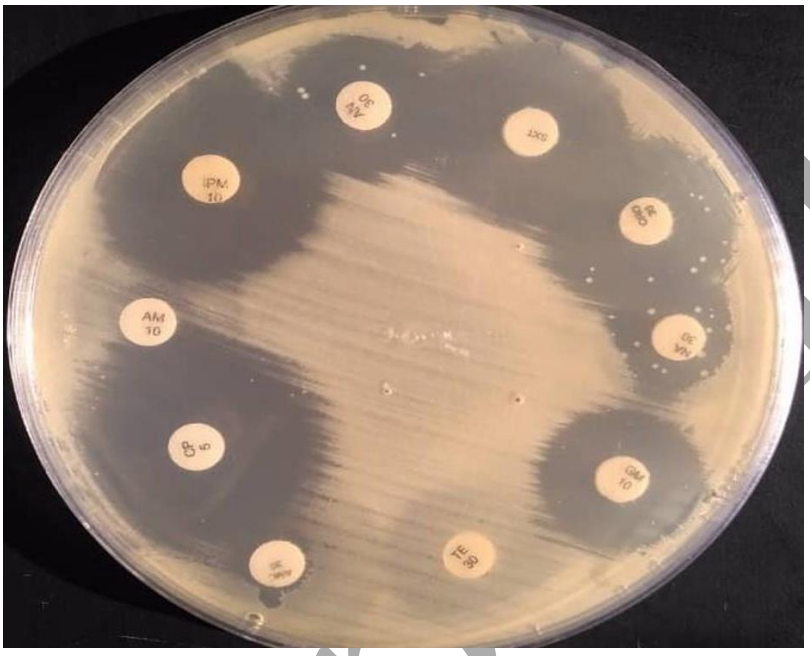

Figure 1. Antibiogram results from cow milk showed that the isolated *Yersinia enterocolitica* strains were susceptible to the antibiotic imipenem (IPM), with a 30 mm inhibition zone, while they exhibited resistance to ampicillin (AM), with a 10 mm inhibition zone.

Table 1. Sensitivity of *Yersinia enterocolitica*. This table summarizes the sensitivity of *Yersinia enterocolitica* isolates to antibiotics in the current study.

Antibiotic	**Sheep**			**Cow**		
	Resistant	Semi-sensitive	Sensitive	Resistant	Semi-sensitive	**Sensitive**
AM	0 (0%)	2 (100%)	0 (0%)	4 (66.7%)	1 (16.7%)	**1 (16.7%)**
AN	1 (50%)	1 (50%)	0 (0%)	3 (50%)	2 (33.3%)	**1 (16.7%)**
SXT	1 (50%)	0 (0%)	1 (50%)	0 (0%)	2 (33.3%)	**4 (66.7%)**
TE	0 (0%)	1 (50%)	1 (50%)	0 (0%)	5 (83.3%)	**1 (16.7%)**
CRO	1 (50%)	0 (0%)	1 (50%)	2 (33.3%)	3 (50%)	**1 (16.7%)**
NA	1 (50%)	1 (50%)	0 (0%)	3 (50%)	1 (16.7%)	**2 (33.4%)**
GM	1 (50%)	1 (50%)	0 (0%)	3 (50%)	2 (33.3%)	**1 (16.7%)**
AMC	1 (50%)	0 (0%)	1 (50%)	2 (33.3%)	2 (33.3%)	**2 (33.3%)**
CP	0 (0%)	1 (50%)	1 (50%)	1 (16.7%)	5 (83.3%)	**0 (0%)**
IPM	**1 (50%)**	**1 (50%)**	**0 (0%)**	**1 (16.7%)**	**3 (50%)**	**2 (33.3%)**

Ampicillin (AM), amikacin (AN), imipenem (IMP), nalidixic acid (NA), ciprofloxacin (CP), amoxiclav (AMC), gentamicin (GM), ceftriaxone (CRO), tetracycline (TE), and sulfamethoxazole (SXT)

Table 2: Antibiotic susceptibility profile of *Yersinia enterocolitica* isolates obtained from raw cow and sheep milk samples.

Source of Sample	Number of Isolates	Antibiotic	Inhibition Zone Diameter (mm) (Mean ± SD)	Susceptibility Status (Based on CLSI)
Cow Milk	6	Imipenem (IPM)	30.2 ± 1.3	Susceptible (S)
		Ampicillin (AM)	10.5 ± 0.7	Resistant (R)
		Ciprofloxacin (CIP)	22.0 ± 1.1	Intermediate (I)
Sheep Milk	2	Imipenem (IPM)	29.0 ± 0.0	Susceptible (S)
		Ampicillin (AM)	11.0 ± 1.4	Resistant (R)
		Ciprofloxacin (CP)	21.5 ± 0.7	Intermediate (I)

CLSI, Clinical and Laboratory Standards Institute. The interpretation of susceptibility (S), intermediate (I), or resistant (R)

4. Discussion

Based on the results of previous studies, it appears that the bacterium *Y. enterocolitica* poses a significant health threat to the public worldwide, and raw milk is considered a potential source of transmission for this bacterium (14). According to recent studies, *Y. enterocolitica* can multiply and cause disease in consumers due to its capacity to grow at low temperatures, even when stored at cold temperatures, and even when kept in a refrigerator. Several studies conducted in various countries have demonstrated that the level of contamination in raw milk with *Y. enterocolitica* is influenced by several factors, including sanitation conditions, production standards, and monitoring methods (15).

In a study conducted in Varamin, Iran, on 446 samples of raw milk collected from tankers between 2008 and 2010, different *Yersinia* species were identified in 29 samples (6.5%), of which 23 were isolated from cow's milk, 5 from sheep's milk, and one from goat's milk (14). The most frequently isolated species was *Yersinia enterocolitica*, which was 65.5% of the isolates and 4.22% of the total samples. In terms of contamination, the results were almost in

line with those of the present study, which reported 4% cases of contamination with this bacterium in raw cow's milk.

In a study conducted by Hanifian and Khani (2012) in northwestern Iran, the prevalence of pathogenic *Y. enterocolitica* strains in raw milk and traditional cheese was investigated (16). The findings of this study showed that although the sample enrichment method followed by PCR for the ail gene detected the presence of bacteria with higher sensitivity (8.66% of all samples), conventional culture was only able to confirm the viability and presence of live bacteria in 2.88% of the samples. These results demonstrate the superiority of the molecular method (PCR) over the culture method in the rapid and sensitive detection of bacteria. However, the lower percentage of isolation by the culture method highlights the importance of paying attention to the presence of live and replicating bacteria as a real threat to food safety.

A study by Alavi et al. (2017) in Shahrekord showed that raw milk from small ruminants (sheep and goat) can be an important reservoir for pathogenic strains of *Y. enterocolitica* (17). Based on the results, 9% of sheep milk samples (and none of goat milk samples) were positive by microbial culture method, of which 5% of the positive samples were confirmed as bioserotype O:3 (including common pathogenic serotypes in humans). Also, the identification of virulence genes ail (in 4 isolates), yadA (in 3 isolates), and virF and ystA (each in 2 isolates) reinforces the potential risk of these isolates to cause disease in humans. These findings, in line with the results of the present study in the Sistan region, emphasize the importance of raw milk as a bacterial transmission agent and the need for more stringent health surveillance.

A study by Sharifi Yazdi et al. (2023) on 360 raw milk samples in Tehran reported a relatively low prevalence (1.1%) of *Y. enterocolitica* and *Y. pseudotuberculosis* (18). Of the positive samples, only three cases (0.83%) were related to Y. enterocolitica, and one case (0.27%) was associated with *Y. pseudotuberculosis*. These results, in comparison with other studies, including the present study in the Sistan region, which showed higher levels of contamination, indicate the positive effect of hygiene control factors such as the use of safe tap water and proper animal husbandry. Additionally, the isolation of other bacterial genera, such as *Klebsiella*, *Serratia*, *Citrobacter*, and *Providencia*, from milk samples underscores the role of poor personal and environmental hygiene in the contamination of raw milk.

There has been a recent increase in concerns about the spread of antibiotic resistance among pathogenic bacteria, making it increasingly important to formulate and implement preventive policies to prevent the spread of *Y. enterocolitica* in the milk and food supply (19). Antibiotic use in the livestock industry needs to be strictly regulated, milk pasteurization should be applied more carefully, and the public needs to be educated about the risks associated with consuming

raw milk, which are inherent to consuming milk that has not been pasteurized. These measures can help reduce the risks posed by this bacterium. Moreover, the development and application of advanced diagnostic methods and techniques, such as more sensitive molecular tests, can also assist regulatory agencies in identifying infected and resistant bacterial strains more rapidly, allowing them to implement appropriate control measures as soon as possible. The establishment of international collaboration in surveillance and control of zoonotic diseases is equally important. Because antibiotic-resistant pathogens do not have a geographically restricted distribution, they can spread rapidly worldwide due to global food trade, travel, and animal movements, which are also important factors in combating them (20). Developing global databases to track resistant strains of *Y. enterocolitica*, facilitating information exchange between countries, and developing coordinated strategies to control infections caused by this bacterium can contribute to reducing the overall impact of this bacterium on public health.

A significant limitation of this study was the limited number of isolates available for antibiotic susceptibility testing, with six obtained from cow's milk and two from sheep's milk. With such a small sample size, the reported percentages of antibiotic resistance or susceptibility cannot be considered precise estimates, and the confidence intervals around these values would be extensive. Therefore, the interpretation of the antibiotic resistance patterns should be made with considerable caution and is primarily exploratory and descriptive in nature. For more robust and generalizable findings regarding the antibiotic resistance patterns of *Yersinia enterocolitica* in the region, future studies on a larger scale and with a substantially higher number of samples are recommended.

Considering the challenges of controlling *Y. enterocolitica* as well as the need to combat antimicrobial resistance, it is recommended that future research should concentrate on developing new methods for preventing and treating this infection. Furthermore, health policymakers should devote more attention to enforcing stricter regulations regarding the use of antibiotics in livestock, as well as improving hygiene practices in the food sector. Increasing public awareness of the risks associated with raw milk consumption and the importance of adhering to hygiene standards can also help reduce the incidence of infections caused by *Y. enterocolitica*.

Declarations and statements

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Author contribution

Conceptualization: [M.A.E., M.R.], …; Methodology: [M.T., M.A.E., M.R., D.S.], …; Formal analysis and investigation: [M.A.E., M.R.], …; Writing - original draft preparation: [A.A.M., M.T., M.A.E.]; Writing - review and editing: [M.T., M.A.E., M.R., D.S.], …; Funding acquisition: [M.A.E.], …; Supervision: [M.A.E.]. All authors checked and approved the final version of the manuscript for publication in the present journal

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The authors declare no conflict of interest.

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Not applicable

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The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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