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Molecular Identification of *Yersinia enterocolitica* Isolated from Different Sources in Erbil City

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ABSTRACT

Yersinia enterocolitica ranks third among the pathogens that often cause the digestive disorder. It is transmitted to humans through food materials, especially contaminated meats. This research aimed to survey the frequency of *Yersinia enterocolitica* in sheep local products, especially meat, in Erbil. To carry out this study, 500 samples of raw milk, soft cheese, ice cream, and meat were collected by random sampling from different shops in Erbil City of Iraq. The samples were divided into four groups, including raw milk, soft cheese, ice cream, and meat. Many microbiological tests (culture, & staining, biochemical Tests, Vitek 2, and species-specific Polymerase Chain Reaction (PCR) amplicon for 16S RNA gene) were performed. Results showed that 5.1% of all of the studied samples were contaminated with *Yersinia enterocolitica*. Analysis of the results showed that meat is more contaminated than other samples. Also, the sequenced DNA evolutionary phylogeny tree of *Yersinia* enterocolitica isolates showed that all bacteria originated from the same genus and species. Therefore, it is recommended to pay special attention to this issue in order to avoid health and economic risks.

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Introduction

Some diseases transfer from contaminated food to the human body which make gastrointestinal diseases. They are classified as intestinal diseases and they exist in developing countries and, also in developed countries with high health standards (1).

Yersinia enterocolitica, is a member of the *Enterobacteriaceae* family and a causative agent of *yersiniosis* that is a zoonotic disease that results in symptoms ranging from mild, self-limiting diarrhea to acute mesenteric lymphadenitis (2). It transmits to humans through food and water, mainly contaminated dairy and meat products (3). Keeping such foods in the refrigerator allows them to multiply (4). Due to the absence of symptoms, consumption of these foods can lead to gastroenteritis and other digestive disorders in consumers. As a global challenge, bacterial infections with different strains are being increased in the society (5). In a study, the effect of the presence of *Escherichia coli* bacteria in raw milk samples on the incidence of diarrhea in children was observed (6).

Generally, humans become infected with *Y. enterocolitica* bacteria by eating red meat contaminated with it (7). Also, a large number of serological studies showed that people who deal with livestock and meat are more susceptible to *Y. enterocolitica* infection than others (8). Intact tissues of healthy animals are free of microbial agents, but during slaughter, bacteria in the digestive tract, the slaughterhouse environment, and the meat preparation process cause meat contamination (9).

Among several pathogenic bacteria that can multiply on meat at refrigerated temperatures, *Y. enterocolitica* is particularly important for two reasons: 1-The ability to grow, multiply and produce enterotoxin during meat storage in the refrigerator without changing the taste and smell of the meat (8), and 2- and their ability to cause serious illness in consumers of contaminated meats such as dysentery, gastroenteritis, autoimmune thyroid disorders, septicemia, mesenteric lymphadenitis, erythema nodosum (EN), and even death in some cases (10,11).

K. pneumonia, as a Gram-negative bacillus, is another member of the *Enterobacteriaceae* family. It causes infections of human nosocomial, pneumonia, septicemia, infections of the urinary tract, diarrhea, meningitis, and infections of soft tissue (12).

This study aimed to isolate and identify *Y. enterocolitica* species from different sources in Erbil city.

Materials and Methods

Five hundred locally food samples of raw milk, locally white soft cheese, meat, and ice cream were randomly selected from different retail shops between April 2021 and August 2021 in Erbil city, and examined for the presence of *Yersinia* species. The samples were aseptically collected in separate sterile containers. All samples were stored in a refrigerator at 4°C before testing. The samples were analyzed on the same day of sampling.

Bacteriological culture

The amount of 25 grams of each sample was homogenized in PBS broth (peptone, sorbitol, and bile salts) (Difco, Heidelberg, Germany) and incubated according to the standard protocol. Then, the samples were streaked into Cefsulodin-Irgasan-Novobiocin (CIN) agar medium plates (Oxoid) and incubated under aerobic conditions for 24 h

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Table1. Sensitivity test for antimicrobial agents.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL			Meropenem	<=0.25	S
Ampicillin	>=32	R	Amikacin	<= 2	S
Amoxicillin/Clavulanic Acid	<= 2	S	Gentamicin	<= 1	S
Piperacillin/Tazobactam	<=4	S	Ciprofloxacin	<= 0.25	S
Cefotaxime	8	S	Norfloxacin	2	S
Ceftazidime	8	I	Fosfomycin	>= 256	R
Cefepime	<= 1	S	Nitrofurantoin	32	S
Ertapenem	<= 0.5	S	Trimethoprim/ Sulfamethoxazole	<= 20	S
Imipenem	<= 0.25	S			

at 30°C. The typical red bull's eye-like colonies (small, with a red center and clear rim) were purified on the surface of soy tryptic agar plates and stored at 4°C for further investigation. Vitek2 bacterial identification system (bio-Merieux) was used according to the manufacturer's instructions (13,14).

Confirmation of the identity of isolates by VITEK 2 System:

The VITEK 2 uses growth-based technology and is more focused on the clinical microbiology laboratory (15–17). The following steps were performed to identify the isolates.

Preparation of Suspension: A sterile stick was applied to transfer colonies from a fresh, pure culture and suspended in sterile saline in a clear plastic test tube.

Inoculation: Identification cards were inoculated with the microorganism suspension. The microorganism suspension in a test tube was placed into a cassette and the identification card was placed in the adjacent slot, and the transfer tube was inserted into the corresponding suspension tube. The filled cassette was automatically transferred to a vacuum chamber station. After applying the vacuum and re-introducing air into the station, the suspension was forced through the transfer tube into microchannels that fill all the test wells.

Card Sealing and Incubation: The inoculated cards were passed by a mechanism that cut off the transfer tube and sealed the gram-negative card before loading it into the incubator. All card types were incubated at 35.5 ± 1.0ºC. There were 48 biochemical tests and one negative control well. Final identification outcomes were achievable in about 10 hours or less. The list of tested substrates was presented in Table 1.

DNA extraction

The DNA extraction of eleven separate soya extract broth medium of *Yersinia enterocolitica* bacterial has been done by DNA extraction kite (Beta Bayern GmbH .90453 Bayern, Germany).

PCR Amplification using 16S ribosomal RNA (16S rRNA)

PCR amplification for 16S rRNA partial gene was done in 50 µl of reaction mixture containing 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 10 Picomol (pmol) of forward primer 16S(AATACCGCATAACGTCTTCG), 10 pmol reverse primer 16S (CTTCTTCTGCGAGTAACGTC), DNase free water and template DNA (Table 2) using Bioresearch PTC-200 Gradient thermocycler.

The temperature profile included step one is a denaturation at 95˚C for 5 min, step two followed by 35 cycles of denaturation at 95˚C for 35 seconds, an annealing step at 58˚C for 35 sec., an extension at 72˚C for 1 min, and the final step is an extra extension at 72˚C for 10 min. PCR amplification was examined by gel electrophoresis. The location of the bands is determined by examining the gel under UV light.

Sequencing of DNA

Promega Wizard® SV Gel and PCR clean-up system (Promega Corporation, USA) was used to purify PCR products according to the manufacturer's protocol. DNA sequencing was performed to determine the nucleotide sequence viral cap protein region. All PCR products were applied to ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogen Center in Korea. Each species was bidirectionally sequenced to obtain the DNA strand sequence based on the forward primer.

Sequence alignment and submission to GenBank

Sequenced data were investigated for quality using BioEdit v.7.0.5 software. Framshifts, stop codons, insertions-deletions, and homology was investigated through NCBI BLAST. BankIt, a web tool was utilized to guide the submission process. The new sequenced data that was annotated and determined were uploaded to GenBank.

Results

Bacteriological culture and the identity of isolates

In this study, a total of 500 samples of raw milk, soft cheese, ice cream, and meat, including 84 samples of raw milk, 50 samples of soft cheese, 50 samples of ice cream, and 316 samples of meats were randomly bought from different shops in Erbil city. From a total of 500 samples, there were 11 positive isolates from meat depending on isolation on CIN agar, not at 37 ºC, but at 28ºC to 30ºC. There were 11 positive isolates depending on Vitek2 com-

Table 2. 16S rRNA PCR Amplification Reagents.

No.	PCR components	Concentration	Volume (µl)
1	Master Mix	2x	25
2	Forward Primer	10 Pmol	3
3	Reverse Primer	10 Pmol	3
4	DNase free Water	-	15
5	Template DNA	50ng/µl	4
Total			50

pact system. Characteristic growth of *Y.enterocolitica* isolate became visible as a deep red center with a clear border or bull's eye on the CIN medium (Figure 1).

DNA Extraction and PCR amplification

The DNA was isolated and the isolated DNA was electrophoresed in 1% Agarose gel. The results indicated 11 bands of DNA Extracted from Y.*enterocolitica* bacteria (Figure 2).

The primers of the 16S rRNA gene were designed for using the sequences of partial gene available in *Y.enterocolitica* bacteria and synthesized by Macrogen Company (South Korea). The primers o16S rRNA could yield a band size ~320bp after the PCR product was electrophoresed and visualized by 1.5% Agarose gel. The results indicated 11 bands of 16S rRNA of *Y.enterocolitica* bacteria with 320 bp (Figure 3).

Data analysis

Results of edited sequences were analyzed using the BLAST server at the NCBI. GenBank accession numbers of *y. enterocolitica* was represented in Table 3.

Phylogenetic analysis

A phylogenetic tree of eleven sequences (marked Isolated) of *Y.enterocolitica* bacteria was constructed through the maximum likelihood method and bootstrap of 200 using MEGA software v.11 (18). A total of 6 sequences accessible in the NCBI GenBank were applied for the comparison. This evolutionary phylogeny showed that all bacteria originated from the same genus and species (Figure 4).

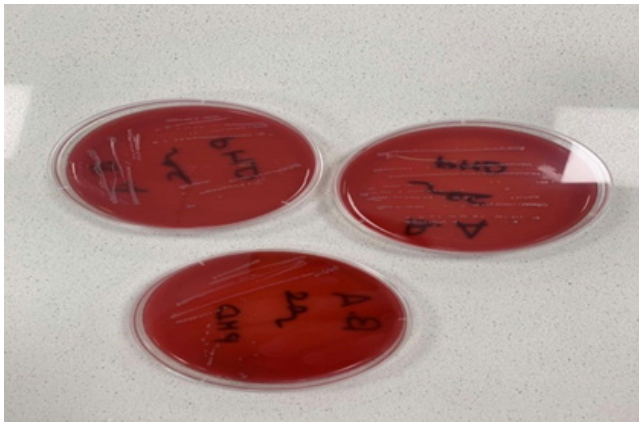

Figure 1. The characteristic growth of y.*enterocolitica* isolate.

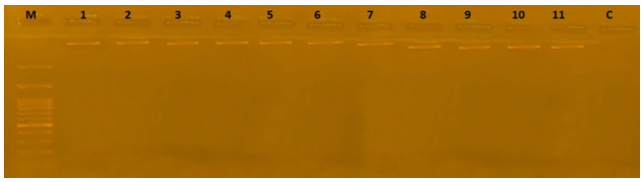

Figure 2. The agarose gel electrophoresis results of isolated DNA (C is negative control and M is the ladder of 3k bp-100 bp).

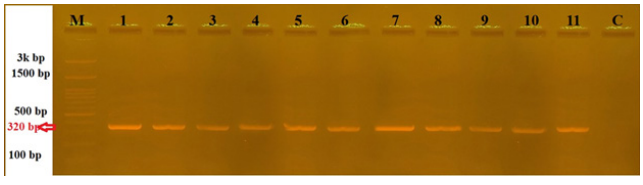

Figure 3. The agarose gel electrophoresis results of PCR product (C is negative control and M is the ladder of 100 bp).

Table 3. GenBank accession no. of *y. enterocolitica.*

Species name	Accession No.
Yersinia enterocolitica	OL685371
Yersinia enterocolitica	OL685372
Yersinia enterocolitica	OL685373
Yersinia enterocolitica	OL685374
Yersinia enterocolitica	OL685375
Yersinia enterocolitica	OM778747
Yersinia enterocolitica	OM778748
Yersinia enterocolitica	OM778749
Yersinia enterocolitica	OM778750
Yersinia enterocolitica	OM778751
Yersinia enterocolitica	OM778752

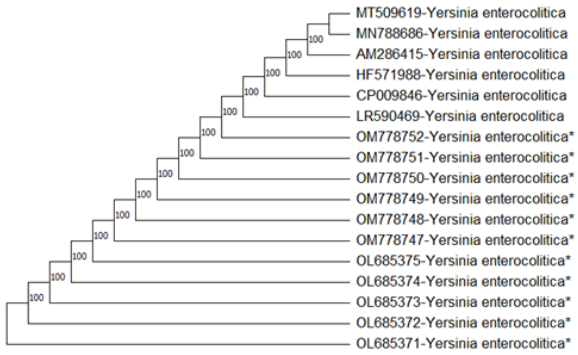

Figure 4. Phylogenetic tree of eleven sequences of *y. enterocolitica* bacteria in this paper (Marked by *) and six other sequences available in the NCBI (On the top of the tree, without any marker).

Discussion

Food contamination with *Yersinia enterocolitica* causes diarrhea, vomiting, and heartache. The main sources of contamination include food (milk, meat and vegetables), water and domestic and wild animals. Sheep was considered as a source of infection with *Yersinia enterocolitica* for other species including humans (19). The bacterial isolates were obtained from 11 out of 500 examined samples in culture media. The low isolation rate of Y. enterocolitica from environmental or food samples may be caused by the limited sensitivity of culture methods. It was confirmed in different studies that a single method is not enough for the trusted identification of *Yersinia enterocolitica.* It is a special challenge for standard biochemical test systems due to its high biochemical similarity and slow growth (20,21). However, conventional biochemical testing was used in this study to confirm the identification of the results of the isolation step. We used the VITEK 2 Compact system which can be considered in clinical microbiology as a first-line technique for the identification of *Y. enterocolitica* isolates (22). The 16S RNA gene PCR amplicon still is the gold standard in microbial identification (23–25).

The results estimated that 5.1% (11/500) out of the whole sample were contaminated with *Y. enterocolitica* depending on PCR amplicon for 16s rRNA gene and the highest isolation parentage was recorded during summer. These results are consistent with the isolation of *Y. enterocolitica* from sheep recorded by Ban and Muhammad (2018) who found a significant increase in the recovery

rate of *Y. enterocolitica* during the cold months as compared with hot months (spring and summer). Outcomes reflect contamination of mincemeat samples with *Y. enterocolitica* in 5.1 percent of cases which is close to their recorded result (26). There have been different studies on the isolation of *Yersinia enterocolitica*. In some studies, the isolation rate of this bacterium is less and in some others it is more than the present study. Also, Piras et al. (2021) detected *Y. enterocolitica* in 4.8% of raw sheep milk samples (27). In a study in Iran, the percentage of contamination of meat types with Yersinia enterocolitica was 4% among 100 meats samples in sheep (28). These findings indicate the existence of complex scenarios of the *Y. enterocolitica* contamination cycle. Contamination may occur at any point from animal farm to markets, shipment of stressed and infected animals, during unhygienic free selling, unknown source of mincemeat, inside abattoirs during slaughtering and evisceration, contaminated utensils, mincing machines, butcher cutting wood surface and knives, and during handling and processing of meat by carrier and infected workers, flies, unclean meat environment, etc. (7,29). Our study applied to the consumer level.

In addition, the difference between the level of contamination in different foods and in different regions of the world is due to several factors, including the methods of isolating bacteria from food, especially in the bacterial enrichment stage, the number of tested samples, the microbial flora and microbial load of the food product, the season and the geographical conditions of the region depend (30).

In summary, about 5.1% of all of the studied samples were contaminated with *Y. enterocolitica*. The analysis of results showed that meat was more contaminated than other samples. Also, evolutionary phylogeny tree of sequenced DNA of *Y. enterocolitica* isolates showed that all bacteria were originated from the same genus and species. According to the obtained results and since this pathogen can be easily transmitted through food and grows easily at 4 degrees Celsius in the refrigerator, it is recommended to pay special attention to this issue in order to avoid health and economic risks.

Acknowledgments
None

Interest conflict
The authors declare no conflict of interest.

Author's contribution
Omar Farooq Yousif and Sawsan M. Sorchee have contributed equally in this research.

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