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Prevalence of Yersinia enterocolitica in raw milk, eggs, Calf and chicken meat in Karbala province

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Abstract **- Yersinia enterocolitica is a foodborne pathogen of added significant public health concern, this study was conducted to detect the prevalence of Yersinia enterocolitica in raw milk, shell eggs, beef, and chicken meat added. 200 samples had been collected from local markets in Karbala province for isolation and identification of Yersinia enteroclolitica . Two methods were used to detect the bacteria: bacterial culture and polymerase chain reaction (PCR). Initial isolation of Y. enterocolitica was performed using Cefsulodin-Irgasan-Novobiocin (CIN) agar medium following enrichment in Peptone Sorbitol Bile Broth. Suspected colonies were confirmed through biochemical tests and polymerase chain reaction (PCR) targeting the 16S rRNA gene. The results of our study have been reveled foodborne pathogen in commonly consumed food products within the Kerbala Provance . A comparison of the accuracy and sensitivity of the two methods was conducted by calculating sensitivity and specificity.**

Results Out of the 200g of tested samples, bacterial growth was observed in (17.5%), with varying prevalence across food types. Raw milk showed the highest contamination rate 34%, while eggs had the lowest prevalence of contamination rate 2%, on the other hand, High sensitivity (88.57%) and specificity (98.18%) were demonstrated by the PCR test when compared to the gold standard culture method

In conclusion: *Yersinia enterocolitica* was successfully isolated from food samples in Kerbala province, highlighting the need for improved food safety measures and The PCR test was found to have high sensitivity (88.57%) and specificity (98.18%) against culture confirmation.

Keywords **- *Yersinia enterocolitica* , Polymerase chain reaction, Ultraviolet radiation.**

INTRODUCTION

Yersinia enterocolitica is a gram-negative, facultative anaerobe that belongs to the family Enterobacteriaceae. It is also considered a significant foodborne pathogen associated with yersiniosis, a gastrointestinal infection that is linked to enterocolitis, mesenteric lymphadenitis, and in a few cases systemic infections (1). The bacteria are often transmitted by eating food contaminated with the bacteria, especially undercooked pork, unpasteurized milk, and contaminated water (2). The clinical symptoms of Y. enterocolitica infection are diarrhea, abdominal pain, fever and vomiting, commonly resembling appendicitis in severe cases, especially in children (3).

The pathogen is a major challenge in food safety and preservation due to its potential ability to grow and multiply at refrigeration temperatures (4). Detection of Y. enterocolitica continues to be problematic because of the use of traditional culture methods which involve tedious growth processes and require specific media like cefsulodin-irgasan-novobiocin (CIN) agar, which highly selective (5). The use of molecular methods, particularly the polymerase chain reaction (PCR), has enhanced the diagnosis of infections by focusing on virulence factors like ail and yadA genes (6). Its potential persistence in the food chain and its tendency to develop resistance to antibiotics necessitates the need yozersiniosis controlling through consistent monitoring and better detection approaches (7). There is limited data on the presence of Y. enterocolitica in various food products in Iraq, particularly in Karbala Governorate. The aim of this study was to determine the prevalence of enteric Yersinia coli in raw milk, egg, beef, and chicken samples collected from local markets in Karbala. The CIN agar selective culture method based on (PCR) amplification of the 16S rRNA gene and molecular techniques were used to detect and confirm the presence Y. enterocolitica. The results of this study will provide valuable information on the potential risk of Y. enterocolitica infection from the consumption of these foods in the region.

MATERIALS AND METHOD

Isolation of Yersinia enterocolitica using CIN medium

Two hundred food samples were randomly collected from local markets, including 50 each of raw milk, shelled eggs, beef, and chicken. The samples were transported to the laboratory under refrigeration within 4 hours. The samples were enriched in peptone-chol broth for 48 hours before initial isolation of Yersinia enterocolitica on CIN (Cefsulodin-Igsan-Novobiocin Agar) medium (Himedia/Indai). Y. enterocolitica colonies on CIN medium were characterized by their reddish-purple appearance surrounded by a transparent halo. Suspected colonies were transferred to confirmatory media and subjected to initial biochemical tests to confirm microbial identity.

Molecular diagnosis of *Y. enterocolitica* by PCR assay

For molecular confirmation, polymerase chain reaction (PCR) was used to detect the specific genes of the bacteria. DNA was extracted from the colonies using a commercial extraction kit, and then PCR was performed using primers specific for the 16S rRNA gene (320 base pairs) and the yst gene (145 base pairs). The reactions were performed in a Thermal Cycler (Biobase/ China), and the reaction products were analyzed by electrophoresis on a 1.5% agarose gel, and the results were photographed using a Gel Documentation device. to evaluate the performance of both the conventional culture method and the PCR technique, both sensitivity and specificity were calculated by comparing the results with reference values, with PCR results considered the gold standard

Sensitivity and specificity of two assay for detection of *Y. enterocolitica* :

The results of the conventional culture method were compared with the PCR results to calculate the sensitivity and specificity of each method, by organizing a confusion matrix that includes the four basic values: true positive, true negative, false positive, and false negative. The PCR results were used as the gold reference for comparison.

Statistical analysis:

The resulting data were analyzed using the chi-square test to determine the presence of statistically significant differences between the detection rates in different food samples. A statistical significance level of ($P < 0.05$) was used as the threshold for accepting or rejecting the null hypothesis. Degrees of freedom (DF) were calculated based on the number of food groups under stud

RESULTS AND DISCUSSION

The study tested 200 different food samples, including raw milk, eggshells, beef, and chicken, for the presence of Y. enterocolitica bacteria. The results showed that raw milk samples recorded the highest level of contamination, with the bacteria found in 34% of samples. This may indicate possible contamination of the milk during milking or storage, especially if it was not heat-treated. The average contamination level in chicken samples was 20%, followed by beef samples at 14%. These results are believed to indicate the potential for contamination of these products at various stages of slaughter or processing. In some cases, the presence of the bacteria may have resulted from improper cooking or refrigeration . Eggshell samples recorded the lowest level of contamination, with the bacteria found in only 2% of samples. This may indicate the infrequent contamination in these samples, or the effectiveness of eggshells in protecting their internal contents from infection . Statistical analysis was performed using the chi-square test, and the results showed significant differences in infection rates across different food types ($X^2 = 18.39$, $DF = 3$, $P < 0.05$). as in Table (1): showed number and percentage of Y.enterocolitica in various food samples.

Table 1. showed number and percentage of *Y.enterocolitica* in various food samples

Food samples	No.	No. of Positive	%
Raw Milk	50	17	34
Egg	50	1	2
Beef	50	7	14
Chicken	50	10	20
Total	200	35	17.50%
Statistical analysis	X^2= 18.39 ; DF= 3; P < 0.05		

Isolation and identification of Y.enterocolitica by using culture assay

Initial isolates of Yersinia coli were obtained from a variety of food samples, including raw milk, beef, eggshells, and chicken. Selective CIN medium was used in this procedure due to its strong ability to inhibit the growth of unwanted colonies and allow the growth of target colonies. Colonies grown in CIN medium exhibit unique characteristics, including a smooth, rounded appearance with regular edges and a distinctive reddish-purple color with a dark center. This color is due to the bacteria's ability to ferment mannitol in the medium and produce acid, which discolors the reagent. This appearance is a typical diagnostic feature of Yersinia coli and has been confirmed by numerous previous studies. In addition, the growth rates of milk

and chicken samples were also higher than those of beef and egg samples in this medium, which may reflect differences in the source of contamination and storage conditions. This observation suggests that raw milk and poultry may be more suitable for the growth of this bacterium under unsanitary conditions (Figure 1).

Figure 1. colonies of *Y.enterocolitica* on CIN agar medium

Molecular assay for detection of *Y.enterocolitica*

Polymerase chain reaction (PCR) was used to detect Yersinia enterocolitica in 200 food samples. The results showed that 34 samples were positive, representing a 17% test rate. This technique relied on detecting two specific genes: the 16S rRNA gene, used for general identification of the bacteria, with the resulting band appearing at 320 base pairs, and the virulence gene yst, responsible for producing an enterotoxin, with its band appearing at 145 base pairs. These results were visually documented through gel electrophoresis, as shown in the figure 2.

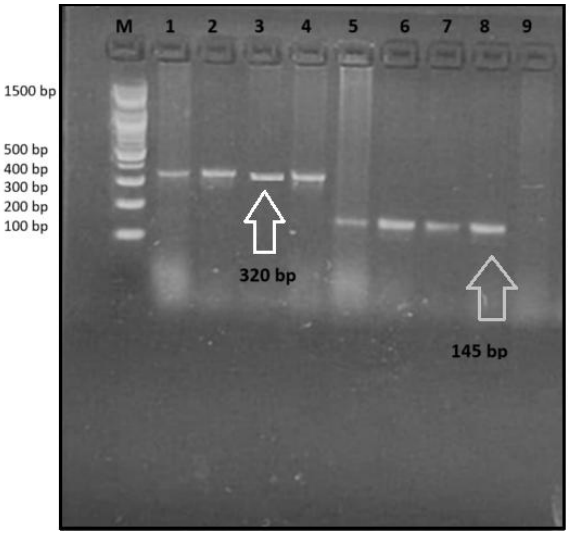

Figure 2. electrophoresis gel for detection of Y. enterocolitica; M DNA ladder marker; 1-4 wells represented 16SRNA as 320 bp and 5-8 wells represented Yst gene as 145 bp and 9 well represented control negative sample

The results of a comparison between bacterial culture and PCR for the diagnosis of Yersinia enterocolitica showed a sensitivity of 88.57%, indicating a high ability of the method to detect positive cases. The confidence interval for the sensitivity ranged between 73.26% and 96.80%, reflecting an acceptable degree of reliability in estimating this diagnostic indicator. The specificity of the test was recorded at 98.18%, a high percentage indicating the accuracy of the method in excluding negative cases. The confidence interval for this value was between 94.78% and 99.62%, enhancing the reliability of the results obtained. The positive predictive value (PPV) was also calculated and reached 91.18%, indicating that the majority of positive results were correct. The confidence interval for the PPV ranged between 76.99% and 96.96%. The negative predictive value (NPV) reached 97.59%, indicating high effectiveness in ensuring the absence of infection in cases with negative outcomes. The confidence interval for this value ranged between 94.15% and 99.03% table 2.

Table 2. Comparsion between Culture and PCR assay for detection of *Yersinia enterocolitica*

PCR Test Result	Disease Present (by Gold Standard)	Disease Absent (by Gold Standard)	Total
Positive	True Positive = 31	False Positive = 3	34
Negative	False Negative = 4	True Negative = 162	166
Total	35	165	200

The results of the current study on the prevalence of Yersinia enterocolitica in raw milk, eggshells, beef, and chicken were compared with previous studies from Iraq, neighboring countries, and other countries worldwide to identify similarities and differences in infection rates, regarding to raw milk, the current study showed an infection rate of 34%. These results were partially consistent with those recorded in a study conducted in Iraq, where the bacteria were detected in 12% of raw milk samples using both conventional and PCR methods, with evidence of virulence genes such as ail and inv. The difference in rates is attributed to differences in local production conditions and testing techniques (8).

In terms of meat, the infection rate was 14% for beef and 20% for chicken. This result is similar to that of a study conducted in Egypt, which showed infection rates of 15.83% for chicken and 10% for beef, with evidence of antibiotic resistance genes, suggesting similar sources of contamination in meat and slaughter methods (9).

As for eggshells, the infection rate reported in this study was low at 2%. Although research in this area is limited, a study conducted in Argentina showed a higher infection rate of 38.65%, suggesting that such samples may be contaminated, although the degree of contamination varies by region and environmental conditions (10). These comparisons indicate that the infection rate of Y. enterocolitica varies by food type and geographical location. area, which highlights the importance of strengthening food safety and quality control measures at all stages of production and distribution to reduce the risk of contamination and the spread of food poisoning. Laboratory results of the isolation of Y. enterocolitica from various foods using CIN medium were compared with previous studies conducted in several countries to determine similarities and differences in colony characteristics and the effectiveness of the culture medium used. A study by (11) developed a CIN medium for the selective isolation of Yersinia enterocolitica from food samples. Colonies on this medium show a characteristic growth pattern. The colonies are small and flat, with a dark red center surrounded by a clear halo called a "bull's eye." This color is due to the acid produced during fermentation of mannitol, which changes the color of the indicator in the medium. This medium is considered effective for distinguishing Yersinia enterocolitica from other enteric bacteria (12).

On the other hand, (13) noted that CIN medium, although effective, may not be sufficient to differentiate Y. enterocolitica from some other mannitol-fermenting bacteria, such as Serratia liquefaciens and Enterobacter agglomerans. Therefore, he recommended the use of additional tests, such as the escin test and other biochemical tests, to improve diagnostic accuracy (14). In a recent study, (11) evaluated the effectiveness of CIN medium after enriching it with ITC broth. The results showed that this method improved the positive bacterial isolation rate in porcine tonsil samples, detecting bacteria in 14% of samples, compared to 9.1% obtained with conventional methods (15). While (16) also noted that CIN medium contains components that inhibit the growth of harmful bacteria, such as crystal violet and bile salts, which increases the selectivity of the medium for Y. enterocolitica. However, some other bacteria may exhibit similar colonies, so additional confirmatory testing is required (17). The results of the polymerase chain reaction (PCR) test for Yersinia enterocolitica were compared with previous studies conducted in different regions to identify similarities andifferences in the effectiveness of bacterial detection techniques in food samples. In a study conducted in Iran, PCR targeting the 16S rRNA gene was used to detect Yersinia enterocolitica in chicken samples. The results showed that 25% of samples were positive, and all positive isolates were identified as belonging to biotype 1A. Studies have shown that an enrichment step prior to PCR can improve detection rates, especially when food samples contain high levels of background bacteria 15). Another study from Italy used real-time PCR to detect the A1 virulence gene in porcine tonsil samples. The results showed that PCR had a higher detection rate than conventional methods, detecting bacteria in 9.8% of samples, while the detection rate in culture was lower. Studies have confirmed that PCR has higher sensitivity, especially in samples with low bacterial counts (18).

The results of Yersinia enterocolitica detection using polymerase chain reaction (PCR) were compared with previous studies from different regions to identify similarities and differences in the effectiveness of this technique in detecting bacteria in food samples. In a study conducted in Iran, PCR targeting the 16S rRNA gene was used to detect Y. enterocolitica in chicken meat samples. The results showed that 25% of samples were positive, with all positive isolates identified as belonging to biotype 1A. The study indicated that the enrichment step prior to PCR contributed to improved detection rates, especially in the presence of large numbers of background bacteria in food samples (19). Another study in Italy used real-time PCR to detect the ail virulence gene in pig tonsil samples. The results showed that the detection rate using PCR was higher than traditional methods, with the bacteria detected in 9.8% of samples using PCR, compared to lower rates using cultural methods. The study confirmed that PCR technology provides higher sensitivity, especially in samples containing low numbers of bacteria (20).

CONCLUSION:

In a study conducted in Iraq, a conventional PCR method was developed and tested for the detection of Y. enterocolitica in various food samples. The method demonstrated high sensitivity, detecting as few as 10 bacterial cells in each PCR reaction. The study also showed that the method was accurate and reliable, with no false positive or negative results when tested on a variety of bacterial strains .

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