PREVALENCE, ANTIBIOTIC SUSCEPTIBILITY, AND VIRULENCE FACTORS OF *YERSINIA ENTEROCOLITICA* ISOLATED FROM RAW MILK IN BASRAH, IRAQ

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**Summary**


A total of one hundred and fifty cow, buffalo, and sheep milk samples were collected from several markets in Basrah, Iraq (50 samples from each species). All milk samples were subjected to enrichment in TSB and cold enrichment in PBS, then cultured on YSA agar in order to obtain *Yersinia* species. The highest growth obtained by TSB enrichment was seen in cow milk (24%) followed by buffalo milk (22%) and sheep milk (12%). The results of PBS enrichment showed the highest growth in cow milk (14%) followed by buffalo (8%) and sheep milk (2%). The results showed that both cow and buffalo milk samples were contaminated by *Y. enterocolitica* at 8% while the prevalence in sheep milk was 4%. Ten isolates from different sources in the current study were examined for their susceptibility to 10 antibiotics. The highest susceptibility (100%) was found towards streptomycin, azithromycin and gentamicin, followed by ciprofloxacin and chloramphenicol, 93.3% for each. Low susceptibility was found toward vancomycin (6.66%) and cloxacillin (33.3%). 16S rRNA sequencing showed homology with previously annotated strains at GenBank of National Centre for Biotechnology (NCBI). Multiple sequence alignments exhibited one difference between the sequences at the locus 764. The phylogenetic tree of the results demonstrated that the local isolates were closely related to strains previously reported from China. All *Yersinia enterocolitica* strains had the *inv* gene. In contrast, the *ail* gene was found in one strain (10%) while the *yad* gene appeared in 50% of the investigated strains.

**Key words**: Basrah, buffaloes, cow, milk, sheep, virulence genes, *Yersinia*

**INTRODUCTION**

*Yersinia enterocolitica* is a Gram-negative, motile, oxidase-negative and facultatively anaerobic coccobacillus. The colonies are dark and red centered (“bulls eye like”) with transparent border on CIN agar. All bacteria of the genus *Yersinia* are catalase-positive, non-spore-forming rods or coccobacilli of 0.5–0.8 × 1–3 µm in size. Strains of *Y. enterocolitica* are urease positive. Most of the strains are motile at 25 °C but non-motile at 37 °C (Cornelis, 1998). These microorganisms
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are psychrotrophic milk-borne enteric pathogens; widely spread in the environment and indigenous to the gastrointestinal tract of warm-blooded animals including dairy cattle (Marshall, 1992).

Chromosome-encoded factors are also needed for pathogenicity. Adherence and invasion to epithelial layers require at least two chromosomal genes, inv (invasion) and ail (attachment invasion locus) (Miller & Falkow, 1988). The invasion (inv) codes for Inv, an outer membrane protein found on the surface of Yersinia, which appears to play a vital role in promoting entry into epithelial cells of the ileum during the initial stage of infection, that is responsible for binding to β1-integrins on the apical surface of M cells and initiating uptake of the organism (Schulte et al., 2000). The attachment invasion locus (ail) codes for the surface protein Ail, which is produced at 37 °C. In contrast to the inv, the ail was shown to be restricted only to serotypes associated with the disease (Jourdan et al., 2000). The presence of pYV plasmid (Yersinia virulence) with approximately 70,000 bp is also one of the basic indicators of virulence. This plasmid encodes YadA adhesin (i.e., the protein of the external membrane that allows adhering to the host cells), Yop outer proteins, which paralyses the immune system, and Ysc proteins composing the secretion system (Bergann et al., 1995; Selma et al., 2006).

Foods of animal origin pose a higher risk of gastrointestinal disease caused by Y. enterocolitica in humans. Milk and dairy products are the main consumed foods of animal origin. In addition, several studies have reported the presence of Y. enterocolitica in milk and dairy products (Rahimi et al., 2013). Antibiotics are commonly used to treat cattle disease especially mastitis in dairy cows and their indiscriminate use leads to the development of multidrug-resistant bacteria rendering antibiotic treatment ineffective (Sadek et al., 2014). Antibiotic therapy for the treatment of yersiniosis in humans is not indicated except in systemic and extraintestinal infection and enterocolitis in immunocompromised patients (Mayrhofer et al., 2004). The emergence of antimicrobial resistance leads to treatment failures and obligate utilisation of expensive and/or toxic alternative drugs (WHO, 2007). The spread of drug resistance among Y. enterocolitica is also of concern for public health. To date, investigation of microbial pathogens in milk in the studied area are available for Brucella (Abbas & Aldeewan, 2009; Abbas & Talei, 2010); E. coli (Abbas et al., 2012a; Abbas, 2013; Abbas et al., 2013a); Staphylococcus aureus (Abbas et al., 2013b; 2014a; 2016), Listeria monocytogenes (Abbas & Jaber, 2012) Bacillus cereus (Abbas et al., 2012b, 2014b). None of these investigations concern Yersinia sp. (Abbas et al., 2017). Therefore, the current study aimed to evaluate the prevalence and resistance of Y. enterocolitica in milk samples from three ruminant species.

MATERIALS AND METHODS

Sample collection

In order to obtain Yersinia isolates, 150 milk samples were collected between October 2017 and February 2018. A total of 50 samples from cows, buffaloes and sheep were collected from several markets in different parts of Basrah province. All samples were transported in an ice box and kept in the refrigerator in plastic bags until use.

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Bacterial isolation

One mL of milk sample was transferred aseptically to 9 mL of Tryptone Soya Broth tubes and incubated at 25 °C for 2 days. For the cold enrichment method, 1 mL of milk was directly inoculated into 9 mL PBS tubes. Tubes were incubated for 21 days at 4 ºC (Green Wood & Hooper, 1989; Baharathy et al., 2015). A loopful of cultures were streaked on Yersinia selective agar (YSA) plates and incubated for 48 h at 25°C.

The dark red colonies surrounded by a transparent border that resembled bull eye were considered as presumptive Y. enterocolitica. Gram stained slides were prepared for these colonies. Yersinia appeared as Gram negative non-spore forming rods or coccobacilli. Biochemical tests including catalase test, oxidase test, Kligler’s iron agar test, motility test and indol test were done.

Extraction of bacterial DNA and PCR amplification

For PCR studies, genomic DNA was extracted from bacterial cultures using a commercial kit, and following the protocol provided by the manufacturer (Gene-Aid). Extracted DNA was stored at –20°C until used. Primers used for investigation of 16Sr RNA, ail, yadA and inv genes of Y. enterocolitica are listed in Table 1.

The detection of the PCR amplified products was done by electrophoresis on 1% agarose gel. DNA ladder marker was used to measure the molecular weight of the PCR products. After 30 min, the PCR amplification products were examined under UV light.

DNA sequencing

DNA sequencing of 16s rRNA was done by sending the PCR products for Macrogen Company, South Korea. Basic local Alignment search tool (blast) sequence analysis was performed by blast algorithm for the sequenced results at National Center for Biotechnology. The website also used for generating a phylogenetic tree and multi-sequence alignments.

Antibiotic susceptibility test of Y. enterocolitica isolates

The testing for susceptibility to antibiotics was conducted according to the method of Bauer et al. (1966). The antibiotics discs were dispensed onto inoculated Mueller-Hinton plates. The plates were inverted and placed in an incubator at 37 °C for 24 h. Inhibition zones were measured in millimeters using a transparent ruler.

Table 1. PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ail</td>
<td>F: TGGTTATGCAGCAAAGCCATGT R: TGGAGTCGAGGTGATGCA</td>
<td>356</td>
<td>Feng et al., 1992</td>
</tr>
<tr>
<td>yadA</td>
<td>F: TAAAGACATGTTCTTGGCGGCA R: TAGTTATTTGCGATCCCCGACCA</td>
<td>747</td>
<td>Kapperud et al., 1993</td>
</tr>
<tr>
<td>inv</td>
<td>F: CAGTGACGGCTCAAGTAACTTG R: CCGTTCCTCCAATGTAGGTATCC</td>
<td>183</td>
<td>Thoerner et al., 2003</td>
</tr>
<tr>
<td>16s RNA</td>
<td>F: AGATTTGGATCCTTGGGTCAG R: GGTACCTTGGTACGACTT</td>
<td>1500</td>
<td>Eden et al., 1991</td>
</tr>
</tbody>
</table>
diameter of the inhibition zone was calculated from the underside of the dish. The results are compared with the minimum inhibition diameter of Bioanalyse Co. (Turkey).

RESULTS

Bacterial isolation

All milk samples were subjected to enrichment in TSB and cold enrichment in PBS, then cultured on YSA agar in order to obtain Yersinia species. Multiple types of colonies appeared on YSA which showed less selectivity. The presumptive Yersinia enterocolitica colonies isolated from selective medium were subjected to further investigation. Dark and red centered colonies with transparent “bulls eye like” border on YSA were picked and streaked individually on the same agar. By Gram staining, the smears showed Gram negative cocobacilli or different irregular shapes. The results of Yersinia enterocolitica biochemical tests showed that this bacterium was catalase positive, oxidase negative, non motile at 37 °C but motile at 25 °C.

The results of bacterial isolation and identification are presented in Table 2. According to TSB enrichment results, the highest prevalence with this method was seen in cow milk (24%) followed by buffalo milk (22%) and sheep milk (12%). The results of PBS enrichment showed the highest prevalence in cow milk (14%) followed by buffalo milk (8%) and sheep milk (2%). All colonies on selective agar with bull eye appearance were subjected to biochemical identification. The results of milk samples showed that both cow and buffalo milk were infected with Y. enterocolitica at 8% followed by sheep milk at 4%.

Antibiotic susceptibility of Y. enterocolitica isolates

In this study, ten isolates from different sources were examined for their susceptibility to 10 antibiotics. The highest susceptibility was found toward streptomycin, azithromycin and gentamicin (100% for each), followed by both ciprofloxacin and chloramphenicol (93.3%). A low susceptibility was demonstrated toward vancomycin (6.66%) and cloxacillin (33.3%) (Table 3).

Identification of strains using 16S rRNA

The DNA was extracted from 10 bacterial strains isolated from milk. DNA concentration and purity were determined by nanodrop 1000 spectrophotometer at 260/280 nm.

Table 2. Number and percentage of milk samples that showed bacterial growth on YSA medium after TSB and PBS enrichments

<table>
<thead>
<tr>
<th>Sample types (number)</th>
<th>TSB enrichments</th>
<th>PBS enrichments</th>
<th>Confirmed Y. enterocolitica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow milk (50)</td>
<td>12 (24%)</td>
<td>7 (14%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Buffalo milk (50)</td>
<td>11 (22%)</td>
<td>4 (8%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Sheep milk (50)</td>
<td>6 (12%)</td>
<td>1 (2%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Total (150)</td>
<td>29 (19.3%)</td>
<td>12 (8%)</td>
<td>10 (6.66%)</td>
</tr>
</tbody>
</table>
All strains were subjected to PCR using 16S rRNA. The PCR results in all strains showed a band size of 1,500 bp (Fig. 1). Three *Yersinia enterocolitica* strains were selected randomly for DNA sequencing. The results of sequencing and related accession numbers in the GenBank are listed in Table 4.

Multiple sequence alignment was done for three strains in the present study (DB30, DB34 and DB37) with two GenBank sequences (JX855135.1 and JX424036.1). Results showed that there was one change between the sequences at the locus 764 (Fig. 2). The phylogenetic tree of the results showed that the local isolates were closely related to those strains previously registered from China (Fig. 3).
Virulence genes

The result of polymerase chain reaction for invasion (inv) gene, Yersinia adhesion A (yadA) and attachment invasion locus (ail) virulence genes showed that all Y. enterocolitica strains had the inv gene (100%) in their genome. In contrast, the ail gene was found in one strain only (10%) while the yad gene appeared in 50% of the investigated strains (Table 5).

DISCUSSION

Milk is a complete food, especially for children and seniors due to high content of proteins, minerals, fats, and vitamins. It is the primary source of nutrients for young mammals before they can digest other types of foods. That is why milk contamination is an important source for infections and illness (Rahimi, 2014).

Yersinia spp, especially Y. enterocolitica comprised strains with different degree of pathogenicity. In 2007, there were 8,792 reported cases of human yersiniosis in the European Union, making Yersinia the third most important zoonotic agent implicated in human enteritis, in terms of the number of cases, after Campylobacter and Salmonella (EFSA-ECDC, 2012; Tan et al., 2014). Y. enterocolitica has a spe-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total strains</th>
<th>Positive result</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inv</td>
<td>10</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>Yad</td>
<td>10</td>
<td>5</td>
<td>50%</td>
</tr>
<tr>
<td>Ail</td>
<td>10</td>
<td>1</td>
<td>10%</td>
</tr>
</tbody>
</table>
cial public health importance, because of its capability of growing in raw milk and viability at refrigeration temperatures for a long time. Therefore, the consumption of milk and dairy products especially raw ones gives a higher chance for infection with *Y. enterocolitica* in humans (Rahimi et al., 2014).

In this study all bacteria recovered after cold enrichment for 21 days and after TSB enrichment for overnight incubation at 30 °C. The psychrotrophic nature of *Y. enterocolitica* is unusual among other Enterobacteriaceae. Consequently, enrichment in different solutions at 4 °C for prolonged periods is used for isolation of *Yersinia* spp. However, the time needed for this method is a disadvantage for routine analysis. In buffalo milk 22% and 8% were enriched by TSB and PBS. Both results were higher than those of Gamal Eldin (2008) – 7% and Khairalla (2006) – 6%. In cow milk results were lower than rate of 39.1%, detected by Askar et al. (2013) and higher than that detected by Ruusunen et al. (2013) and El-Prince & Elsyad (2002). The prevalence in sheep milk was 12% but *Y. enterocolitica* were not isolated by El-Leboudy & Gamel (1994). In a study in Morocco (Hamama et al., 1992), *Yersinia* spp. was recovered from 11 of 30 raw milk (36.6%), 1 of 20 pasteurised milk (5%), 15 of 63 traditional fermented milk (23.8%), 7 of 94 cheeses (7.44%), and 1 of 20 cream samples (5%). The overall incidence of *Y. enterocolitica* in milk and dairy products at previous study was 6.6%, which was similar to our results (6.66%). Another study showed that the prevalence of *Y. enterocolitica* was 24.1% in raw buffalo milk; however, no isolation was made from the pasteurised milk samples (Toora et al., 1989). Sheep usually have a mass of fat attached to the back of their rump and it is in touch with faecal contamination. Thus sheep milk is likely to be contaminated by faeces during milking. This contamination is different than that observed in the survey previously conducted in Iran and other countries on several kinds of dairy products. In Turkey, 47.3% prevalence of *Y. enterocolitica* contamination was observed (Yucel & Ulusoy, 2006). In Mexico, Bernardino-Varo et al., (2012) reported a 34.92% prevalence of *Y. enterocolitica* contamination. In another study 9 sheep milk samples (9%) were positive for *Y. enterocolitica*. In Iran, (Jamali et al., 2015) showed that *Y. enterocolitica* contamination of sheep milk samples was 3% and that of goat milk samples was 2.4%.

Virulence genes, such as *ail* and *yst*, are located on the bacterial chromosome. The Ail protein is encoded by the *ail* gene and occurs only in pathogenic *Y. enterocolitica*; it helps the bacterial adhesion to the host cell as well as intensifies resistance to the bactericidal effects of complement (Hanifian & Khani, 2012; Jamali et al., 2015). In this study the result of presence of virulence genes differ from research data obtained by Saberianpour et al. (2014). In that study, multiplex PCR assay results showed that chromosomal virulence genes included *inv* (100%), *ail* (50%) and *ystA* (51.85%), and plasmid-encoded virulence factors included *yadA* (44.74%) and *virF* (35.18%). Those results agree with prevalence of *inv* gene in the present study (100%), and was lower than prevalence of *yadA* (26%). In another study, the *ail* gene was found in 100% of pathogenic *Y. enterocolitica* strains and the *yadA* gene – in only 86% of pathogenic *Y. enterocolitica* strains, but neither of the genes were detected in nonpathogenic strains of *Yersinia* spp. (Blais & Phillippe, 1995). In a previous research
multiplex PCR test results showed the *ail* gene found in 4 (4%) isolates, the *yadA* factor in 3 (3%), the *virF* and *ystA* genes in 2 (2%) of *Y. enterocolitica* strains while in present study, *Inv* gene found in 100% of the strains, *YadA* in 50%, and *ail* gene in 10% of the investigated strains. However, the *ail* gene was only detected from biotype 4/O:3 and all isolates of *Y. enterocolitica* biotype 1A only harboured the *ystB* gene and the *ystA* gene was not observed in all isolates. Hanifian & Khani (2012) reported 2.26% isolation of the *ail* gene of raw milk and cheese samples.

In this study all tested isolates were susceptible to streptomycin, azithromycin and gentamycin, 93.3% – to ciprofloxacin and chloramphenicol. Only fewe were susceptible to vancomycin (6.66%) and cloxacillin (33.3%). These rates differ from results of Soltan-Dallal et al. (2004) who reported that all samples were resistant to ampicillin and sensitive to tetracycline. In another study in Sardinia, all isolates were susceptible to cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, nalidixic acid, sulphonamide, tetracycline and trimethoprim-sulphamethoxazole. Resistances to ampicillin and cefalothin were the most common (100%), followed by amoxicillin/clavulanic acid (83.0%) and streptomycin (4.3%).

**CONCLUSION**

On the basis of presented results, we conclude that the higher occurrence of *Yersinia enterocolitica* in cow milk, cow cheese samples in comparison with buffaloes milk, buffaloes cheese, sheep milk and sheep cheese samples. The isolated bacteria containing virulence genes and enterotoxin gene that helps in pathogenicity of these bacteria which showed resistance toward some commonly used antibiotics.

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