ISOLATION AND MOLECULAR IDENTIFICATION OF YERSINIA ENTEROCOLITICA IN SHEEP IN SOUTH REGION OF IRAQ

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ABSTRACT
The study was conducted to find the infection rate of Yersinia enterocolitica in sheep in the south region of Iraq, based on molecular identification of the bacteria and specific three virulence genes ail, inv, yst A. A total 1200 fecal samples were collected randomly from sheep in the period from July 2016 to June 2017. Fecal samples were cultured by using cold enrichment Yersinia broth and selective media agar (Cefsulodin Irgasan Novobiocin (CIN). The suspected colonies were subjected to PCR technique for identification of the encoding targeting gene 16s rRNA, and three virulent genes: ail, inv, yst A. Sequencing had been carried out for all identified genes for 12 isolates to discriminate the pathogenic strains. The total rate of infection was 5.16%. A successful amplification of 16s rRNA gene was done at 1485 bp fragment. Seven isolates showed 100% compatibility, the bite score 1663 and expect 0.0 with standard gene of Gene Bank, five isolates showed 99% compatibility with variant transition and transversion in the several nucleotide regions. The existence rate of the three virulence genes was 77.41%, the unique ail, inv, yst A genes were present in the Yersinia isolates at rates: 82.25%, 100%, 90.3% respectively, the variations in sequences of the ail, inv, yst A genes from that strain at the Gene Bank were recorded. It was concluded that sheep may act as a potential source of infection to human and other animal species and the variations in genotype within the same strain of Y. enterocolitica were present among isolates from different localities.

KEYWORDS: CIN, Yersinia isolates, molecular, identification.

INTRODUCTION
Yersinia enterocolitica was discovered by Schleifstein and Coleman in 1939, but publications were delayed until 1960s (Schiemann, 1982). It is considered the most common zoonotic disease after salmonellosis and compaylo-bacteriosis in European Union (EFSA, 2012). Yersiniosis is increased in the last 2 decades in human (Fredriksson-Ahomaa and Korkeala, 2003). Beside to the enteric infection, Y. enterocolitica may infect many organs and tissues causing different diseased conditions (Bin-Sagheer et al., 1997). Yersinia enterocolitica has ability to grow and multiply in cold environment (refrigerator), so it is mostly responsible for food borne illness (Ray et al., 2004). The epidemiological of Y. enterocolitica is complex and remains poorly understood (Fredriksson-Ahomaa et al., 2006). The clinical signs in human patient mainly consist of diarrhea, fever and abdominal pain (Savin et al., 2012), whereas in sheep fever, diarrhea, dehydration and weight loss (Sakai et al., 2005). However the severity of infection in animals varies according to host age, management, breed and virulence of pathogen (Bottone, 1977). The virulence of Y. enterocolitica is depending on existence of genes in both chromosomes and plasmids, besides that the virulence genes had been used widely in identification of pathogenic strains (Nakajima et al., 1992), also the pathogenic Y. enterocolitica that infects human being, had been isolated from sheep in Great Britain (McNally et al., 2004), the genotype relation –ship between human and sheep strains, might indicate sheep act as potential source of infection. Furthermore, indistinguishable genotypes had also been found between human and strains isolated from other animal species (dogs, cats, sheep and wild rodents’), indicating these species are possibly considered other sources of infection (McCarthy and Fenwick, 1990). The rapid and accurate methods are required for detecting pathogenic Y. enterocolitica in natural samples; several DNA-based methods had been used for identifying presumptive types of Y. enterocolitica (Sachdeva and Virdi, 2004). A duplex-PCR assay was designed simultaneously to detect Y. enterocolitica and discriminate pathogenic and nonpathogenic strains (Aarts et al., 2001). Also a colony hybridization method by using probes targeting the chromosomal ail and inv genes were developed (Goverde et al., 1994). Moreover amplification of rfbC gene of Y. enterocolitica O: 3 in fecal sample had been achieved (Weynants et al., 1996). In addition, it was reported that the TaqMan assay is more sensitive than traditional PCR assay (Boyapalle et al., 2001). Because of possible plasmid loss on subculture and storage, PCR methods for targeting chromosomal virulence genes had also been created for natural samples (Falcao et al., 2004). The ail gene, located in the chromosome of pathogenic Y. enterocolitica strains, is the most frequently used as target. Furthermore, some PCR assays had been designed to detect the inv and yst A genes also. However, a PCR method targeting the 16s rRNA gene combined with sequencing had been described (Neubauer et al., 2000). In addition several studies had been conducted to investigate the distribution of different virulence genes (ail, inv, yst,
Isolation and molecular identification of *Yersinia enterocolitica* in sheep

*yadA, virF* and *yopT* among *Y. enterocolitica* strains by PCR (Gürtler et al., 2005). In the field, PCR is considered the most reliable and plausible method for detecting nucleic acids in a variety of samples, due to high specificity and sensitivity in addition to save time and labor consumed (Fredriksson-Ahomaa and Korkeala, 2003). Sequencing allows the cataloguing of all genetic variables, providing knowledge about bacterial pathogenicity and help for better understand the origin and spread of microbial diseases (Kotetishvili et al., 2005). The present work was aimed to detect the pathogenic *Yersinia enterocolitica* in sheep by amplification of virulence genes, in the south region of Iraq and study the sequences of some virulence genes in many selected isolates.

**MATERIALS & METHODS**

**Primers and PCR amplification condition**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer name</th>
<th>Sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16srRNA</td>
<td></td>
<td>GGTTACCTTGTTACGACTT</td>
<td>Srinivasan et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGTTACCTTGTTACGACTT</td>
<td></td>
</tr>
<tr>
<td>Ail</td>
<td>Ail1</td>
<td>ACT CGA TGA TAA CTG GGG AG</td>
<td>Falcao et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Ail2</td>
<td>CCC CCA GTA ATC CAT AAA GG</td>
<td></td>
</tr>
<tr>
<td>Inv</td>
<td>YC1</td>
<td>CTG TGG GGA GAG TGG GGAAGT TTGG</td>
<td>Falcao et al., 2004</td>
</tr>
<tr>
<td></td>
<td>YC2</td>
<td>GAA CTG CTT GAA TCC CTGAAA ACCG</td>
<td></td>
</tr>
<tr>
<td>yst</td>
<td>Pr2a</td>
<td>A ATG CTG TCT TCA 1TT GGA GCA</td>
<td>Falcao et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Pr2c</td>
<td>ATC CCA ATC ACT ACT GAC TTC</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1:** Primers used in this study to detect the 16srRNA, *ail, inv* and *yst* genes in *Y. enterocolitica*

**TABLE 2:** 16s rRNA gene was amplified by PCR using the following condition.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Tm (°C)</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C</td>
<td>1 min</td>
<td>35 cycle</td>
</tr>
<tr>
<td>Extension-1</td>
<td>72°C</td>
<td>1:40hour</td>
<td></td>
</tr>
<tr>
<td>Extension-2</td>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 3:** *ail, inv* and *yst A* genes were amplified by PCR using the following condition.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Tm (°C)</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>45 sec</td>
<td>35 cycle</td>
</tr>
<tr>
<td>Extension-1</td>
<td>72°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Extension-2</td>
<td>72°C</td>
<td>7 min</td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were separated by 5% agarose gel electrophoresis and visualized by exposure to UV light after staining with a red stain.

**PCR products sequence analysis**

Sequencing and sequence alignment of 16s rRNA, *ail, inv, yst A* genes were performed by Macro gene company, USA. Homology search was conducted using Basic Alignment Search Tool (BLAST) program which is available at National Center Biotechnology Information (NCBI) online at http:// www.ncbi.nlm.nih.gov and BioEdit program. The results were compared with the data obtained from Gene Bank published ExPASY program which is available at the NCBI online.

One thousand and 200 fecal swabs were taken randomly from sheep in the south region of Iraq: (Thi-Qar, Basrah, Missan, Al-Muthana governorates), in the period from July 2016 to June 2017. *Yersinia* enrichment broth (pH 7.4) were added to each sample and kept at 4°C for 48 hours. Culturing was done by streaking on selective Cefsulodin Irgasan Novobiocin (CIN) agar and incubated at 25°C for 48 hours (Van Noyen et al., 1981). The *Y. enterocolitica* strains were identified by PCR for presence of 16srRNA gene and for more confirmation, *ail, inv, yst A* genes to discriminate pathogenic from non pathogenic strains. Twelve isolates were selected for sequencing study on the base of presence three virulence genes and severity of clinical signs that appeared on the infected sheep.

**DNA extraction**

DNA was extracted from fresh *Y. enterocolitica* colonies, using G- spin DNA extraction kit (Intron, Korea), according to the manufactured company instructions.

**Statistical analysis**

Data were analyzed statistically by using (SAS) version 9.1. Chi- square test was used for comparison (SAS, 2010).

**RESULTS**

Suspected isolates of *Y. enterocolitica* was 13.83% (166/1200) by culturing method, depending on the morphological and biochemical characters, however this rate was reduced significantly to 5.16% (62 isolates) by PCR technique. A successful amplification of encoding targeting gene 16srRNA with its single band was achieved at 1485 bp fragment (Fig.1).
FIGURE 1: PCR product of 16s rRNA gene, the band size 1485bp. The product was separated by electrophoresis on agarose, 62 samples were positive visualized under U.V light after staining with red stain.

Sequencing of 16s rRNA gene was done for twelve isolates; those were selected as high virulent pathogens isolated from seven clinical cases and on the bases of presence three virulence genes. Seven isolates showed 100% compatibility, with gene of the Gene Bank (Sequence ID: KJ606906.1), there was no variation from 109-1008 number of nucleotide from gene of the Gene Bank (Fig. 2:A ). Whereas sequences alignment of 5 isolates showed 99% compatibility: two isolates of *Y. enterocolitica* shows 99% compatibility, score 1834 and expect 0.0 with the gene of Gene Bank (Sequence ID: KJ606906.1), from 110 to 1108 number of nucleotide, there is transition in (1093 C>T), transversion in (1105 G>C) Fig. 2:B, also the sequences of 16S rRNA gene belonged to the another two isolates shows 99% compatibility, score 1657 and expect 0.0 with standard *Y. enterocolitica* at the Gene Bank (Sequence ID: KJ606906.1), from 108 to 1007 number of nucleotide, there is transition in (989 G>A) Fig. 2:C, and the sequences 16s rRNA gene owing to the fifth isolate shows 99% compatibility also, score 1424 and expect 0.0 with the gene of Gene Bank (Sequence ID: KJ606906.1), from 109 to 906 number of nucleotide, multiple transversions were observed: transversion in 245 C>A, transversion in 263 G>T, transversion in 304 C>G, transversion in 309 C>A, transversion in 311 G>C, transversion in 315 C>A, transversion in 316 C>A, transition in 806 G>A (Fig. 2: D).
Isolation and molecular identification of *Yersinia enterocolitica* in sheep

The pathogenic virulence gene *ail*, was successfully amplified using specific PCR primers at 170 bp, the *ail* gene was existed in 51 isolates (82.25 %) of *Y. enterocolitica* (out of 62) Fig. 3.

The sequencing alignment of *ail* gene of only one isolate had 99% similarity, score 197 and expect 3e-47 with the gene of Gene Bank (Sequence ID: AJ344214.2), from 9516 to 9627 number of nucleotide, there is transversion in 9622 A>T (Fig. 4).

Amplification of *inv* gene at 570 bp with an existence rate of this gene was 100% (62/62) Fig.5
Moreover, sequencing alignment for inv gene from three isolates showed various sequences alignment: inv gene of one isolate showed 100% similarity, score 888 and expect 0.0 with the gene of Gene Bank (Sequence ID: CP009367.1) from 2005318 to 2005755 number of nucleotide (Fig.6:B), and the inv gene from third isolate shows 99% compatibility, score 904 and expect 0.0 with the gene of Gene Bank (Sequence ID: CP009367.1) from 2005265 to 2005768 number of nucleotide, the transversion was in (2005274 C>A) Fig. 6:C.

FIGURE 5: PCR product of inv gene, the band size 570bp. The product was separated by electrophoresis on agarose, (62 isolates) visualized under U.V light after staining with red stain.

FIGURE 6: Partial sequence of inv gene of Y.enterocolitica isolate(A);(B):100% compatibility. (C) 99% compatibility with Gene Bank.

Amplification of ystA gene at 145bp and an existence rate of the yst A virulence gene was 90.32% (56/62) Fig. 7.

FIGURE 7: PCR product of ystA gene, band size145bp. The product was separated by electrophoresis on agarose, 62 samples (56 positive ystA gene) visualized under U.V light after staining with red stain.

The sequencing alignment for the yst A gene of two isolates were also showed 100% compatibility, score 183 and expect 6e-43 with the gene of Gene Bank (Sequence ID: CP009846.1) from 1303083 to 1303183 number of nucleotide (Figure 8).
Yersinia enterocolitica (Bucher et al., 1977). In Turkey, Y. enterocolitica were isolated from diarrheic patient in 1998 (Özbas et al., 2000). It was reported that virulence inv gene showed high existence rate (100%) in Y. enterocolitica strains, other virulence genes (93%) ystA (Wang et al., 2009), moreover this result is close to our finding (90.3%) the high rate of ystA gene presence in isolates might explained the high number of diarrheic sheep in our study.

However, the rate of existence of the three virulent genes (ail, inv, ystA) in the isolated Y. enterocolitica was 77.41% (48 out of 62), this in turn indicates high spillover of pathogenic Y. enterocolitica in sheep feces. Also the feces of sheep was mostly used as an organic fertilizer in home gardens and other agriculture fields, beside that the grazing animals will spread the yersinia pathogens in pastures, these contribute actively in threatening human and animal populations, particularly the increase spreading of pathogenic organism in cold and wet weather was reported previously (Saleh and Zenad, 2017).

CONCLUSION

The sheep and their products were considered a potential source of infection to human. The PCR technique is highly acceptable method for rapid detection of the pathogenic yersinia species, and suitable for surveillance studies, the sequences of pathogenic strains provide a useful tool for genetic diagnosis.

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Isolation and molecular identification of *Yersinia enterocolitica* in sheep


