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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 *Yrsinia 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 *16srRNA* 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,*

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

One thousand and 200 fecal swabs were taken randomly from sheep in the south region of Iraq: (Thi-Qar, Basrah, Messan, 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.

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

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

TABLE 2: *16srRNA* gene was amplified by PCR using the following condition

Phase	Tm (°C)	Time	No. of cycle
Initial Denaturation	94°C	3 min	
Denaturation	94°C	1 min	
Annealing	62°C	1 min	35 cycle
Extension-1	72°C	1:40hour	
Extension-2	72°C	10 min	

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

Phase	Tm (°C)	Time	No. of cycle
Initial Denaturation	94°C	3 min	
Denaturation	94°C	45sec	
Annealing	60°C	45sec	35 cycle
Extension-1	72°C	45sec	
Extension-2	72°C	7min	

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 alalysis

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.

Statistical analysis

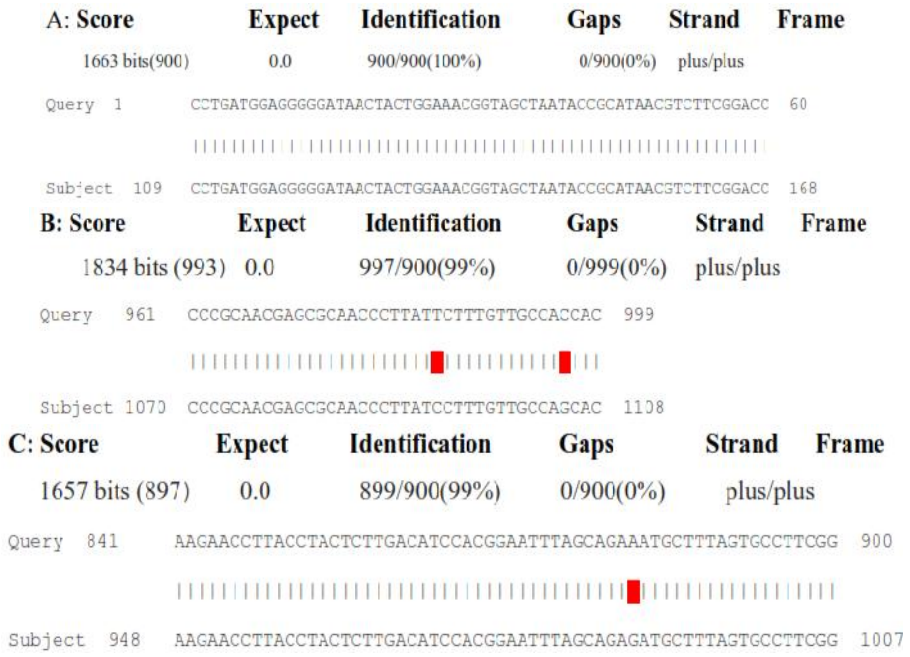
Data were analyzed statistically by using (SAS) version 9.1. Chi- square test was used for comparison (SAS, 2010).

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 *16srRNA* 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 sever 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).



D :	Score	Expect	Identification	Gaps	Strand	Frame
	1424 bits (771)	0.0	789/798(99%)	0/798(0%)	plus/plus	

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Query  121  GTGGGGTAATGGCTCACCTAGGCGAAGATCCCTATCTGGTCTGAGAGGATGACCAGCCAC  180
Subject  229  GTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCAC  288

Query  181  ACTGGAACTGAGACAGGGTCAACACTAATACGGGAGGCAGCAGTGGGGAATATTGCACAA  240
Subject  289  ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA  348

Query  661  CCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAAGTTGTGCCCTTGAGGCGTGGCT  720
Subject  769  CCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCT  828
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FIGURE 2: Partial sequence of 16srRNA gene of *Y. enterocolitica* isolates (A):100% compatibility. (B); (C); (D) 99% compatibility with gene of the Gene Bank

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. entercolitica* (out of 62) Fig. 3.

FIGURE 3: PCR product of *ail* gene at the band size 170bp. The product was electrophoresis on agarose, (2, 3, 4, 5, 6, 9, 10, 13, 14, 15, 16, 18, 19,21, 22, 23, 24, 25, 26, 28 positive *ail* gene); (1, 7, 8, 11, 12, 17, 20, 27 negative *ail* gene) visualized under U.V light after red stain staining

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).

Score	Expect	Identification	Gaps	Strand	Frame
197 bits (218)	3e-47	111/112(99%)	0/112(0%)	plus/plus	

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Query  61    TAATGCAGTCGATAAAGGCTAATCGTGCCAGCCGTGGCTTTACCTTTATGGA  112
Subject  9576  TAATGCAGTCGATAAAGGCTAATCGTGCCAGCCGTGGCTTTACCTTAATGGA  9627
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FIGURE 4: Partial sequence of *ail* gene of *Y. enterocolitica* isolate 99% compatibility with gene of the Gene Bank.

Amplification of *inv* gene at 570 bp with an existence rate of this gene was 100% (62/62) Fig.5

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.

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 2005277 to 2005768 number of nucleotide (Fig.6:A). Whereas sequences alignment of (*inv* gene) second isolate showed 100% similarity, score 791 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 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.

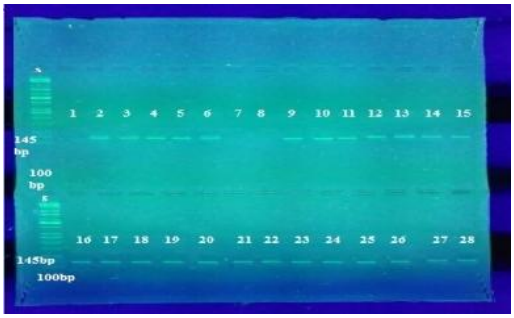

FIGURE 7: PCR product of *ystA* gene, band size145bp. The product was separated by electrophoresis on agarose, 62 samples (56 positive *ystA* gene) visualized under U.V light after staining with red stain.

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).

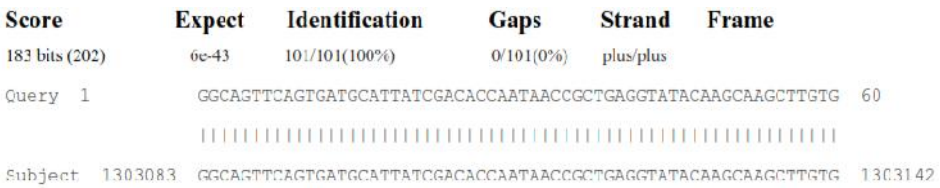

FIGURE 8: Partial sequence of *ystA* gene of *Y. enterocolitica* isolate100% compatibility with gene of Gene Bank.

DISCUSSION

It was found the presence of pathogenic strains of *Y. enteocolitica* in fecal samples of sheep was high; this was in agreement with (Fredriksson-Ahomaa *et al*., 2006). The traditional cultural method showed high false positive fecal sample as this confirmed by PCR analysis, the PCR technique facilitates epidemiological studies, tracing the suspected infection source, ecology of isolated bacteria and conducted the presence of *Y. enterocolitica* in sheep fecal samples (McLellan *et al*., 2001). Also a successful amplification of 16s rRNA gene was performed at different (1285 bp) base pair fragments (Okwori, 2008). The variance in the number of the base pair fragment in different strains might be due to an environmental pollution or/and geographical heterogeneity (Siriken, 2004). Sequencing of this gene (16s rRNA) was performed to detect variations between local isolated *Y. enterocolitica* strains and strain from the Gene Bank. Nevertheless, the results of present study showed the genome sequences of *Yersinia enterocolitica* comparing with the gene of Gene Bank with accession numbers KJ606906.1 were show 99% DNA sequence homology with strain isolated from human stool at 1466 bp in USA (Cunningham, 2015), in fact this is supporting that sheep considered as a source of infection to human being. Moreover *Yersinia enterocolitica* were isolated from diarrheic patient in the Northern of Iraq (Kanan and Abdulla, 2009) in which heavy sheep population reared. The successful amplification of *ail* gene at 170 bp was achieved, in accordance with many authors (Fredriksson- Ahomaa and Korkeala, 2003; Falcao *et al*., 2006; Bhadari and Cotteren, 1998), although other workers succeeded in amplification of the same gene (*ail*) at various fragments: 425bp, 351bp, 163bp (Wim *et al*., 2001; Amany *et al*., 2015; Wang *et al*., 2009 respectively), and the rate of presence (82.25%) *ail* gene in *Y. enterocolitica* isolate was close to that recorded in (94%) China (Wang *et al*., 2009), and higher than the existence rates of *ail* gene in isolates from fecal samples in Nigeria 1% (Okwori *et al*., 2009) and in (0%) German (Bucher *et al.*, 2008), the later authors found *Y. enterocolitica* isolated from tonsil samples of sheep, showed low existence rate (5%) of *ail* gene. The variation in the presence of *ail* gene is referring to the uniqueness of this virulence gene only among pathogenic strains of *Y. enterocolitica* (Bucher *et al.*, 2008; Schulte *et al*., 2000) and many authors referred to the presence of *ail* gene indicates the virulence of strain (Grant *et al*., 1998), also the variant rates of presence *ail* gene were recorded in the variety of samples and strains from different localities. The genetic variations among the same strain were reported (Neubauer *et al*., 2001). The *inv* gene was also amplified previously at the same band size (576bp) from *Y. enterocolitica* isolated from human and different animal species (including sheep) as well as various types of food origin in Brazil (Falcao *et al*., 2006), and also from *Y. enterocolitica* isolated from raw milk samples in Turkey (Özbas *et al*., 2000). It was reported that virulence *inv* gene showed high existence rate (100%) in *Y. enterocolitica* strains, than 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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