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IDENTIFICATION AND CHARACTERIZATION OF VIRULENT *YERSINIA ENTEROCOLITICA* IN LOCALLY PRODUCED SOFT CHEESE IN IRAQ

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Abstract

This study was aimed to identification and characterization of virulent *Yersinia enterocolitica* in in locally produced soft cheese in Iraq. Virulent *Yersinia enterocolitica* isolates have been recovered from cheese samples. They were found in 11 (4.5%) samples of the total 240 tested samples. Those positive isolates showed successful amplification to 16s rRNA at 1485 bp. The PCR method confirmed these isolates which showed results for chromosomal and plasmid encoded virulence markers in both phenotypic and genotypic assays. In case of phenotypic tests there were only 5 isolates revealed to contain chromosomal-encoded virulence markers and only 8 revealed to contain plasmid -encoded virulence markers while in case of molecular investigation all the 11 isolates revealed to harbor chromosomal-encoded virulence gene (Ail gene) while only 7 isolates was harbored plasmid encoded virulence marker (YadA). *Yersinia enterocolitica* occurrence approved to be present as contaminant in cheese. The pathogenicity of Yersinia isolates should always be assessed which is considered an important evidence of a risk to human health.

Keywords: *Yersinia enterocolitica*; cheese; 16s rRNA, Ail, Yad A.

Introduction

Y. enterocolitica is a food-borne infectious agent pathogen and may cause abundant gastrointestinal diseases in humans, ranging from mild diarrhea to mesenteric gland inflammation, the evocation of appendicitis and septicemia in children and individuals with underlying disease, symptoms may be severe. (Bottone, 1997). Yersiniosis among the numerous food borne illness, yersiniosis is listed in third place after campylobacteriosis and salmonellosis (Bolton and McDowell, 2013). In Iraq, there is presently a scarcity of data on the occurrence of food borne diseases including. *Y. enterocolitica* is known to be the foremost common cause of bacterial enteritis in Northern and Western Europe. The number of cases of infection detected in North America has minimized over the previous couple of years. *Y. enterocolitica* is transmitted by all kinds of food including milk and dairy products (Harakeh *et al.*, 2012). The possession of psychrotrophic nature of *Y. enterocolitica* and its ability to multiply and grow within the storage temperature that used for keep perishable food of animal origin such as milk and dairy products, this reveal that the likelihood of such foods to act as sturdy reservoir for *Y. enterocolitica* if its antecedently contaminated with the pathogen before storage. (Kapperud, 1991). The virulent strains of *Y. enterocolitica* have been isolated from raw milk and dairy products (Hanifian & Khani, 2012., Soltan-Dallal *et al.*, 2004; Hamama *et al.*,1992;) A problem with the detection of strains of *Y. enterocolitica* is that they are only pathogenic to man in the presence of Bacterial strains, cultivation and enumeration specific virulence factors. A set of three tests has been projected to separate pathogenic from non-pathogenic Yersinia trains; Pyrazinamidase activity, Esculin hydrolysis and Salicin fermentation The virulence of *Y. enterocolitica* depends on possession of both chromosomal and plasmidial genes because they are essential to act as pathogen. These genes are responsible for the assembly of the proteins Inv (invasin), Ail (attachment invasion locus), Yst (*Yersinia* stable toxin) and YadA (*Yersinia* adhesion) (Hallanvuo, 2009). Several studies targeted those genes to verify the pathogenicity of *Y. enterocolitica* isolates in which Polymerase Chain Reaction (PCR) have been used (Darwish *et al.*, 2015; Khalid and abbas, 2019). Difficulties associated with the isolation of pathogenic *Y.*

enterocolitica stem from the small number of pathogenic strains in the samples and the large number of organisms in the background fiora, particularly in food and environmental samples. Direct isolation, even on selective media, is seldom prospering and time-consuming enrichment steps are required. No single procedure is presently out there which is able to recover all pathogenic serotypes (Fredriksson & Korkeala 2003). In Iraq, the consumption of raw milk and derived products (cheese) has traditionally been a very common practice in cities and countryside areas. However, very few information is available on the occurrence of *Y. enterocolitica* in dairy products in Iraq. Therefore, the aim of this study was to identify the virulence of *Y. enterocolitica* in locally produced soft cheese using conventional culture method and PCR assay as a method is very needed because the culture methods are complex in addition to time consuming and have low specificity for pathogenic strains of *Y. enterocolitica*.

Materials and Methods

Collection and Processing of Samples

A total of 240 soft cheese samples with whey included 167 cow cheese and 73 sheep cheese were collected. Each sample with volume ranged (250-500 g). They were collected randomly from markets at different nine regions of Baghdad province and four provinces at the middle Euphrates (Karbala, Babylon, Al-Najaf and Al Qadysia) during December 2018 till May 2019. In which they collected aseptically in sterile non-permeable & non-durable plastic bags (500-1000 L) and transported in ice box to a milk hygiene laboratory as soon as possible, then macerated manually with its own whey (original sample), then 10 gm sample from a whole homogenized cheese lobes (original sample) were diluted decimally with 10 parts 90ml of 2% (buffered sodium citrate solution) emulsifying cheese lobules to a tiny portions to extract hidden cells, then homogenized with stomacher for (5-3) minutes. The samples were analyzed and processed according to different standard reference food microbiological which most recommended and used for isolation *Y. enterocolitica* from animal food products with some modifications.

Processing of Samples Unit

All the samples subjected to pre-enrichment by using a modified typtone soy broth (MTSB), double strength power containing tryptone soy broth (TSB) plus yeast extract (YE), one part of raw milk sample (25 ml) to 9 parts (225 ml) of (MTSB-YE) to get (1:10) (dilution food standard formula), then homogenized with stomacher for (3-5) minutes after that incubated at 25ºC for 24 hours for resuscitation of stressed or sub lethally damaged cells. After pre enrichment the samples underwent cold enrichment in different *Yersinia* selective broths which are recommended by ISO10723:(2003) for the detection of Pathogenic *Y. enterocolitica* in food. PSB (peptone Sorbitol bile broth), ITC (Irgasan/Triclosan Ticarcillin/ Chlorate broth base) for (48-72) hours at 4°C then the cold enriched samples were subjected to alkali treatment (0.5) ml of each. The culture was transferred for 20 seconds into 4.5 ml of 0.5% KOH in 0.5% NaCl) mixed by vortexing for (2-3) seconds, then a loopful was streaked onto MacConkey and CIN (Cefsulodin-Irgasan-Novobiocin) agar plates which supplemented with Cefsulodin-irgasan-novobiocin antibiotics which simultaneously and incubated at 25°C for (24-48) hours. The plates were examined for the presence of specific colonies of Yersinia species morphology, Isolates showing both Non Lactose Fermenting (NLF) colonies on MacConkey agar and bull's eye colonies with deep red center and clear colorless periphery on CIN agar plates were considered presumptive *Yersinia* species. The suspected colonies were picked and purified then stained with Gram's technique and examined microscopically after that the isolates were subjected to multiple traditional lab biochemical tests for preliminary detection for *Y. enterocolitica* (oxidase, catalase, urease, triple sugar iron test and citrate utilization test). The presumptive *Yersinia* isolates subjected to the identification at species level by using VITEK2 compact system the confirmed isolates of *Y. enterocolitica* by VITEK2 identified by conventional PCR assay

DNA extraction

Bacterial DNA was extracted from fresh *Y. enterocolitica* colonies grown in the broth containing presumptive pure culture colonies of *Y. enterocolitica* by using Geneaid Presto™ Mini DNA Bacteria Kit (Taiwan) following manufacturer's instructions for DNA extraction protocol extracted DNA was stored at –20°C until used.

Identification using a PCR assay targeting the 16S rRNA gene

Y. enterocolitica isolates were identified by PCR assay targeting the 16S rRNA gene region, which is conserved in *Y. enterocolitica*. Primers: (forward 5¹AGAGTTTGATCCTGGCTCAG-¹3 reword 5¹-GGTTACCTTGTTACGACTT-¹3) were used to amplify a 1485 bp fragment of the 16S rRNA gene found in *Y. enterocolitica*.

Testing for virulence Markers

The pathogenicity of all presumptive *Y*

.enterocolitica isolates were studied by phenotypic and genotypic methods. Phenotypic tests including Temp-Dependent autoagglutination (25°C-35°C) in Methyl Red-Voges Proskauer broth and pyrazinamidase production, were performed three times for all study isolates as described by Yang and Fang, 2003.

Genotypic tests including the chromosomal virulence gene: Ail(attachment invasion locus gene) and the plasmid-borne virulence gene Yad A (*Yersinia* adhesin A) gene comprised in the analysis. The primers based on the sequences of the Ail (5'-TAATGTGTACGCTGCGAG-3 and 5'- GACGTCTTACTTGCACTG-3') and YadA (5'CTTCAGATACTGGTGTCGCTGT-3' and 5'-ATGCCTGACTAGAGCGATATCC -3') were used to amplify a 351pb- and a 849-bp fragment, respectively. The primers were lyophilized and dissolved in free deionized distill water (ddH_2O) to get a final concentration of 100 pmol/µl as stock solution which was kept at -20°C to prepare10 pmol/µl concentration as work primer suspended, 10 µl of the stock solution in 90 µl of free deionized distill water (ddH_2O) to reach a final volume 100 µl.

Primer sequences, their references, product lengths and specific targets are listed in table 1. The PCR conditions for the primers listed in table 2, 3 and 4, respectively.

Afterwards, 10 µl of PCR amplicon were analyzed by electrophoresis on a 2.5% agarose gel. Gels were visualized under a UV illuminator and photographed through gel documentation system. A 100-bp DNA ladder (Bioneer, Korea)was used as DNA molecule size marker.

Results and Discussion

According to the results that revealed by the culture method on CIN that used in the study of 240 cheese samples there were 17 (7%) positive cheese samples suspected to be infected with *Y. enterocolitica* that shown the typical bull's eye character on the surface of agar medium. After that those suspected isolates were purified and gram stained and considered as presumptive *Yersinia* spp. Then subjected to identification to species level by VITEK2 colorimetric compact system in which 12(5%) of presumptive isolates were identified as *Y. enterocolitica* according to the data base of VITEK2 system while the identification by molecular level by targeting the 16s rRNA gene region result revealed that only 11(4.5%) isolates showed successful amplification to 16s rRNA at 1485 bp (as illustrated in Fig. 1). The data summarized in table 5. The molecular confirmed *Y. enterocolitica* isolates were tested for occurrence of pathogenic markers by phenotypic biochemical lab tests using two tests the Temp-Dependent autoagglutination (25°C-35°C) and the pyrazinamidase production test. the results showed that 8 of the tested isolates were positive for plasmid encoding marker while only 5 isolates were negative (virulent *Yersinia enterocolitica* is negative for this tests) for chromosomal encoding marker. The genotypic investigation of pathogenic markers showed the existence of the Ail gene (chromosomal virulence gene) in all the 11 *Y.enterocolitica* isolates while the Yad A (plasmid virulence gene) exist in only 7 of *Y. enterocolitica* isolates. As illustrated in Fig. 2 and 3 respectively.

Table 1: Primers used in this study to detect the16srRNA, ail and Yad A genes in Y. enterocolitica.

Primer	Size in BP	Sequence (5 ¹-3 ¹)	Reference
16s rRNA	1485	5'AGAGTTTGATCCTGGCTCAG-3' 5'-GGTTACCTTGTTACGACTT-3'	Hao *et al.*, 2016
Ail	351	5'-TAATGTGTACGCTGCGAG-3' 5'- GACGTCTTACTTGCACTG-3'	Thoerner, *et al.*, 2002
YadA	849	5'CTTCAGATACTGGTGTCGCTGT-3' 5'-ATGCCTGACTAGAGCGATATCC -3'	Thoerner, *et al.*, 2002

Table 2: Amplification of 16s rRNA gene was achieved using the following condition.

Phase	Tm(°C)	Time	No. of cycle
Initial Denaturation	94 °C	5 min	1 cycle
Denaturation	94 °C	30 sec	35 cycles
Annealing	62 °C	30 sec	
Extension-1	72 °C	1 min	1 cycle
Extension-2	72 °C	5 min	

Table 3: Amplification of Ail gene was achieved using the following condition.

Phase	Tm (°C)	Time	No. of cycle
Initial Denaturation	94°C	5 min	1 cycle
Denaturation	94°C	30 sec	35 cycles
Annealing	57°C	30 sec	
Extension-1	72°C	30 sec	1 cycle
Extension-2	72°C	5 min	

Table 4: Amplification of: YadA gene were achieved using the following condition.

Phase	Tm (°C)	Time	No. of cycle
Initial Denaturation	94°C	5 min	1 cycle
Denaturation	94°C	30 sec	35 cycles
Annealing	60°C	30 sec	
Extension-1	72°C	1 min	1 cycle
Extension-2	72°C	5 min	

Table 5: Identification of *Yersinia enterocolitica* in cheese samples.

Positive isolates by culture method (study sample code)	VITEK2 compact system	16s rRNA
CHBABGB8	*Y. enterocolitica*	*Y. enterocolitica*
CHBALTB10	N.D	N.D
CHBALSHB7	*Y. enterocolitica*	*Y. enterocolitica*
CHBALFOB7	N.D	N.D
CHBALNAB3	*Y. enterocolitica*	*Y. enterocolitica*
CHKB27	*Y. enterocolitica*	*Y. enterocolitica*
HCHB7	N.D	N.D
HCHB22	*Y. enterocolitica*	*Y. enterocolitica*
CHNB23	*Y. enterocolitica*	*Y. enterocolitica*
CHDB5	*Y. enterocolitica*	*Y. enterocolitica*
CHDB16	*Y. enterocolitica*	*Y. enterocolitica*
CHDB27	N.D	N.D
CHBALSO2	*Y. enterocolitica*	*Y. enterocolitica*
CHBALFOO4	*Y. enterocolitica*	*Y. enterocolitica*
HCHO13	*Y. enterocolitica*	N.D
CHDO1	*Y. enterocolitica*	*Y. enterocolitica*
CHDO6	N.D	N.D

N.D: not detected

Table 6: Identification of virulence markers in the study isolates.

PCR confirmed isolates	pyrazin-amidase prod-uction	Auto-aggl utin-ation test	Ail	YadA
CHBABGB8	+	+	+	+
CHBALTB10	-	-	+	-
CHBALSHB7	+	+	+	+
CHBALFOB7	+	+	+	+
CHBALNAB3	-	+	+	-
CHKB27	-	+	+	+
HCHB7	+	+	+	+
HCHB22	-	-	+	-
CHNB23	+	+	+	+
CHDB5	-	+	+	+
CHDB16	-	-	+	-

The data summarized in table 6.

Discussion

In Iraq the prevalence of *Y. enterocolitica* in foods and its significance in public health remain unidentified. Although, in some assessment investigations the prevalence rate of *Y.enterocolitica* as causative agent of gastroenteritis has been evaluated, no clear knowledge of its quota in intestinal infections is available (Kanan and Abdulla. 2009) Moreover, eating habits in Iraq are unalike from developed countries, as most commonly prefer to consume traditional and home-made foods rather than industrially-produced types. This is especially true about consumption of raw milk, traditional local produced cheese in addition to butter and cream. Therefore, investigations dealing with the identification of virulent

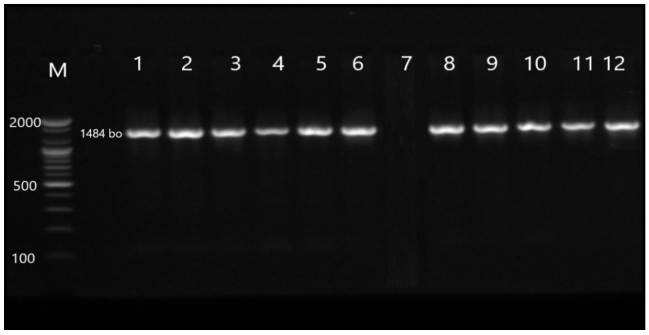

Fig. 1: Identification species specific of 16s rRNA gene of *Y. entercolitica* PCR product at the band size 1485 bp.Lane M =DNA ladder (100 pb), lane (1-12) represent gene of 16s rRNA of *Y. enterocolitica* which appear in11 isolates, lane 7 negative of 16s rRNA gene of *Y. enterocolitica*.

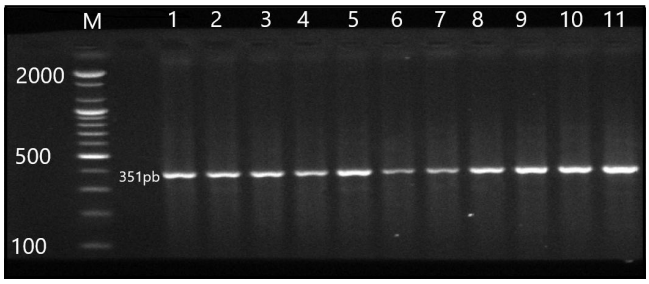

Fig. 2: Identification of Ail gene the PCR product at band size 351 bp (Lane M:100 bp DNA marker, Lane (1-11) represent Ail gene of *Y. enterocolitica* appeared in all 11 of *Y. enterocolitica* isolates visualized under ultraviolet; light (after ethidium bromide staining).

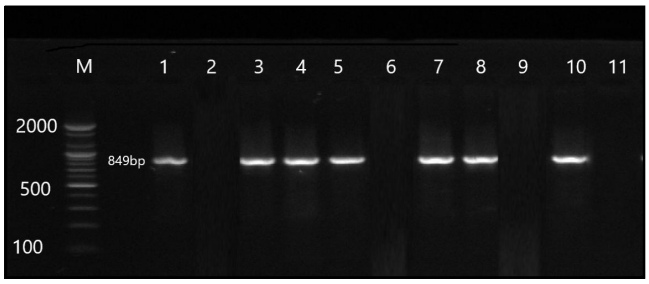

Fig. 3: Identification of Yad A gene the PCR product at band size 849 bp (Lane M:100 bp DNA marker ,Lane (1,3,4,5,7,8,10) represent Yad Agene of *Y.enterocolitica* appeared in 24 of *Y.enterocolitica* isolates lane(2,6,9,11) negative of Yad A gene of *Y.enterocolitica.* visualized under ultraviolet light (after ethidium bromide staining.).

Y.enterocolitica in traditional dairy products seem to be of overriding importance.

According to the investigation of occurrence of pathogenic *Y.enterocolitica* in locally produced 240 soft cheese samples that retailed in several public markets in Iraq the results subsequently *Y.enterocolitica* was isolated by the culture method and confirmed by the second PCR method by species specific 16s rRNA target gene which revealed 11 (4.5%) positive isolates.

Several researchers investigate for occurrence of *Y.enterocolitica* in cheese samples some recorded it in low percentage like (10.5%, 9.2%, 7%) (Hanifan and Khani 2012), Bonardi, 1978; Hamama *et al.,* 1992). Other recorded higher 24.4%,35.7% Report in cheese samples by Harakeh *et al.,* (2012) and Yucel and Ulusoy, (2006). Our results showed a low level of contamination. This result may be ascribed to heat treatment of the raw milk that used for manufacturing of cheese which is one of processing steps of cheese making in our country, but this didn't decrease the risk of such contaminated food on public health so unrestricted hygienic monitoring systems and food policies like absence of biosafety and hazard analysis critical control points during production and handling of healthy cheese. The contamination may occurred from human handlers, the eventual contamination environment and water. Also, storage conditions could also increase contamination since *Yersinia* spp. and other pathogens can grow at refrigeration temperature (Palumbo, *et al.,* 1986; Tibana, *et al.,* 1987).

In numerous investigations, the occurrence of virulence *Y. enterocolitica* in foods samples has been assessed by both PCR assay and culture methods. (Bonardi, *et al.,* 2018, Özdemir & Arslan (2015)., Ye *et al.,* (2014). Several genes such as ail, yadA, yst, Inv and etc were used. According to our results in addition to these results, the efficiency of PCR method is much more reliable than that of the culture method in detection of the virulent *Y.enterocolitica.* Direct isolation; even on selective media is time-consuming yet rarely successful. Besides, there is no comprehensive culture method which could identify all pathogenic strains. Therefore, the low rates of isolation of pathogenic *Y. enterocolitica* in natural samples may be due to the limited sensitivity of culture methods (Fredriksson and Korkeala, 2003). Likewise, results of this study suggested that the pathogenic *Y. enterocolitica* can be detected more rapidly and with a higher degree of sensitivity through the use of PCR method.

Although PCR assay can be highly effective with purified bacterial isolates, its sensitivity is reduced when it is applied directly to the natural samples. This can be attributed to the complexity of composition of such samples (e.g., cheese, chicken, meat and stool) as well as the low bacterial log may lead to decrease the efficiency of PCR (Fredriksson- and Korkeala, 2003; Rossen *et al.,* 1992). Therefore, an enrichment step prior to PCR, which increases the quantity and ensures the detection of viable cells, has been applied in most procedures (Lantz *et al.,* 1998; Fredriksson and Korkeala, 2003).

In public health point of view any food samples in which detected to be contaminated by indicator bacteria particularly pathogenic species this food considered unfit for man consumption and may act as risk for man health if accidentally consumed. Since this study was conducted closed to consumer level and the detection focused on the occurrence of virulence isolates of *Y. enterocolitica* which had been detected in the tested cheese samples that retailed to the public markets so that active deed should be pragmatic to reduce or eliminate the risks posed by this organism and this may involve a number of stages of the food chain. These include the application of good agricultural practice, good manufacturing practice and hazard analysis of critical control points at every stage of the food supply chain, from the farm to the retailer to eliminate the pathogen in food reaching to the consumer.

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