DETECTION OF ENTEROTOXIN GENES AND ANTIBIOTIC SUSCEPTIBILITY PATTERN IN *YERSINIA ENTEROCOLITICA* ISOLATED FROM CHEESE IN BASRAH

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(Received 9January 2019 ,Accepted 20 March 2019)

Key Words: Yersinia, enterotoxine, Cheese.

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ABSTRACT

One hundred fifty cheese samples were collected between 8 October 2017 to February 2018. Fifty samples from each cows , buffalos and sheep. The sample transferred to TSB (tryptone soy broth) and PBS (Phosphate buffer saline) for enrichment and cooled enrichment procedure respectively. Using *Yersinia* selective agar TSB enrichment showed high percentage of suspected *Yersinia* isolation. Eleven isolates from cow cheese (22%), 10 isolates from buffaloes cheese (20%) and 8 isolates from sheep cheese (12%). In contrast PBS enrichment showed better selectivity to reduce bacterial number other than suspected *Yersinia enterocolitica* isolates. The results indicate there were 8 isolates from cow cheese (22%), 9 isolates from buffaloes cheese (20%) and 7 isolates from sheep cheese (16 %). The suspected colonies that grow on selective agar and having bull eye appearance were subjected to biochemical identification. The results showed that cow and buffaloes cheese were contaminated with this bacterium at the percentage of 8% and 6% respectively. Sheep cheese were also contaminated with *Yersinia enterocolitica* at the percentage of 4% . The total percentage of isolation of *Yersinia enterocolitica* from all animals were 6.%**.**. The isolated strains were highly susceptible toward azithromycin, streptomycin, and Gentamycin, followed by Ciprofloxacin and Chloramphenicol (93.3%). The low susceptibility were found toward vancomycin (6.66%) followed by Cloxacillin (33.3%). The result of polymerase chain reaction (PCR) for enterotoxin genes, *yst*A and *yst*B were investigated by PCR using a pair of primers for each. The results

showed that *yst*A gene was absent in all nine investigated strains while *yst*B gene was present in four strain at a ratio 44.4%.

INTRODUCTION

Yersinia enterocolitica is a food borne pathogenic bacteria. This bacterium causing a sever gastrointestinal problems in human (1). Food-borne diseases are causing an international health illnesses particularly in developing countries. After campylobacteriosis and salmonellosis yersiniosis is listed in third place among the various food borne disease (2). Several studies reported presence of *Y. enterocolitica* in milk and milk products (3). Ackers *et al.,* (4) reported the three yersiniosis outbreaks occurred by milk. The psychrotropic nature of this microorganism allowing its growth at low temperature (5). Strains of *Y. pseudotuberculosis* have a virulence plasmid pYV. This plasmid is important for bacterium survival and multiplication in lymphoid tissues. Plasmid pYV encodes a type III secretion system and the outer membrane protein YadA (*Yersinia* adhesin A), which has been found to play a functions in the pathogenesis (6;7). The enterotoxin produced by *Y. enterocolitica* is thermostable. Thermostable enterotoxin can be degraded only by heating at 100 to 120 ^{0}C for 20 to 15 minutes respectively (8; 9). Heating susceptibility is strain-dependent: The time required at a certain temperature to kill 90% of the organisms (D values) in milk range from 0.7 to57.6 second (10). The presence of pYV plasmid (*Yersinia* virulence) with approximately 70,000 bp is considered one of the basic indicators of virulence. This plasmid encodes, the protein allowed adhering to the host cells (*YadA* adhesin), outer proteins that affect the immune system (*Yop)*, and secretion system protein (Ysc), (6; 7). To date, investigation of microbes in milk at the studied area were applied for *Brucella* (11,12); *E. coli* (13,14,15); *Staphylococcus aureus* (16, 17, 18), *Listeria monocytogenes* (19) *Bacillus cereus* (20, 21, 22, 23). None of those investigations were deal with *Yersinia* sp. (24).

MATERIALS AND METHODS

Sample's collection: In order to obtain *Yersinia*isolate different sample were collected from several regions in Basrah province. 150 cheese samples were collected between 8 October 2017 to February 2017 . Fifty samples from each cows , buffalos and sheep. The samples were collected from several markets in different parts of Basrah province. All dairy product were transported to the central laboratory of

College of Veterinary Medicine with ice box and kept in refrigerator in plastic bags until use.

Bacterial Isolation: Twenty five grams of homogenized cheese also aseptically added to 100 ml of Tryptone Soya Broth flasks and incubated at 25˚C for 2 days. For cold enrichment method, 25 gm of homogenized cheese samples were added to 100 ml flasks of PBS. Above flasks were incubated for 21 days at 4 ºC (25; 26). Aloopful of cultures were streaked on *Yersinia* Selective agar (YSA) plates which incubated for 48 hr at 25˚C.

Laboratory diagnosis: The dark red colonies surrounded by a transparent border that resembled bull eye were considered as suspected *Y. enterocolitica.* Gram stain slides were prepared for suspected colonies. *Yersinia* appeared as Gram negative non-spore-forming rods or coccobacilli.Biochemical testes were done including catalase test, oxidase test, Kligler'siron agar test, motility test and indole test.

Extraction of bacterial DNA and PCR amplification: For PCR studies genomic DNA was extracted from bacterial cultures using commercial kit, and following the protocol provided by the manufacture (Geneaid, South Korea). Extracted DNA were stored at -20°C until used. The following primers in the table,1 were used for investigation of 16Sr RNA, *ystA and ystB,* genes of *Y. entereocolitica*. Detection of the PCR amplified product was done by electrophoresis on the agarose gel at 1.5%, for 30 min . DNA ladder was used to measure the molecular weight of PCR product. The PCR amplification products were examined under UV light.

DNA sequencing: DNA sequencing of 16s rRNA were 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.

Antibiotic susceptibility test of *Y. enterocolitica* isolates

The antibiotics susceptibility testing done according to method of Bauer and Kirby (27).The antibiotics discs dispensed onto inoculated Mueller-Hinton plates. The plates were inverted and place in an incubator at 37˚C for 24hrs. Inhibition zone was measured by millimeter. The results are compared with minimum inhibition diameter of Bioanalysis Co.(Turkey).

Table. 1. PCR primers used in this study

Primers	Primer sequence	Product size	Reference
Ysta	F: ATCGACACCAATAACCGCTGAG R: CCAATCACTACTGACTTCGGCT	79	Thoerner *et.al.,* 2003(28)
Ystb	F: GTACATTAGGCCAAGAGACG R: GCAACATACCTCACAACACC	164	Thoerner *et.al.,* 2003(28)
16rDNA	F: AGAGTTGATCCTGGCTCAG R: GGTTACCTTGTTACGACTT	1500	Eden *et.al.*,.1991(29)

RESULTS

Bacterial isolation according to TSB and PBS enrichment

The highest percentage of growth were obtained in cows cheese (22%) followed by buffaloes cheese (20%) and sheep cheese (16%) respectively. At PBS enrichment the highest percentage of growth were obtained in buffaloes cheese (18%) followed by cow cheese (16%) and sheep cheese (14%) respectively, (table 2).

Table (2):Number and percentage of cheese samples that showed bacterial growth on YSA medium after TSB enrichments and PBS enrichment.

Sample types	No. of samples	enriched by TSB		enriched by PBS	
		No.	%	No.	%
Cow Cheese	50	11	22	8	16
BuffalosCheese	50	10	20	9	18
Sheep cheese	50	8	16	7	14
total	150	29	19.3	24	16

$X^2 = 0.298$ ⟶ 0.05

Bacterial diagnosis

Since YSA showed multiple growth resembled *Yersinia enterocolitica* colonies, further biochemical and molecular identification were done. The suspected colonies of *Yersinia enterocolitica* isolated from selective medium were subjected to further investigation. Dark and red centered colonies with transparent border "Bulls eye like" on YSA were picked and streaked individually on same agar (Fig.1). By Gram stain, the smear of suspected colonies showed Gram negative, cocbacilli or different irregulars shape. Different biochemical tests were done for identification of bacteria. These including catalase test, oxidase test, motility test, and Kligler's Iron Agar test. The results showed that this bacterium was catalase positive, oxidase negative , non-motile on 37^{o}C, motile on 25^{o}C and alkaline/acid (Table 3).

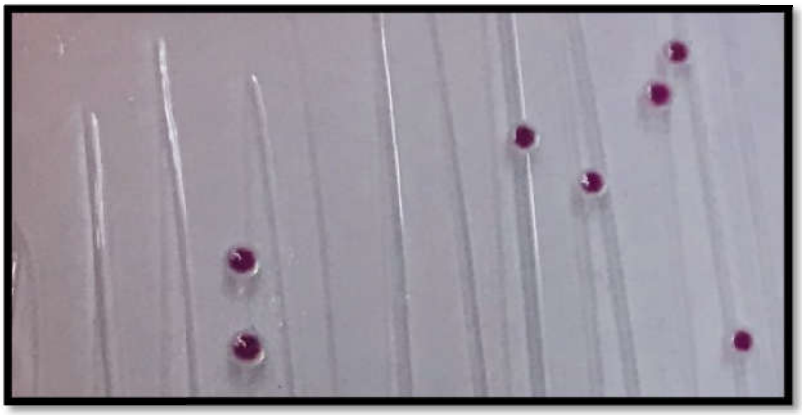

Figure 1. Culture of *Yersinia enterocolitica* on YSA showing colonies of *Yersinia enterocolitica* with bull eye appearance.

Table 3. The biochemical tests of *Yersinia enterocolitica* and their result

Test	**Results**
Catalase	+
Oxidase	-
Kligler's Iron Agar test	K/A (alkaline/acid)
Motility on 37^{o}C	- (non-motile)

Motility on 25°C	+ (motile)

Number of confirmed *Y. enterocolitica* isolates according to animal: All colonies appeared on selective agar having bull eye appearance were subjected to biochemical identification. The results of cheese samples showed that cow's and buffaloes cheese were infected with this bacterium at the percentage of 8% and 6% respectively. Sheep cheese were infected with *Yersinia enterocolitica*at the percentage of 4% (table 4)**.** No significatly difference were obserbed regarding animal samples.

Table 4. The number and the percentage of *Yersinia enterocolitica* isolated from cheese samples.

Sample types	No. of total samples	No. of culture positive	*Y.enterocolitica*	
			No.	%
Cow cheese	50	11	4	8
Buffalo cheese	50	10	3	6
Sheep cheese	50	8	2	4
total	150	29	9	6

X^2= 0.709 → p>0.05

Antibiotic susceptibility of *Y. enterocolitica* isolates: Nine isolates from different sources in current study were examined for their susceptibility to 10 antibiotics. The complete susceptibility were found toward streptomycin ,azithromycin, Gentamycin, and Ciprofloxacin. The low susceptibility were found toward vancomycin and Cloxacillin. The susceptibilitypattern of *Yersinia enterocolitica* against these antibiotics were illustrated in figure 2.

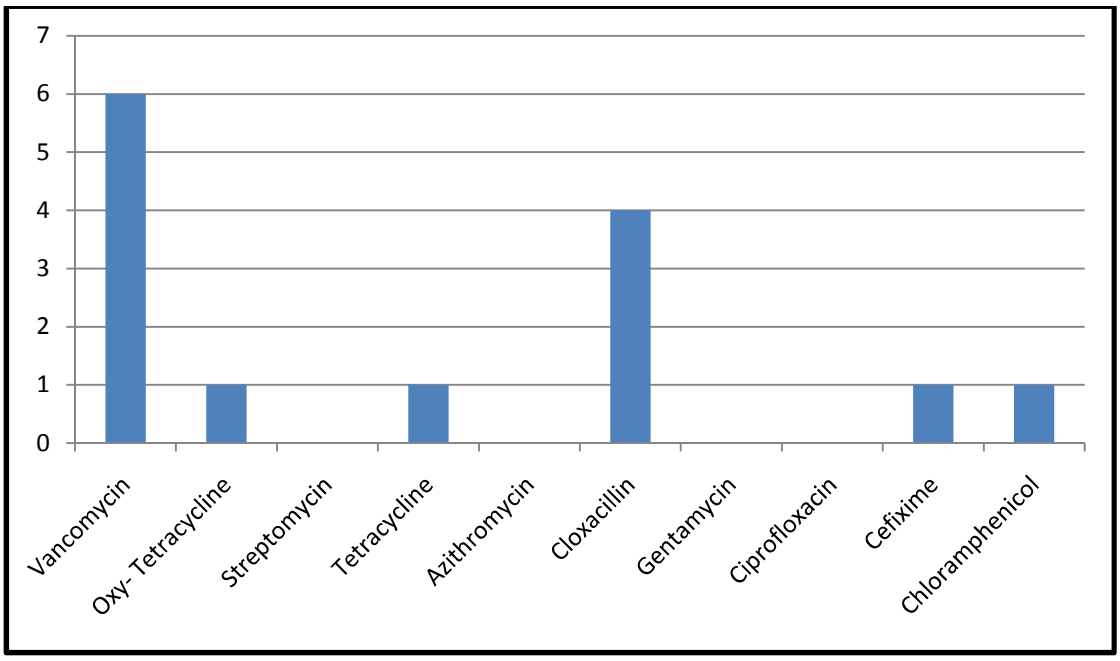

Fig. 2. Susceptibility toward antibiotics in *Yersinia enterocolitica*

Molecular study

DNA extraction: The DNA was extracted from 9 bacterial strains isolated from milk and cheese. Extraction result were accepted for concentration and purity which were determined by Nanodrop.

DNA sequencing: All strains were subjected to PCR using 16s rDNA. The PCR result indicated in all strains with a band size 1500 bp (fig.3).Three strains of *Yersinia enterocolitica* were selected randomly for DNA sequencing. The results of sequencing showed a 100% homology with a strain of *Yersinia enterocolitica*with accession numbers JX424036.1 in the GenBank.

Polymerase chain reaction results of enterotoxin genes: Enterotoxin genes namely *yst*A and *yst*B were investigated by PCR using pair of primers for each. The results showed that *yst*A gene was absent in all 9 investigated strains while *yst*B gene was significantly difference than *YstA* which present in four strain at a ratio 44.4% (Figure 4, table 5).

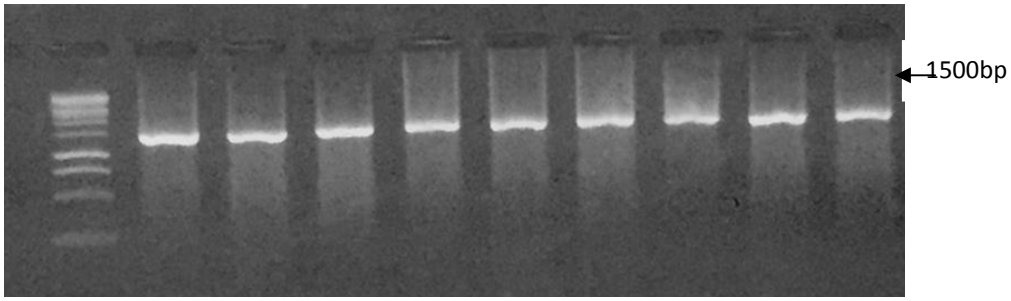

Fig. 3. Gel electrophoresis in 1% agarose of 16S rDNA of *Yersinia enterocolitica* isolates. M lane = 250bpDNA ladder, other lane positive strains.

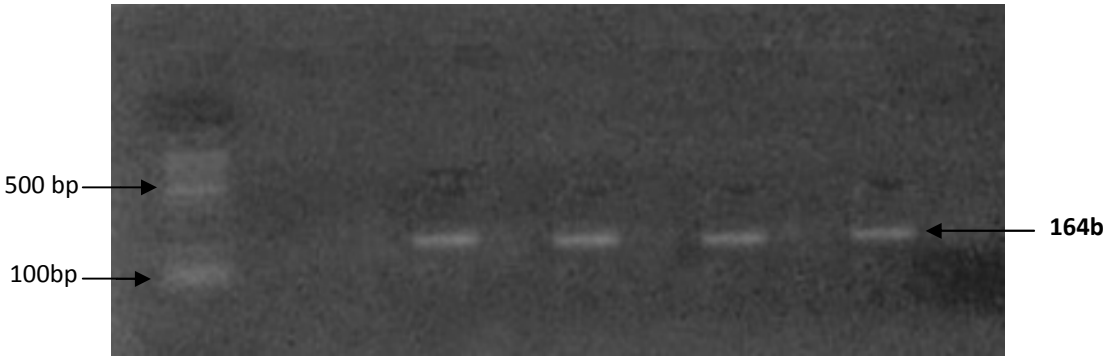

Fig. 4. PCR amplification of *Yersinia enterocolitica ystb* gene (164 bp)

Table 5. Frequency of *ystA* and *ystB* genes in isolated *Y. enterocolitica*

Gene	Total Strains	Positive result	Percentage %
ystA	9	0	0%
ystB	9	4	44.4%

$X^2 = 22.167 \longrightarrow p>0.05$

DISCUSSION

In this study 150 cheese samples were collected from different markets. Presence of *Y. enterocolitica* investigated by using two different enrichment procedures. An overnight TSB enrichment and cold enrichment in PBS were applies. All of samples recovered after cold enrichment for 21 days, TSB for overnight incubated in 30°C. *Y. enterocolitica* showed a psychrotrophic nature that is uncommon among family Enterobacteriaceae. The enrichment procedure at 4°C for long periods has been used for isolation of *Yersinia* spp. However, the disadvantage of this procedure is time consuming. In this study, suspected *Yersinia enterocolitica* was isolated from cheese is higher than that obtained by (30) ,4% and (31) 5.5% and lower than that obtained by (32) 8%. *Yersinia enterocolitica* could not detected in samples of locally manufactured cheese (33)**.** In other study, *Yersinia* spp. was recovered from 7 of 94 cheeses samples (7.44%), and the overall incidence of *Y. enterocolitica* in milk and dairy products was 6.6% (31), which was slightly higher than our results (5.07%). The sheep milk would be more contaminated by faeces. This is because the sheep have a fat mass attached to the rump. This *Y. enterocolitica* recovered from sheep during this study is vary from other studies in different countries (34).

The pathogenic mechanism if *Yersinia* are not fully understood. However, there are a multiple factors play a role in this mechanism. These factors are encoded by both chromosomal and plasmid genes. The infections with *Y. enterocolitica* are usually acquired by ingestions of contaminated food or water. After ingestion the Yst protein (thermostable enterotoxin) facilitates the invasion of the pathogen into tissues which encodes by *yst* gene. PCR results showed that ystA found in 51.85% of isolates (35). In another study, *ail* gene and the yadA gene was found in 100% and 86% of

pathogenic strains of *Y. enterocolitica* respectively. None of the genes were detected in nonpathogenic strains of *Yersinia* spp. (36). In this research, results indicate the *ail* gene found in 4 (4%) of isolates, the *yadA* gen in 3 (3%), the *virF* and *ystA* genes in 2 (2%) of *Y. enterocolitica* strains. Our result of virulence genes included *ystA* (4%) and *ystA* (0%) . Results of Jamali *et. al*, (37), showed that *ystA* gene was found in all *Y. enterocolitica* bioserotypes 1B/O:8 or 4/O:3. Moreover, all isolates of biotype 1A contain only the *ystB* gene. In our study *ystA* gene was not observed the in all isolates.

In this study The high susceptibility was found toward streptomycin ,azithromycin, Gentamycin and Ciprofloxacin. The low susceptibility was found toward vancomycin and Cloxacillin. That differ from other research when all sample resistant to ampicillin ,sensitive to tetracycline (38). Study in Sardinia (39) susceptiblity toward most antibiotics were observed but it was resistant toward ampicillin and cefalothin and amoxicillin/clavulanic acid. Variation in antibiotic susceptibility of *Y. enterocolitica* isolates may be may be due to geographical differences or usage of antibiotics (40). *Y. enterocolitica* are mostly susceptible to the wide range of antibiotics especially in developing countries (41).

التحري عن جينات السمية المعوية والحساسية للمضادات الحيوية في جرثومة يرسينيا القولون والمعزولة من الاجبان في البصرة

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الخلاصة

تم جمع مائة وخمسين عينة من الجبن في الفترة من ٨ تشرين الاول ٢٠١٧ إلى شباط ٢٠١٨. وكانت بواقع خمسون عينة من كل الأبقار والجاموس والأغنام. هذه العينات نقلت الى وسط تريبتون سويابروث و فوسفيت بفر سلاين لتنشيط البكتيريا وتنشيطها بالتبريد ايضا . أخذت مسحه من وسط التنشيط وزرعت بالتخطيط على وسط انتقائي لليرسينا *Yersinia enterocolitica*. وظهرت في أحد عشر عزلة من جبن البقر (٢٢ ٪) ، ١٠ عزلات من جبن الجاموس (٢٠ ٪) و ٨ عزلات من جبن الأغنام (١٢ ٪). في المقابل ، أظهر تخصيب PBS انتقائية أفضل لتقليل عدد البكتيريا بخلاف عزلات *Yersinia enterocolitica*

المشتبه بها. تشير النتائج إلى وجود ٨ عزلات من جبن البقر (٢٢٪) و ٩ عزلات من جبن الجاموس (٢٠٪) و ٧ عزلات من جبن الأغنام (١٦٪). تعرضت المستعمرات المشتبه بها التي تنمو على أجار انتقائي وله مظهر عين الثور للاختبارات البايوكياوية. أظهرت النتائج أن جبن البقر والجاموس ملوث بهذه البكتيريا بنسبة ٨٪ و ٦٪ على التوالي. وتلوث جبن الغنم أيضًا بـ *Yersinia enterocolitica* بنسبة ٤٪. كانت النسبة المئوية الكلية لعزل *Yersinia enterocolitica* من جميع الحيوانات ٦٪. وكانت السلالات المعزولة شديدة الحساسية تجاه الأزيثروميسين والستربتومايسين والجنتاميسين ، تليها السيبروفلوكساسين والكلورامفينيكول (٩٣.٣٪). تم العثور على حساسية منخفضة تجاه فانكومايسين (٦.٦٦ ٪) تليها كلوكساسيلين (٣٣.٣ ٪). تم دراسة نتيجة تفاعل سلسلة البلمرة (PCR) لجينات معوية السموم ، ystA و ystB بواسطة PCR باستخدام زوج من البرايمرات لكل منهما. أظهرت النتائج أن جين ystA كان غائبًا في جميع السلالات التسعة التي تم فحصها بينما كان جين ystB موجودًا في أربع سلالات بنسبة ٤٤.٤٪.

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