Outer membrane protein C (*ompC*) gene as target for diagnosis of *Salmonella* spp. using polymerase chain (PCR) reaction

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Abstract

A total of 1200 different specimens collected from human and animals sources. 600 specimens from patients suffering from diarrhea who were admitted to Al-Diwaniya Teaching Hospital for Maternity and Children. 600 specimens from gall bladder (bile) of cattle from Al-Diwaniya slaughter house. This study was conducted during the period that extended from May 2013 to April 2014. *Salmonella* spp. were isolated and identified using bacterial culturing on selective media, biochemical, API 20E, serotyping by polyvalent and conformation by PCR. Polymerase chain reaction (PCR) was used to detect *ompC* gene encoding for biosynthesis of outer membrane protein C of Salmonella genus. The results revealed that the rate of *Salmonella* isolates was 0.5% (3/600) from human and 1% (6/600) from animals. The PCR technique revealed that 9 isolates of *Salmonella* spp. were contain *ompC* gene (DNA amplification showed one distinct band with molecular weight of 204 bp when electrophorised on agarose gel).The results of this study revealed that the PCR technique had a high specificity in detection of *Salmonella* spp., in comparison to culture and biochemical test, Mini API 20 E and serological tests. The present study found no significant differences between human and animal isolates.

Key words: Salmonella, API20e, antisera, *ompC* gene, PCR.

مورث بروتين الغشاء الخارجي (ج) هدف لتشخيص جنس السالمونيلا باستخدام تفاعل البلمرة

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الخلاصة

جمعت 1200 عينة مختلفة من الانسان والحيوان، منها 600 عينه تم جمعها من الاطفال المرضى الراقدين والمراجعين إلى مستشفى النسائية والأطفال التعليمي واللذين يعانون من الإسهال و600 عينه مراره ماشيه من مجزرة لحوم الديوانية. أجريت الدراسة للمدة من شهر ايار 2013 ولغاية شهر نيسان 2014. تم عزل بكتريا السالمونيلا من خلال الزرع البكتيري للعينات على أوساط زرعيه أغنائية وانتقائية وشخصت من خلال الاختبارات الكيميوحيوية واستعمل Mini API 20E و كما تم استخدام اختبار التلازن باستعمال المصول المضادة متعددة التكافؤ. استعملت تقنية التفاعل التضاعف لسلسلة الدنا (PCR) للكشف عن وجود الجين (*ompC*) المشفر لبروتين الغشاء الخارجي نوع C الخاصة بجنس السالمونيلا *Salmonella* spp. اظهرت النتائج ان نسبة عزل بكتريا السالمونيلا *Salmonella* spp. من عينات براز الأطفال كانت 0.5(3/600)% ، وكانت نسبة العزل من الحيوان 1(1/600)% إذ وجدت فروق معنوية عند مستوى احتمالية (P<0.01) في نسبة العزل عند استخدام الطريقة المظهرية التقليدية والجينية (PCR). واظهرت نتائج استعمال Mini API 20E و نتائج استخدام اختبار التلازن باستعمال المصول المضادة متعددة التكافؤ. وتقنية التفاعل التضاعفي لسلسلة الدنا (PCR) للكشف بان هنالك 9 عزلات للسالمونيلا .أظهرت تقنية الـPCR المفردة للكشف عن الجين المشفر لتصنيع الهدف (*ompC*) ان جميع عزلات السالمونيلا تمتلك هذا الجين فقد تظهرت حزمة واحدة ناتجة من عملية التضخيم للـDNA وكان حجمها 204 زوج قاعدي على هلام الاكاروز. أظهرت نتائج هذه الدراسة إن تقنية الPCR نوعية عالية مقارنة بالفحوصات الأخرى الزرعية والكيموحيوية والفحوصات المصلية وكذلك Mini API 20E.

الكلمات المفتاحية: السالمونيلا ، ابي 20 ي ، مصل مضاد ، مورث بروتين خارجي ، دنا.

Introduction

Enteric pathogens such as *Salmonella enterica* cause significant morbidity and mortality. *S. enterica serovars* are a diverse group of pathogens that have evolved to survive in a wide range of environments and across multiple hosts (1). Infection begins with the ingestion of contaminated food or water so that salmonellae reach the intestinal epithelium and trigger gastrointestinal disease. In some patients the infection spreads upon invasion of the intestinal epithelium, internalization within phagocytes and subsequent dissemination (2). It has been estimated that there are more than 3 million deaths associated with Gram-negative enteric pathogens worldwide due to diarrhea and enteric fever each year (3). The use of selective and differential plating media is a simple method for the isolation of *Salmonella* spp. A wide variety of selective and differential media has been developed for this purpose, including xylose lysine desoxycholate agar (XLD), Hektoen enteric (HE) agar, and bismuth sulfite (BS) agar(4). There has been a general move toward molecular methods of *Salmonella* detection and typing, PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy (5).This study aim to evaluate the rapid detection test to identify salmonellosis using the PCR technique for the detection of *ompC* gene of *Salmonella* genus, and the purpose of this study was to compare the PCR with traditional isolation and characterization methods currently used in diagnostic laboratories.

Materials and methods

Samples collection:

Weighing out 25 g of human stool with a sterile wood spatula, or animal bile directly injecting into an Erlenmeyer flask and add 225 ml buffered peptone water to obtain 1 part sample + 9 part buffer. Mixing, incubation at 37°C overnight (16-20 hours). transfer 1 ml of the pre enrichment broth to 10 ml Tetrathionate broth incubated at 37°C overnight (18-24 hours) and transfer 0.1 ml (100 uL) of the pre-enrichment broth to 10 ml Rappaport-Vassiliadis soy peptone (RVS) broth incubated 41°C overnight.

Isolation and identification of *Salmonella* isolates:

Spreading a 10 µl loop full from the inoculated and incubated Tetrathionate broth (I) and RVS broth (II) on XLD and on BGA agar plates and incubation at 37°C overnight (18-24 hours). Plating two suspect colonies from XLD agar and BGA onto non-selective media (nutrient agar) for biochemical confirmation and serotyping (6). Colonies that showed biochemical characteristics similar to that of *Salmonella* spp. were tested by Mini API20 E then serotyping, the isolates identified by PCR with *ompC* primers for detection of *Salmonella* genus (7).

PCR method:

DNA extraction and purification:

The DNA of all isolates were extracted and purified using DNA extraction and purification kit was provided by geneaid, Taiwan.

Primers:

Specific primers used for the detection specific sequence of *ompC* gene coding for biosynthesis of outer membrane protein C of *Salmonella* genus (7), which is provided by Bioneer co. (S. Korea) (Table 1).

Table(1):Specific primers used for the detection specific sequence of *ompC* gene.

Sequence (5'-3')	PCR product Size (bp)
ATC GCT GAC TTA TGC AAT CG	204
CGG GTT GCG TTA TAG GTC TG	

These primers were prepared by dissolving each primer in 1000 µl of deionized distilled water to obtain stocks in concentration 12 picomole / µl of each of the PCR primers.

Detection of *ompC* gene:

For the detection of *Salmonella* genus by PCR. The PCR amplification mixture (25µl) which was used for the detection each gene includes 12.5 µl of (Green master mix, 2x, which provided by promega, U.S.A.) include: bacterially derived Taq DNA polymerase; dNTPs which include: 400 µM of each

dATP, dGTP, dCTP, dTTP; 3mM of Mgcl2; Yellow and blue dyes as loading dye), 2.5 µl of template DNA, 1.25 µl of each forwarded and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to 25 µl.

Table (2): PCR amplification program for *ompC* gene.

Step	Temperature (C°)	Time (second)	No. of cycle
Initial denaturation	95	120	1
Denaturation	95	60	30
Annealing	57	60	
Extension	72	120	
Final extension	72	300	1

The PCR tubes containing amplification mixture were transferred to thermocycler and started the programs as in table (2). After PCR, the profiles of amplification products were detected by gel electrophoresis

Five microliters of total reaction mixture was loaded on a 1.5 % agarose gel and electrophoresed at 100Vat 70 mA for 60 min. The amplified DNA fragments were visualized by UV illumination after agarose gel electrophoresis and ethidium bromide staining by standard procedures.

Statistical Analysis:

The results were statistical analysis by using chi-square calculation method (8).

Results

The percentage of *Salmonella* spp. isolation was 0.5% (3/600) from human and 1% (6/600) from animals by using the conventional culture methods on enrichment and selective media (Table 3).

Biochemical confirmative tests using Mini API20E system:

The confirmed diagnosis of *Salmonella* spp. were performed by Mini API 20 E system and the results were obtained depending on interpretation kit chart and result entry to Mini API 20 E system (Table 4).

Validity of Lab Techniques in Diagnosis of *Salmonella* isolates:

9 isolates gave positive results for culturing and biochemical tests, Mini API20E (at likelihood 99.9%), serotyping by polyvalent antisera and gave positive results for PCR tests (Table 5).

Amplification of target DNA (*ompC* gene):

The results of PCR amplification which was performed on the DNA extracted from all the studied isolates were confirmed by electrophoresis analysis. By this analysis, the strands of DNA resulted from the successful binding between specific primers and isolates extracted DNA. These successful bindings appeared as single bands under the U.V light using ethidium bromide as a specific DNA stain. The electrophoresis was also used to estimate DNA weight depending on DNA marker (8000 bp DNA ladder) and the result of this estimation revealed that the amplified DNA is 204 bp for *ompC* gene (Fig. 1).

Table (3): Percentage of *Salmonella* spp. isolation by culture methods

	No.	Positive results	Negative results	X^2 value
Human	600	3	597	Calculated X^2=1.008 Tabulated X^2 =6.6349 df= 1 No significant (P<0.01)
Animal	600	6	594	
Total	1200	9	1191	

Table (5): Different laboratory techniques used for *Salmonella* spp.

Test	Culture and biochemical	Mini API20E	Serotyping	PCR
	+ve isolates	+ve isolates	+ve isolates	+ve isolates
Study group	9	9	9	9

Table (4): Analytical profile index (API) 20 E system of *Salmonella* spp. isolats

Test / Isolate No.	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
1	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
2	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
3	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
4	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
5	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
6	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
7	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
8	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+
9	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+

1: ONPG: beta- galactosidase; 2: ADH: arginine dihydrolase; 3: LDC: Lysine decarboxylase; 4: ODC: Ornithine decarboxylase; 5: CIT :Citrate utilization;6: H_2S: H_2S production; 7: URE: Urea hydrolysis; 8: TDA: Tryptophan deaminase; 9: IND :Indole production; 10: VP : acetoin production; 11 GEL: gelatinase; 12: GLU glucose Fermentation; 13: MAN mannitol Fermentation; 14: INO: inositol Fermentation; 15: SOR: Sorbitol Fermentation; 16: RHA Rhamnose Fermentation; 17: SAC: Sucrose Fermentation; 18: MEL :Melibiose Fermentation; 19: AMY: Amygdalin Fermentation; 20 :ARA: Arabinose Fermentation; 21: OX: Oxidase Fermentation.

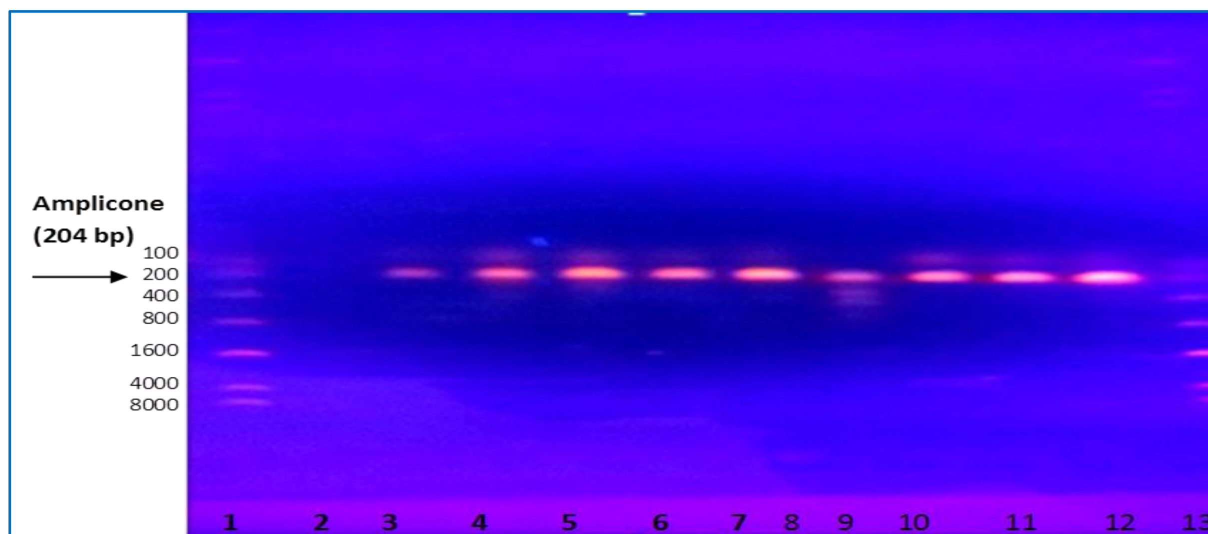

Fig.(1): DNA amplification of a 204 bp of *Salmonella* spp. detecting *ompC* gene using PCR. Lane1: ladder, lane2 negative, lane 3, 4, 5, 6, 7, 8, 9, 10, 11 positive results as *Salmonella* spp., lane 12 :8000bp marker (Ladder).

Discussions

In this study, we found that *Salmonella* spp. infection is considered one of the causes of diarrhea. This may reflect the fact that *Salmonella* spp. is one of etiologic agents of diarrhea that infect human and animals especially during the summer, and that *Salmonella* is a zoonotic bacterial agent, and *S. Typhimurium* is the most common serovar found in animals and in human. In this study, we found 0.5% (3/600) from human and 1% (6/600) from animals by using the conventional culture methods on enrichment and selective media. The present study found no significant differences between human and animal isolates. Traditional *Salmonella* detection methods are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests (**7**). Other studies also conducted in Al- Diwaniya province revealed the prevalence of *Salmonella* spp. there were: 7.9%, 8.47%, and 10% (9,10,11) respectively. *Salmonella* detection by using conventional media, such as *Salmonella*-

Shigella agar (SS), is based on lactose fermentation and H_2S production, the number of false-positive results with these media may be occur, necessitates time-consuming and expensive additional testing (12). According to the reading of Mini API 20E system: All the 9 isolates (by culture and biochemical tests) were detected as *Salmonella* spp. isolates at likelihood levels 99.9 %. Other study evaluated API 20E and PCR for the identification of Salmonella spp. isolates, they found that API 20E had the highest agreement with PCR tests at the 99.9% likelihood level. Validation of both PCR and API 20E (at the 99.9% likelihood level) as accurate diagnostic tests (13). In this study, 9 isolates belong to *Salmonella* spp. (by using PCR detecting *ompC* gene) detected as *Salmonella* isolates.

The specific PCR product is an 204-bp fragment which was visualized by gel electrophoresis and ethidium bromide staining. All *Salmonella* isolates gave positive results by the PCR, the *ompC* gene contains sequences unique to *Salmonella* isolates and demonstrate that this gene is a suitable PCR target for detection of *Salmonella* strains. This study conclude the polymerase chain reaction (PCR) technique gave a high specificity in comparison with other done test, with its advantages of greater speed and effectiveness than conventional detection method, this study recommend the PCR technique provides a new strategy for rapid and sensitive detection of *Salmonella* strains PCR is suitable, highly specific, test and can be used as a basis for future application.

References

1-Foley SL, Johnson TJ, Ricke SC, Nayak R, Danzeisen J (2013) Salmonella Pathogenicity and Host Adaptation in Chicken-Associated Serovars. J. Microbiol. & Mole Biol. Rev.77(4): 582- 607.

2-Fàbrega A, Vila J (2013) Salmonella enterica Serovar Typhimurium Skills To Succeed in the Host: Virulence and Regulation. J. Clin. Microbiol. Rev. 26(2): 308 –341.

3-Donnenberg MS (2000) Pathogenic strategies of enteric bacteria. Nature.406:768–774.

4-Park SH, Ryu S, Kang DH (2012) Development of an Improved Selective and Differential Medium for Isolation of Salmonella spp. J. Clin. Microbiol. 50 (10): 3222-3226.

5-Pickup RW, Rhodes G, Hermon-Taylor J (2003) Monitoring bacterial pathogens in the environment: advantages of a multilayered approach. Curr. Opin. Biotechnol. 14:319–325.

6-WHO (2010) Laboratory Protocol "Isolation of Salmonella spp. From Food and Animal Feces" 5th ed.

7-Alvarez J, Sota M, Vivanco A B, Perales I, Cisterna R, Rementeria A, Garaizar J (2004) Development of a Multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. J. Clin. Microbiol. 42(4):1734–1738.

8-Belle GV, Fisher LD, Heagerty PJ, Lumley T (2004) Biostatistics A Methodology for the Health Sciences. 2nd.ed. A John Wiley & Sons, Inc., Publication. U.S.A.

9-Jawad AA (2010) Detection of *fim* A, *rfbj* (B) and *fim*C Genes of *Salmonella* Isolates Using Single-plex and Multiplex Polymerase Chain Reaction. M.Sc. Thesis, College of Medicine, Al-Qadisiya University.

10-Al–Janabi JK (2006) Microbiological study of some causative agents of diarrhea in children under five year of age in Al – Diwaniya city. Ph.D. Thesis. College of Education, Al - Qadisiya University.

11-Al–Karawiy HAM (2008) Isolation and identification of *Salomonella typhimurium* and detection of gene encoded type -1- fimbria by using polymerase chain reaction. M.Sc. Thesis, College of Veterinary Medicine, University of Basrah.

12-Dijk SV, Bruins MJ, Gijs JH, Ruijs M (2009) Evaluation and implementation of a chromogenic agar medium for *Salmonella* detection in stool in routine laboratory diagnostics J. Clin. Microbiol. 47 (2): 456-458.

13-Nucera DM, Maddox CW, Hoien-Dalen P, Weigel RM (2006) Comparison of API 20E and *invA* PCR for identification of *Salmonella enterica* isolates from swine production units. J. Clin. Microbiol. 44 (9) : 3388–3390.