Detection of *fimC* gene as fingerprinting for *Salmonella typhimurium* isolates by using Polymerase Chain Reaction

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Abstract

A total of 480 fecal samples were collected from children (less than 3 years old) , of both sexes suffering from diarrhea who admitted to The Teaching Hospital of Maternity and Pediatrics in Al- Diwaniya governorate, *Salmonella* spp. were isolated and identified using bacterial culturing on selective media, in addition to, biochemical and Mini API 20E and serotyping by monovalent antisera. Polymerase chain reaction (PCR) was used to detect *fimC* gene encoding for biosynthesis of *fimC* of *Salmonella typhimurium*. The results revealed that the rate of *Salmonella* isolates in fecal samples of patients were (38/480) 7.9% using cultural and Mini API20 E, The results of serotyping revealed that isolates there were 34 belong to *Salmonella* spp. Of these isolates 30 belong to *S. typhimurium*, while the remaining belong to *S. enteritidis* (2 isolates) and *S. meunchen* (2 isolates), when the PCR technique was used to detect the presence of *fimC* gene, 32 *Salmonella* isolates were belong to *S. typhimurium* appeared to contain this gene . The results of this study revealed that the PCR technique had a high specificity (100%) in detection of *S. typhimurium* in comparison to serotyping.

Introduction

Salmonellosis is one of the most common infectious diseases in the world in both humans and animals⁽¹⁾. *Salmonella enterica* is a significant food-borne pathogen of humans transmitted via the consumption of meat, animal products, and food products (fruits and vegetables) contaminated with animal waste⁽²⁾. *Salmonella enterica* is the common cause of human gastroenteritis and bacteremia worldwide, and a wide variety of animals, particularly food animals, have been identified as reservoirs for non typhoidal *Salmonella*⁽³⁾. Food-borne diseases caused by zoonotic *Salmonella enterica* species that represent an important public health problem worldwide ⁽⁴⁾. *S. typhimurium* causes a host-dependent range of diseases from self-limiting to life-threatening systemic infections⁽⁵⁾. Traditional *Salmonella*

detection methods are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests. These methods are generally time-consuming ,Therefore, a rapid method is necessary for the identification of *Salmonella* spp. from clinical specimens^(6) . There has been a general move toward molecular methods of *Salmonella* detection and typing, which are based less on phenotypic features and more on stable genotypic characteristics⁽⁷⁾. PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy⁽⁸⁾ . The study aimed to evaluate the rapid diagnostic test to identify salmonellosis using the PCR technique for the detection of *fimC* gene of *Salmonella typhimurium*.

Materials and Methods

-Samples Collection: A total of 480 stool samples were collected from inpatients and outpatients (both sexes) with diarrhea who were admitted to Al-Diwaniya Teaching Hospital for Maternity and Children. One gram of stool sample was placed in 5 ml of Selenite broth, labeled and transported to

the laboratory in portable container, then incubated for 18-24 hrs at 37C^o⁽⁹⁾. This study was conducted during the period that extended from November 2008 to October 2009.

-Isolation and Identification of *Salmonella typhimurium*: After culturing

on Selenite broth, a loopful of broth was streaked on surface of S.S, XLD and B.G agar plates and then incubated at 37°C for 24 hrs. The biochemical characters of non – lactose fermenting bacteria was determined by using TSI agar and Urease test and other biochemical tests⁽⁹⁾. Colonies that showed biochemical characteristics similar to that of *Salmonella* spp. were tested by Mini API20 E then serotyped by monovalent antisera and the confirmation was identified by PCR with *fimC* genes primers for detection of *Salmonella typhimurium*⁽¹⁰⁾

PCR method:

DNA Extraction and Purification: The DNA of all isolates were extracted and purified using genome DNA purification kit (DNA- sorb-B) provided by Sacace biotechnologies, Italy).

Primers: Specific primers used for the detection specific sequence of *fimC* gene coding for biosynthesis of *fimC* of *Salmonella typhimurium*⁽¹⁰⁾, which is provided by Alpha DNA company (Canada).

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

Sequence	Orientation	Position	Size of PCR product(bp)
5'-AGCGAGCCCCAAAAGTGAAA-3'	Forward	224-262	257bp
5'-ATCTTGAGATGGTTGCCAC-3'	Reverse	450-481	

These primers were prepared according to the information of company by dissolve each primer in 1000 µl of deionized distilled water to obtain stocks in concentration 40,50 picomol / µl of each of the PCR primers.

The *fimC* Gene Detection: For the detection of *Salmonella typhimurium* by PCR the specific primers of *fimC* gene which is responsible for biosynthesis of *fimC* gene of *Salmonella typhimurium* were used. The PCR amplification mixture (25µl) which was used for the detection *fimC* 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 forward and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to 25 µl. The PCR tubes containing amplification mixture were transferred to thermocycler and started the program as in the following reaction : 94°C for 1min as initial denaturation then 35 cycles each cycle consisted of the following: 90°C for 30 sec (denaturation), 54°C for 30 sec

(annealing), and 72°C for 1 min (extension), and final extension 72°C for 480 sec. After PCR, the profiles of amplification products were detected by gel electrophoresis. Ten microliters of total reaction mixture was loaded on a 2% agarose gel and electrophoresed at 100V at 70 mA for 45 to 60 min. The amplified DNA fragments were visualized by UV illumination after agarose gel electrophoresis and ethidium bromide staining by standard procedures.

Statistical Analysis: All results were performed by Chi square test at the level of significant when P-value < 0.01. The specificity, sensitivity and diagnostic accuracy of the results were also calculated by applying the following equations:

$(a / a + b) \times 100 = \text{sensitivity}$, $(d / d + c) \times 100 = \text{specificity}$. diagnostic accuracy = $(a+d / a+b+c+d) \times 100$. also calculated positive predictive value (PPV) = $(a / a + c) \times 100$ and negative predictive value (NPV) = $(d / b + d) \times 100$. (a = the total number of positive cases, b = false positive those bearing positive reading from negative samples, d = total number of true negatives, c = those with negative reading from positive cases⁽¹¹⁾).

Results

Culture and biochemical tests:

The percentage of *Salmonella* spp. isolation was 7.9% (38/480) by using the conventional culture methods of stool samples on enrichment and selective

media.(Table2).There was a significant differences ($P < 0.01$) between the positive and negative results.

Table(2): Percentage of *Salmonella* spp. isolated by using culture methods and biochemical tests.

Isolation results	No.	%	X ² value (p<0.01)
Positive results	38	7.9	Calculated X ² =340.033 Tabulated X ² =6.6349 df= 1 (significant)
Negative results	442	92.1	
Total	480	100	

Validity of the Traditional Techniques in Diagnosis of *Salmonella* spp.

Out of the 38 cases of the study group, 38 cases (100%) gave positive results for culturing and biochemical tests, 38 Mini API20E (at likelihood 99.9% and 95.5%), 34 cases (89.5%) gave positive

results for serotyping as *Salmonella* spp. and 4 cases (10.5 %) gave negative results for serotyping. In this study, 12 cases of non *Salmonella* spp. (control group) gave negative results for culture, Mini API 20E, and serotyping.

Table (3): Different traditional techniques used for *Salmonella* spp.

Test	Culture and biochemical		Mini API20E		Serotyping	
	+ve	-ve	+ve	-ve	+ve	-ve
Study group	38	0	38	0	34	4
Control	0	12	0	12	0	12

Table(4):Relationships among serotyping and other used tests for detection *Salmonella* spp.

Tests		serotyping				Total		X ² value (p<0.01)
		+ve		-ve				
		No.	%	No.	%	No		
Culture method	+ve 38	34	89.5	4	10.5	50		Cal. X ² =33.553 Tab. X ² =6.634 df=1 (Significant)
	-ve 12	0	0	12	100			
MiniAPI20E	+ve 38	34	89.5	4	10.5	50		Cal. X ² =33.553 Tab. X ² =6.634 df=1 (Significant)
	-ve 12	0	0	12	100			

Serotyping Results Versus Selective Media and Biochemical Tests:

The sensitivity, specificity, accuracy PPV and NPV of the serotyping test were (89.5%,100%,92%,100%,75%), respectively while for culture and biochemical tests, there were (100%,75%,92%,89.5%,100%) respectively.

Serotyping Results Versus Mini API 20E Test:

The sensitivity, specificity, accuracy, PPV and NPV of the serotyping test were (89.5%,100%,92%,100%,75%) respectively while for Mini API20 E test, there were (100%,75%,92%,89.5%,100%) respectively.

Relationship Between PCR and Serotyping for Detecting *S. typhimurium*:

Out of 34 isolates as *Salmonella* spp. that gave positive on serotyping, there were 30 isolates (88 %) positive for *S. typhimurium* and 4 isolates were 2 of *S. enteritidis* and 2 of *S. meunchen*, while in PCR, there were 32 (94%) isolates

positive for *S. typhimurium* and the other 2(6%) isolates were negative to *S. typhimurium*. All the 12 isolates that gave negative results in serotyping were gave negative results for PCR detection. There were significant differences ($P < 0.01$) between the PCR and serotyping methods in diagnosis *S. typhimurium*.

Table (5): Relationship between PCR and serotyping for detection *S. typhimurium*:

Tests		PCR method by <i>fimC</i>				Total		X ² value (p<0.01)	
		+ve		-ve					
		No.	%	No.	%	No		Cal.X ² =37.1 Tab.X ² =6.634 df=1 (Significant)	
Serotyping method	+ve 32	32	94	2	6	46			
	-ve 12	0	0	12	100				

PCR Results Versus Serotyping Test for Detecting *S. typhimurium*:

The sensitivity, specificity, accuracy, PPV and NPV of the PCR test were (94%, 100%, 95.6%, 100%, 85.7%), respectively, while for serotyping they were (100%, 85.7%, 95.6%, 94%, 100%), respectively.

PCR Results:

DNA Extraction: The DNA of all isolates were extracted and purified using genome DNA purification kit. The results were detected by electrophoresis on 1% agarose gel and exposed to U.V light in which the DNA appeared as compact bands (figure 1).

Fig1: Total genomic DNA extracted of isolates using 1 % agarose gel electrophoresis.

Amplification of target DNA (*fimC* 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 (100 bp DNA ladder) and the result of this estimation revealed that the amplified DNA of 257 bp (Figure2).

Figure 2:DNA amplification of a 257 bp of *S. typhimurium* detecting *fimC* gene using PCR. Lane 1: Control positive, lane 2:control negative, lane:3,4,5 +ve results as *S. typhimurium*., lane 6 :100 bp marker.

Discussions

In this study, we found that *Salmonella* spp. infection in Al- Diwaniya Governorate 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 infants and young adults especially during the summer, and that *Salmonella* is a zoonotic bacterial agent, and *S. typhimurium* is the most common serotype found in animals and humans⁽¹²⁾. In this study, we found (38/480)7.9% suspected isolates of *Salmonella* spp. on culture and biochemical tests. Traditional *Salmonella* detection methods are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests⁽⁶⁾. Other studies also conducted in Al- Diwaniya province revealed the prevalence of *Salmonella* spp. there were: 14.47%, 8.47% respectively^(13, 14). *Salmonella* detection in stool using conventional media, such as *Salmonella-Shigella* agar (SS), is based on lactose fermentation and H₂S production. The number of false-positive results with these media necessitates time-consuming and expensive additional testing⁽¹⁵⁾. because the conventional methods for *Salmonella* spp., have very poor specificity, and there were numerous false- positive results^(16, 17). According to the reading of Mini API 20E system: All the 38 isolates (by culture

and biochemical tests) were detected as *Salmonella* isolates at likelihood levels 99.9% and 95.5%, the present study found significant differences between serotyping and Mini API 20E test at $p < 0.01$ for diagnosis of *Salmonella* spp. In this study, 32 isolates belong to *S. typhimurium* (by using PCR detecting *fimC* gene). The specific PCR product is an 257-bp fragment which was visualized by gel electrophoresis and ethidium bromide staining. All *S. typhimurium* isolates gave positive results by the PCR, the *fimC* gene contains sequences unique to *S. typhimurium* isolates and demonstrate that this gene is a suitable PCR target for detection of *S. typhimurium*⁽¹⁰⁾. Non amplified DNA fragment were obtained from non *S. typhimurium*. There were significant differences between PCR and serotyping for the diagnosis of *S. typhimurium*. The PCR is more specific than serotyping in the diagnosis of *S. typhimurium*, while serotyping is the method of choice to identify and discriminate isolates of *S. enterica*. But, the serotyping has a number of deficiencies, including the inability to serotype 5 and 8% of isolates and the incorrect typing due to the loss of surface antigens^(18,19). While other study used *fimA* gene which contains sequences unique to *Salmonella* isolates and demonstrate that

this gene is a suitable PCR target for detection of *Salmonella* strains⁽²⁰⁾.

Conclusions:

1. 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; it was successfully used to identify the *Salmonella typhimurium*.
2. Using PCR technique for direct stool samples without Lab. culture procedure gave a good result in direct diagnosis of *S. typhimurium*.

3. We can detect *Salmonella* isolates based on *fimC* gene as *S. typhimurium*.

Recommendations:

1. PCR is suitable, highly specific, test and can be used as a basis for future application.
2. Further study is suggested to test different version of the PCR methods using different samples, to select the most sensitive and specific method.
3. The PCR technique provides a new strategy for rapid and sensitive detection of *Salmonella* strains.

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الخلاصة

جمعت 480 عينة براز من الأطفال دون سن ثلاث سنوات من الذكور والإناث الراقدين والمراجعين إلى مستشفى النسائية والأطفال التعليمي في الديوانية والذين يعانون من الإسهال. أجريت الدراسة للمدة من تشرين الثاني 2008 ولغاية شهر تشرين الأول 2009. تم عزل بكتريا السالمونيلا من خلال الزرع البكتيري للعينات على أوساط زرع أغانيه و انتقائية وشخصت من خلال الاختبارات الكيميوحيوية واستعمل Mini API 20E و كما تم استخدام اختبار التلازن باستعمال المصل المضاد أحادية التكافؤ. استعملت تقنية تفاعل سلسلة البلمرة (PCR) للكشف عن الجين المشفر للهدب (*fimC*) الخاص ببكتريا *S. typhimurium*. أظهرت النتائج ان نسبة عزل بكتريا السالمونيلا *Salmonella* spp. من عينات براز الأطفال كانت 7.9% (38/480)، إذ وجدت فروق معنوية عند مستوى احتمالية ($P < 0.01$) في نسبة العزل عند استخدام الطريقة المظهرية التقليدية والتنميط المصلي. أظهرت نتائج التنميط المصلي لعزلات السالمونيلا باستعمال المصل المضاد أحادي التكافؤ ان 30 من أصل 34 عزلة جرثومية مختبرة (88%) في الأطفال تعود الى النمط المصلي *S. typhimurium* و عزلتين تعودان الى كل من *S. enteritidis* و *S. muenchen*. أظهرت تقنية الـ PCR للكشف عن الجين المشفر لتخليق الهدب (*fimC*)، أن 32 عزله سالمونيلا تعود إلى النمط المصلي *S. typhimurium*. لاحتوائها على هذا الجين و ظهور حزمة واحدة ناتجة من عملية التضخيم الـ DNA وكان حجمها 257 زوج قاعدي عند ترحيلها على هلام الاكاروز. كشفت نتائج هذه الدراسة ان تقنية الـ PCR أظهرت نوعية (Specificity) عالية (100%) في الكشف عن النمط المصلي *S. typhimurium* مقارنة بالفحوصات المصلية.