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Detection of *fimA* and *fimC* genes of *Salmonella* isolates by using Polymerase Chain Reaction

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Summary:

A total of 480 fecal samples were collected from children under 3 years of age of both sexes, suffering from diarrhea who admitted to the Teaching Hospital of Maternity and Pediatrics, in Al-Diwaniya governorate from November 2008 till the end of October 2009.

Salmonella spp. were isolated and identified using bacterial culturing on selective media, in addition to, biochemical, Mini API 20 E system and serotyping monovalent antisera. Polymerase chain reaction (PCR) was used to detect *fimA* gene encoding for biosynthesis of fimbriae A of *Salmonella* spp., and *fimC* gene encoding for biosynthesis of fimbriae C of *Salmonella typhimurium*. The results revealed that the rate of *Salmonella* isolates in fecal samples was (38/480) 7.9%. The results of serotyping *Salmonella* isolates by using monovalent antisera revealed that 30 out of 34 isolates that belong to *S . typhimurium*, while the remain belongs to *S. enteritidis* (2 isolates) and *S. meunchen* (2 isolates).

The PCR technique revealed that 34 isolates of *Salmonella* spp. contained *fimA* gene (DNA amplification showed one distinct band with molecular weight of 85 bp when electrophorised on agarose gel), while for *fimC* gene, 32 *Salmonella* isolates were *S. typhimurium* appeared to contain this gene (DNA amplification showed one distinct band with M. Wt. of 257 bp).The results of this study revealed that the PCR technique had a high specifity (100%) in the detection of *Salmonella* spp., *Salmonella typhimurium* in comparison to culture and biochemical test, Mini API 20 E and serological tests.

Introduction:

Salmonellosis is one of the most common infectious diseases in the world in both humans and animals[1]. *Salmonella* are among the leading causes of community that acquired food borne bacterial gastroenteritis worldwide[2]. *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[3].

S. typhimurium causes a host-dependent range of diseases from self-limiting to life-threatening systemic infections[4].

Traditional *Salmonella* detection methods are based on cultures using selective media and the 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* isolates from

clinical specimens[5].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[6].PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy[7].

The aim of study was to evaluate the rapid diagnostic test to identify salmonellosis using the PCR technique for the detection of *fim*A and *fimC* genes of *Salmonella* spp. and *Salmonella typhimurium*.

The purpose of this study was to compare the sensitivity and specificity of PCR with traditional isolation and characterization methods currently used in diagnostic laboratories.

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˚[8].This study was conducted during the period that extended from November 2008 to October 2009.

- Isolation and Identification of *Salmonella* isolates: 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 37C˚ for 24 hrs. The biochemical characters of non – lactose fermenting bacteria was determined by using TSI agar and Urease test and the other biochemical tests[8]. Colonies that showed biochemical characteristics similar to that of *Salmonella* spp. were tested by Mini API20 E and the confirmation was identified by PCR with *fim* A and *fimC* genes primers for the detection of *Salmonella* spp.[9]and *S. 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 *fim*A gene coding for biosynthesis of fimbriae A of *Salmonella* spp.[9] , which is provided by Bio Corp company (Canada) (Table 1). and *fimC* gene coding for the biosynthesis of fimbriae C of *S. typhimurium*[10], which is provided by Alpha DNA company (Canada) (Table 2).

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

Nucleotide Sequence	Orientation	Position	Size of PCR product
5-CCT TTC TCC ATC GTC CTG AA-3	Forward	586-605	85 bp
5-TGG TGT TAT CTG CCT GAC CA-3	Reverse	651-670	

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

Table (2): Specific primers used for the detection specific sequence of *fimC* gene

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

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

Detection of *fim*A and *fim*C genes:

For the detection of *Salmonella* spp. and *S. typhimurium* 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. The PCR tubes containing an amplification mixture were transferred to thermocycler and started the program for amplification of the *fim*A gene as follows: 20 cycles of PCR, with 1 cycle consisting of 1 min at 94C˚ (denaturation), 30 s at 58 C˚ (annealing), and 1 min at 72 C˚ (primer extension). An additional cycle of 1 min at 94 C˚, 30 s at 58 C˚, and 5 min at 72 C˚ was also included[9].While PCR amplification program for *fimC* gene detection was carried out in a thermal cycler with a temperature programme consisting of the initial denaturation (1 min at 94 C˚), 35 amplification cycles (30 s at 90 C˚, 30 s at 54 C˚ , 60 s at 72 C˚), and the final extension (8min at 72 C˚)[10].

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 significance 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) x 100 = sensitivity, (d / d + c) x 100 = specificity. diagnostic accuracy = (a+d / a+b+c+d)x100 . also calculated positive predictive value (PPV) =(a / a + c)x 100 and negative predictive value (NPV) =(d / b + d)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.samples on enrichment and selective isolation was 7.9% (38/480) by using themedia.(Table 3).There were significant conventional culture methods of stool

differences (P < 0.01) between the positiveand negative results.

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

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 Used Lab Techniques in Diagnosis of *Salmonella* isolates:

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

Table (4): Different laboratory techniques used for *Salmonella* spp.

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

Table (5): Relationships among PCR technique (using *fimA* gene) and other used tests for detection *Salmonella* spp.

Tests		PCR method				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	+ve 34	34	100	0	0	50	Cal. X^2 =50 Tab. X^2=6.634 df=1 (Significant)
	-ve 16	0	0	16	100		

PCR results (using *fimA* gene) Versus culture and Biochemical Tests:

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

(100%,75%,92%,89.5%,100%) respectively.

PCR Results (using *fimA* gene) Versus Mini API 20E Test:

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

PCR Results (using *fimA* gene) Versus Serotyping Test:

The sensitivity, specificity, accuracy, PPV and NPV of the PCR and serotyping tests were 100% for all the above statistic parameter.

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. All the 12 isolates that gave negative results in serotyping gave negative results for PCR detection.There were significant differences (P<0.01) between the PCR and serotyping methods in the diagnosis of *S. typhimurium*.

Table (6): Relationship between PCR and serotyping for detection *S. typhimurium*:

Tests		PCR method by *fimC*				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.

Amplification of target DNA (*fim*A gene):

The results of PCR amplification which was performed on the DNA extracted from all the studied isolates were confirmed by the 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 is 85 bp for *fimA* gene (Fig.1) and 257 bp for *fimC* gene (Fig.2).

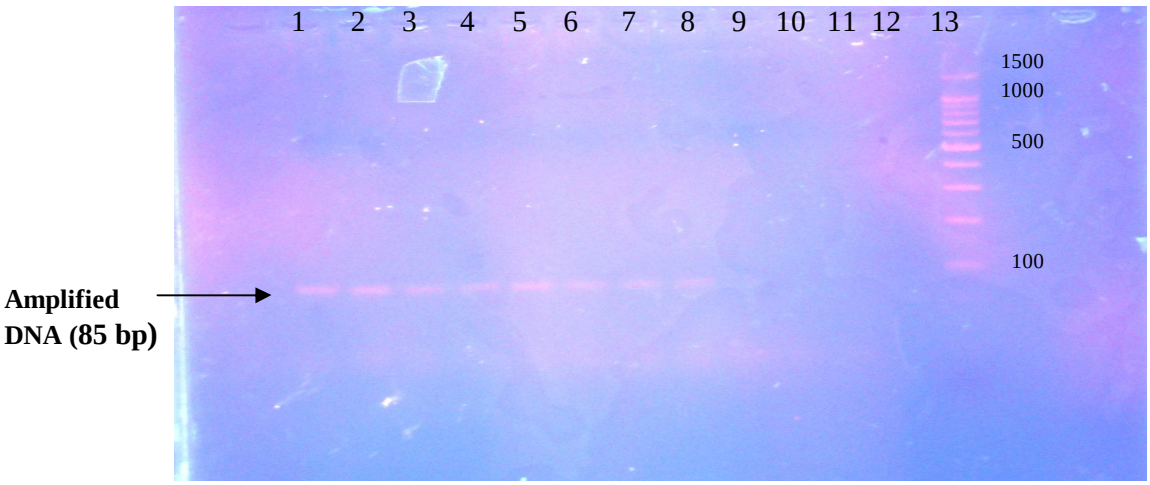

Fig.(1): DNA amplification of a 85 bp of *Salmonella* spp. detecting *fimA* gene. Lane1: control positive, lane2 ,3,4,5,6,7,8, positive results as *Salmonella* spp., lane 9, 10,11, negative results, lane 12 control negative, Lane 13:100bp marker.

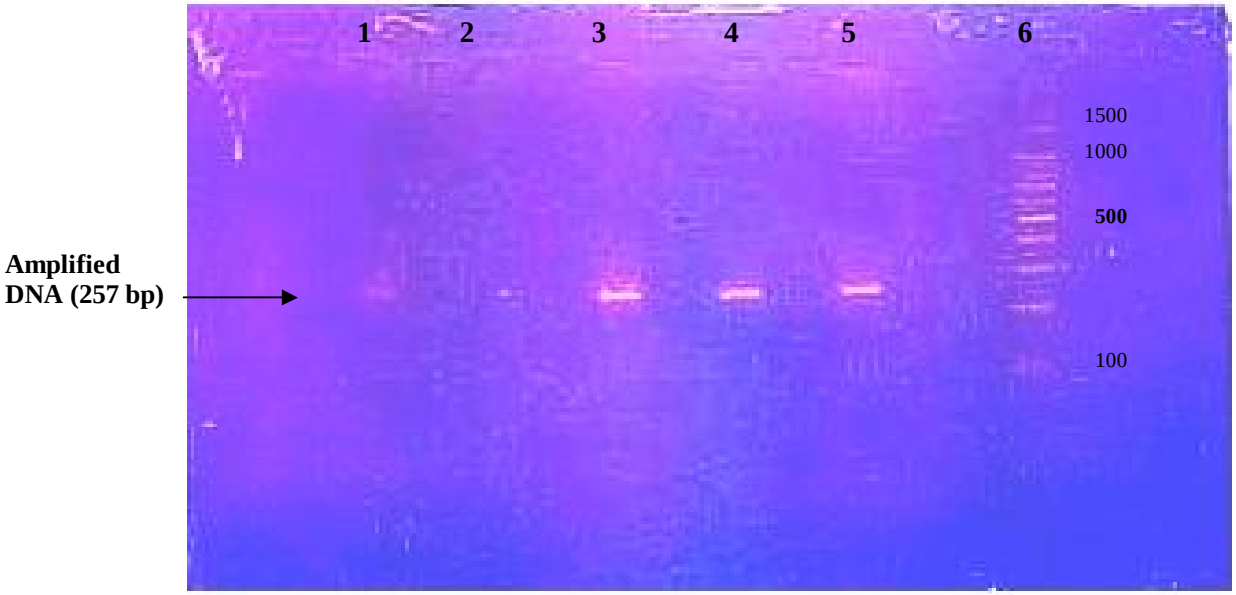

Fig.(2):DNA amplification of a 257 bp of *S. typhimurium* detecting *fimC* gene using PCR. Lane 1: Control positive, lane 2:control negative, lane:3,4,5 +ve results as *S. typhimurium*., lane 6 :100 bp marker.

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. enterica* serotype *typhimurium* is the most common serotype found in animals and in 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 the characterization of suspicious colonies by biochemical and serological tests[5]. Other studies also conducted in Al-Diwaniya province revealed the prevalence of *Salmonella* spp. There were: 10%, 14.47%, 8.47% respectively[13,14,15]. *Salmonella* detection in stool 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 necessitates time-consuming and expensive additional testing[16]. There were significant differences ($P < 0.01$) between conventional methods and PCR for the diagnosis of *Salmonella* spp. and we also found that PCR is more specific than conventional method, because the conventional methods for *Salmonella* spp., have very poor specificity, and there were numerous false- positive results[17,18]. 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 PCR for detection *fim*A gene at $p< 0.01$, and Mini API 20E test. While other studies evaluated API 20E and *invA* PCR for the identification of *S. enterica* isolates, and they found that API 20E had the highest agreement with PCR tests at the 99.9% likelihood level. The validation of both *invA* PCR and API 20E (at the 99.9% likelihood level) as accurate diagnostic tests, *invA* PCR is able to identify only *S. enterica* and not other *Enterobacteriaceae* as does API 20E, thus limiting its identification to one specific pathogen, both *invA* PCR and API 20E (at the 99.9% likelihood level) were demonstrated to be accurate methods for *S. enterica* identification and the results of animal samples production systems were in general agreement with those samples collected for the diagnosis in both veterinary and human medicine, thus indicating wide applicability of both diagnostic tools for *Salmonella* identification[19].

In this study, 34 isolates belong to *Salmonella* (by using PCR detecting *fimA* gene) detected as *Salmonella* isolates. The specific PCR product is an 85-bp fragment which was visualized by gel electrophoresis and ethidium bromide staining. All *Salmonella* isolates gave positive results by the PCR, the *fimA* gene contains sequences unique to *Salmonella* isolates and demonstrate that this gene is a suitable PCR target for the detection of *Salmonella* strains[9]. Non amplified DNA fragment was obtained from non *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 the detection of *S . typhimurium*[10]. Non amplified DNA fragment was 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[20,21].

Conclusions:

1. The polymerase chain reaction (PCR) technique gave a high specificity in comparison with the other who have done the test, with its advantages of greater speed and effectiveness than conventional detection method; it was successfully used to identify the *Salmonella* isolates.
2. Using PCR technique for direct stool samples without Lab. culture procedure gave a good result in the direct diagnosis of *Salmonella* spp.
3. We can detect *Salmonella* isolates based on *fim*A gene as *Salmonella* spp. and on *fimC* gene as *Salmonella 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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ألخلاصه:

جمعت 480 عينة براز من الأطفال دون سن ثلاث سنوات من الذكور والإناث الراقدين والمراجعين إلى مستشفى النسائية والأطفال التعليمي وكانوا 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 زرعية أغنائيه و انتقائية وشخصت من خلال الاختبارات الكيميوحيوية واستعمل Mini API 20E و كما تم استخدام اختبار التلازن باستعمال المصول المضادة أحادية التكافؤ . استعملت تقنية التفاعل التضاعفي لسلسلة الدنا (PCR) للكشف عن وجود الجين (*fimA*) المشفر للأهداب الخاصة بجنس السالمونيلا .*Salmonella* spp والجين(*fimC*) المشفر للأهداب الخاصة بالنمط *S. typhimurium*

اظهرت النتائج ان نسبة عزل بكتريا السالمونيلا .*Salmonella* spp من عينات براز الأطفال كانت (38/480)7.9% ، إذ وجدت فروق معنوية عند مستوى احتمالية ($P<0.01$) في نسبة العزل عند استخدام الطريقة المظهرية التقليدية والجينية (PCR) .

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. entritidis* و *S. muenchen*.

أظهرت تقنية الـــPCR المفرده للكشف عن الجين المشفر لتصنيع الهدب (*fimA*) ان جميع عزلات السالمونيلا تمتلك هذا الجين فقد ظهرت حزمة واحدة ناتجة من عملية التضخيم للـــDNA وكان حجمها 85 زوج قاعدي على هلام الاكاروز، فيما تبين من خلال الكشف عن الجين المشفر لتصنيع الهدب *fimC* إن 32 عزلة سالمونيللا تعود الى *S .typhimurium* لاحتوائها على هذا الجين اذ ظهرت حزمة واحدة ناتجة من عملية التضخيم للـــDNA وكان حجمها 257 زوج قاعدي.

كشفت نتائج هذه الدراسة إن تقنية الـــPCR أظهرت نوعية (Specificity) عالية (100%) في الكشف عن السالمونيلا (.*Salmonella* spp), *S. typhimurium* مقارنة بالفحوصات الأخرى الزرعية والكيموحيوية، فحص Mini API 20E وكذلك الفحوصات المصلية.