The use of *fim*A gene primers for detection of *Salmonella* spp. isolated from children with diarrhea.

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الخلاصه

جمعت 480 عينة براز من الأطفال دون سن ثلاث سنوات من الذكور والإناث الراقدين والمراجعين إلى مستشفى النسائية والأطفال التعليمي الذين كانوا 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 زرعيه أغنائيه و انتقائية وشخصت من خلال الاختبارات الكيميوحيوية واستعمل Mini API 20E و استعملت تقنية التفاعل التضاعفي لسلسلة الدنا (PCR) للكشف عن وجود الجين (*fimA*) المشفر للأهداب الخاصة بجنس السالمونيلا *Salmonella* spp. .

أظهرت النتائج إن 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 تقنية الـPCR ألمفرده للكشف عن الجين المشفر لتخليق الهدب (*fimA*) ان جميع عزلات السالمونيلا قيد الدراسة تمتلك هذا الجين فقد تظهرت حزمة واحدة ناتجة من عملية التضخيم للـDNA وكان حجمها 85 زوج قاعدي على هلام الاكاروز.

كشفت نتائج هذه الدراسة ان تقنية الـPCR أظهرت نوعية (Specificity) عالية (100%) في الكشف عن السالمونيلا (*Salmonella* spp.) . مقارنة بالفحوصات الأخرى الزرعية والكيموحيوية، فحص Mini API 20E.

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. Polymerase chain reaction (PCR) was used to detect *fim*A gene encoding for biosynthesis of *fim*A of *Salmonella* spp.

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The results revealed that the rate of *Salmonella* isolates in fecal samples of patients were (38/480) 7.9% using cultural and MiniAPI20 E, when the PCR technique was used to detect the presence of *fim*A gene,34 *Salmonella* isolates belong to *Salmonella* spp. appeared to contain this gene since DNA amplification showed one distinct band (size 85 bp) when electrophorised on agarose gel. The results of this study revealed that the PCR technique had a high specifity (100%) in detection of *Salmonella* spp. in comparison to cultural and biochemical and Mini API20E 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 acquired food borne bacterial gastroenteritis worldwide[2] . More than one-third of salmonellosis cases occur in children younger than10 years old, and the incidence in children younger than 1 year old is 10 times higher than in the general population[3]. 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[4] . 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[5] . PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy[6].

The aim of study

was to evaluate the rapid diagnostic test to identify salmonellosis using the PCR technique for the detection of *fim*A gene of *Salmonella* spp. 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°[7].This study was conducted during the period that extended from November 2008 to October 2009.

- Isolation and Identification of *Salmonella* spp. : 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 other biochemical tests[7]. 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 genes primers for detection of *Salmonella* spp.[8].

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 *fim* A of *Salmonella* spp.[8] , which is provided by Bio Corp company (Canada).

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

Sequence	Orientation	Position	Size of PCR product (bp)
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.

The *fim*A Gene Detection

For the detection of *Salmonella* spp. by PCR the specific primers of *fim*A gene which is responsible for biosynthesis of *fim*A gene of *Salmonella* spp. were used. The PCR amplification mixture (25µl) which was used for the detection *fim*A 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 amplification mixture were transferred to preheated thermocycler and started the program as in the following reaction was taken through 20 cycles in a DNA thermal cycler. Each cycle consisted of the following: 94°C for 1 min (denaturation), 58°C for 30 sec (annealing), and 72°C for 1 min (extension),and additional cycle consist of the following: 94°C for 1 min (denaturation), 58°C for 30 sec (annealing), and 72°C for 300 sec (extension).

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) 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[9].

Results

Clinical manifestations

Several distinct clinical syndromes were showed in patients infected with nontyphoidal *Salmonella* depending on host factors and the specific serotype involved. After an incubation period of 6-72 hrs (mean, 24 hrs), there was an abrupt onset of nausea, vomiting and crampy abdominal pain primarily in the periumbilical area and right lower quadrant, followed by mild to severe watery diarrhea and sometimes by diarrhea containing blood and mucus, fever (38.5-39 C^{o}), some children developed severe disease with high fever, headache, drowsiness, confusion meningism, seizures and abdominal distension, abdominal examination revealed some tenderness. Symptoms subside within 2-7 days in healthy children, fatalities were rare.

Laboratory investigations:

1: 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	

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 (3) Different laboratory techniques used for *Salmonella* isolates.

Test	Culture and biochemical		Mini API20E		PCR	
	+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 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		

PCR Results Versus Selective Media 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 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:

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).

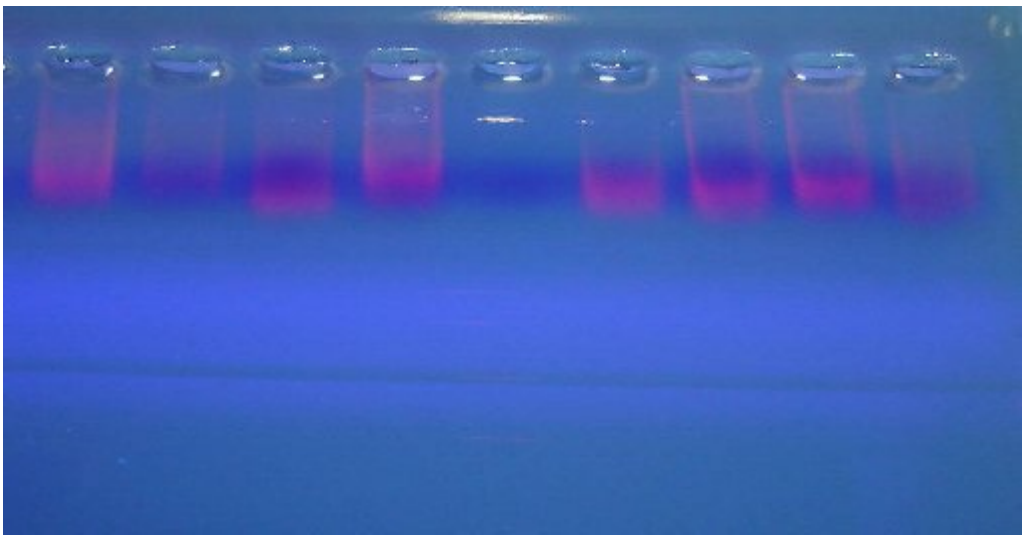

Figure1: Total genomic DNA extracted of isolates using 1 % agarose gel electrophoresis.

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 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 85 bp (Figure2).

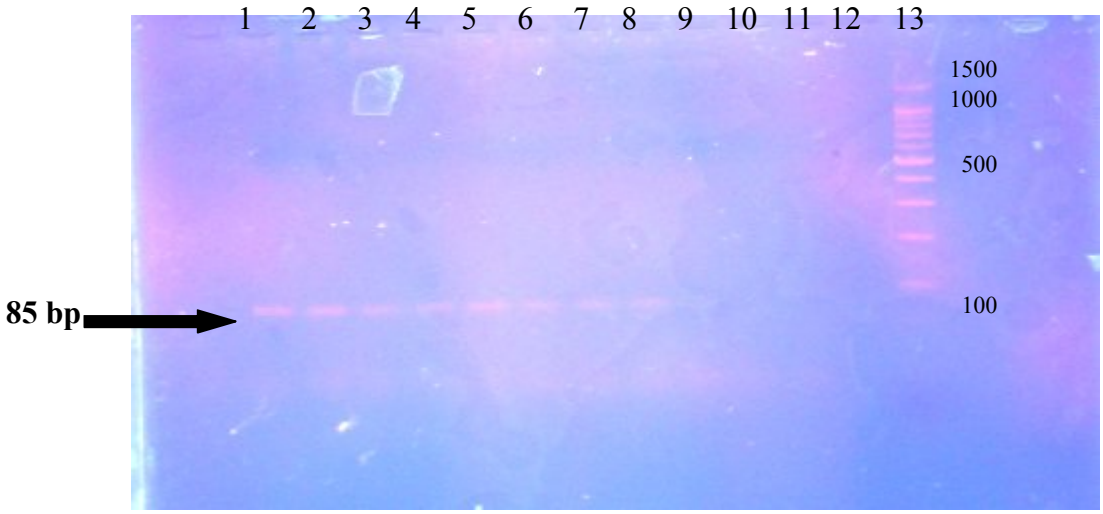

Figure (2) DNA amplification of a 85 bp of *Salmonella* spp. detecting *fimA* gene. Lane1: control positive, lane 2, 3, 4, 5, 6, 7, 8, positive results as *Salmonella* spp., lane 9 ,10, 11, negative results, lane 12 control negative, Lane 13:100bp marker (Ladder)

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[(10)] . 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[(4)] .

Other studies also conducted in Al- Diwaniya province revealed the prevalence of *Salmonella* spp. there were: 10%,

14.47%, 8.47% respectively [11, 12, 13] . *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[14]. We found a significant differences (P < 0.01) between conventional methods and PCR for 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[15,16]. 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 study 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. 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 diagnosis in both veterinary and human medicine, thus indicating wide applicability of both diagnostic tools for *Salmonella* identification[17].

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 detection of *Salmonella* strains[8] . Non amplified DNA fragment were obtained from non *Salmonella*

spp. The PCR technique provides a new strategy for rapid and sensitive detection of *Salmonella* strains[18].

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 spp*.
2. Using PCR technique for direct stool samples without Lab. culture procedure gave a good result in direct diagnosis of *Salmonella* spp.
3. We can detect *Salmonella* isolates based on *fim*A gene as *Salmonella* spp.

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.

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