Detection of *Rfbj (B)* Gene of *Salmonella* Serogroup-B Isolated from Patients with Salmonellosis

Alaa Abdel-Kadhim Jawad , Adnan Hamad Al-Hamadani and Aqeel Reheem Hassan

**College of medicine /Al-Qadisiya University*

Summary

A total of 480 fecal samples were collected from patients (less than 18 years old), of both sex suffering from diarrhea who admitted to Al-Diwaniya Teaching Hospital and the Teaching Hospital of Maternity and Pediatrics in Al- Qadisiya governorate. *Salmonella* spp. was isolated and identified using bacterial culturing on selective media, in addition to, biochemical and serotyping by monovalent antisera. Polymerase Chain Reaction (PCR) was used to detect *rfbj (B)* gene encoding for biosynthesis of LPS of group B *Salmonella*. The results revealed that the rate of *Salmonella* isolates in fecal samples of patients were 7.9% using cultural and biochemical methods .The result of *Salmonella* isolates by serotyping using monovalent antisera revealed that 30 out of 34 isolates (88 %) belong to *S.* Typhimurium serotype , while the remain belong to *S.* Enteritidis (2 isolates) and *S.*Meunchen (2isolates). when the PCR technique was used to detect the presence of *rfbj*(B) gene, 34 *Salmonella* isolates belong to *Salmonella* serogroup (B) appeared to contain this gene since DNA amplification showed one distinct band (882 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* serogroup B in comparison to cultural, biochemical 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 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* serotypes 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)]. Serotyping of *Salmonellae* is a valuable phenotypic subtyping tool for understanding the epidemiology of this important food-borne pathogen[(7)]. Serotyping have limitations; production and quality control of the hundreds of antisera required for serotyping is difficult and time consuming , and not all *Salmonella* strains are typeable[(8)]. *Salmonella* isolates are serotyped using the Kauffmann-White scheme according to their O , H , and Vi antigens[(9,10)] .Many of the genes required for O-antigen biosynthesis are organized in a large regulon termed the *rfb* gene cluster [(11)]. *rfb* gene clusters have been

characterized from a growing number of gram-negative bacteria; this operon is located between *galF* and *gnd* in *Salmonella enterica* and *Escherichia coli*[12]. Typically, three classes of genes are found in *rfb* clusters: (i) genes for synthesis of nucleotide sugars specific to the respective O antigen , (ii) sugar transferase genes to build the O subunit , and (iii) the O-antigen polymerase (*wzy*) and transport protein (*wzx*) genes for assembly of the O subunit into the O antigen[12]. The aim of study was to evaluate the rapid diagnostic test to identify salmonellosis using PCR technique for the detection of *rfbj(B)* gene to differentiate among *Salmonella* serogroups.

Materials and Methods

-**Samples Collection:** A total of 480 stool samples were collected from inpatients and outpatients (both sex) with diarrhea who were admitted to Al-Diwaniya Teaching Hospital and 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°[13]**.**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 Salmonella-Shigella agar (S.S), Xylose-lysine deoxycholate (XLD) and Brilliant green agar (BGA) plates and then incubated at 37C° for 24 hrs. The biochemical characters of non – lactose fermenting bacteria was determined by using Triple Sugar Iron agar (TSI) and Urease test [13]. Colonies that showed biochemical characteristics similar to that of *Salmonella* spp. were tested by serogrouped using monovalent antisera in central health laboratory/ Baghdad and the confirmation was identified by PCR with , *rfbj(B)* genes primers for detection of *Salmonella* serogroup B[14].

PCR method: DNA Extraction and Purification: The DNA of all isolates were extracted using genome DNA purification kit.(DNA-sorbB) provided by Sacacebiotechnologies, Italy. **Primers**: The primers were used for the detection of *rfbj* (B) gene coding for biosynthesis of lipopolysaccharide (LPS) of group B *Salmonella* according to [14], which is provided by Alpha DNA company (Canada)(Table 1).

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

Nucleotide sequence	position	Tm(**C**°)	Size of PCR products(bp)
Forward 5′-AGAATATGTAATTGTCAG-3′	15-32	48	~882
Reverse 5′-TAACCGTTTCAGTAGTTC-3′	880-897	50	

The *rfbj (B)* Gene Detection:

For the detection of *Salmonella* serogroup B by PCR the specific primers of *rfbj (B)* gene which is responsible for biosynthesis of lipopolysacharide (LPS) of *Salmonella* serogroup B were used. The PCR amplification mixture (25µl) which was used for the detection *rfbj(B)* gene includes 12.5 µl of (Green master mix, 2x,which provided by promega ,U.S.A)include: 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 25 cycles in a DNA thermal cycler. Each cycle consisted of the

following: 94°C for 1 min (denaturation), 45°C for 1 min (annealing), and 72°C for 2 min (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 110V 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 [14].

Amplification of target DNA (*rfbj*(B) gene):The PCR amplification which was performed on the DNA extracted from all the studied isolates were confirmed by electrophoresis analysis. The electrophoresis was also used to estimate DNA weight depending on DNA marker (100 bp DNA ladder).

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[15].

Results

Laboratory investigations: Culture, serotyping and PCR: 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 (Table 2).There was a significant differences (P<0.01) between the positive and negative results.

Table 2. Percentage of *Salmonella* spp. isolation 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, 38cases (100%) gave positive results for culturing and biochemical tests, 34 cases (89.5%) gave positive results for serotyping and 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, serotyping and PCR (Table 3

Table 3. Different laboratory techniques used for *Salmonella* spp. and non-*Salmonella* isolates .

Test	Culture		serotyping		PCR	
	+ve	-ve	+ve	-ve	+ve	-ve
Study group	38	0	34	4	34	4
Non *Salmonella* isolates	0	12	0	12	0	12

PCR Results: The result of the amplified DNA was 882 bp when compared with 100bp marker.

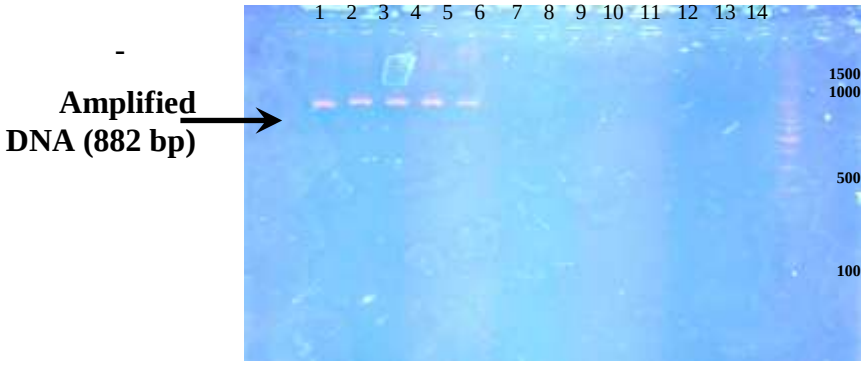

Figure 1. PCR amplification of a882 bp of *Salmonella* Serogroup B *rfbj*(B) gene. Lane 1:control positive , lane 2,3,4,5, positive results, lane 6,7,8,9,10,11,12, negative results, lane 13 : control negative , lane 14:100bp DNA marker

Comparison of PCR technique and Other Used Tests for Detection of *Salmonella* spp; Out of 38 cases that gave positive results as *Salmonella spp* on culture media and biochemical tests, 34 cases (89.5 %) were positive for serotyping and PCR techniques and 4 cases (10.5 %) were negative for PCR and serotyping tests (Table 4). Out of 12 cases that gave negative results in culture and biochemical tests, these 12 cases (100%) gave negative for PCR, serotyping tests. There were significant differences (P<0.01) between the PCR and culture media methods and serotyping in diagnosis *Salmonella* spp. (table 4).

Table 4. Comparison of PCR technique and other used tests for detection *Salmonella* spp.and *Salmonella* serogroup -B

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	100	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	100	Cal. X^2 =50 Tab. X^2=6.634 df=1 (Significant)
	-ve 16	0	0	16	100			

PCR results Versus Selective Media and Biochemical Tests: The sensitivity, specificity, accuracy PPV and NPV of 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 Serotyping Test The sensitivity, specificity, accuracy, PPV and NPV of the PCR and serotyping tests were 100% for all the above statistic parameter.

Comparison between PCR and Serotyping tests for Detecting *Salmonella* serogroup B:

Out of 34 cases from *Salmonella* spp. that gave positive on serotyping , there were 30 cases positive for *Salmonella* serogroup B and 4 cases (2 cases *S*.Enteritidis and 2 cases *S*.Meunchen).In PCR, there were 34 cases positive to group B *Salmonella* which gave positive results to *rfbj*(*B*). Out of 16 cases that gave negative results in serotyping also gave

negative results for PCR test. There were significant differences (P<0.01) between the PCR and serotyping methods in diagnosis *Salmonella* serogroup B (Table 5).

Table 5.Relationship between PCR and serotyping for detection *Salmonella* serogroup B

Tests		PCR method				Total		X^2 value (p<0.01)
		+ve		-ve				
		No.	%	No.	%	No	%	
Serotyping method	+ve 30	30	100	0	0	50	100	Cal.X^2=35.29 Tab.X^2=6.634 df=1 (Significant)
	-ve 20	4	0	16	100			

PCR Results Versus Serotyping for Detecting *Salmonella* sergroup B :The sensitivity, specificity, accuracy, PPV and NPVofPCRtestwere (100%, 80%, 92%, 88.2%, 100%) respectively while for serotyping, there were (88.2%, 100%, 92%,100%, 80%) respectively.

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 in humans[16]. In this study, we found 38(7.9%) suspected isolates of *Salmonella* spp. on culture and biochemical tests. Other studies also conducted in Al-Diwaniya province revealed the prevalence of *Salmonella* spp.: 10%, 14.47%, 8.47% which were reported by**,** [17,18,19] respectively. *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[20].The study revealed that significant differences (P < 0.01) between conventional methods and PCR for diagnosis of *Salmonella spp*. and the 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[21,22]. Furthermore, serotyping of isolates by monospecific antisera, we found 30 (88 %) *S.*Typhimurium isolates, 2 (6 %) *S.*Enteitidis and 2(6 %) *S.*Meunchen; while according to the results of PCR, 32isolates (94 %) were *S.*Typhimurium and 2 isolates (6 %) belong to *Salmonella* group B but no *S.* Typhimurium. The results revealed that there were significant differences between PCR and serotyping for the diagnosis of *Salmonella* serogroup *B* and we found PCR is more specific than serotyping in the diagnosis of *Salmonella* serogroup B, 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[23,24]**.**The results showed that all *Salmonella* spp. isolates 34(100%) belong to serogroup B by using specific primer set to confirm the isolation as positive *Salmonella* by detecting *rfbj*(B) gene. The selected *rfbj* gene sequences were proved to be useful DNA-based markers for identification and differentiation among *Salmonella* serogroups A, B, C2, and D[14], all PCR products of isolates screened by PCR results in 882 bp amplified fragment.

Non amplified DNA fragment were obtained from non *Salmonella* serogroup B.

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 classify *Salmonella* isolates based on, rfbj *(B)* gene as *Salmonella* serogroup B.

Recommendations

1. PCR is very 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**.**

References

1. Stone, G . G.; Oberst, R. D.; Hays, M. P.; McVey, S. and Chengappa, M. M. (1994). Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. J. Clin. Microbiol. 32:1742–1749.
2. Todd, E.C. (1997). Epidemiology of foodborne diseases: a worldwide review. World Health Stat Q .50: 30–50.
3. United States Department of Agriculture. Report to Congress. (1998). FoodNet: An Active Surveillance System for Bacterial Foodborne Diseases in the United States.
4. Alvarez, J.; Sota, M.; Vivanco, A. B.; Perales, I.; Cisterna, R.; Rementeria, A. and 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.
5. Arrach, N.; Porwollik, S.; Cheng, P.; Cho, A.; Long, F.; Choi, S. and McClelland, M. (2008). *Salmonella* Serovar Identification Using PCR-Based Detection of Gene Presence and Absence .J. Clin . Microbiol .46(8): 2581–2589.
6. Pickup, R. W.; Rhodes, G. and Hermon-Taylor, J. (2003). Monitoring bacterial pathogens in the environment: advantages of a multilayered approach. Curr. Opin. Biotechnol. 14:319–325.
7. Fitzgerald, C.; Collins, M.; van Duyne, S.; Mikoleit, M.; Brown, T.and Fields, P. (2007). Multiplex, Bead-Based Suspension Array for Molecular Determination of Common *Salmonella* Serogroups J. Clin.Microbiol.45(10) 3323–3334
8. Fitzgerald, C.; Gheesling, L.; Collins, M. and Fields. P. I.(2006). Sequence analysis of the *rfb* loci, encoding proteins involved in the biosynthesis of the *Salmonella enterica* O17 and O18 antigens: serogroup-specific identificationby PCR. Appl. Environ. Microbiol. 72:7949–7953.
9. Brenner, F. W. and McWhorter-Murlin. A. C. (1998.) .Identification and serotyping of Salmonella. Centers for Disease Control and Prevention, Atlanta, GA.
10. Popoff, M. Y.; Bockemuhl, J.;Brenner, F. W. and L. L. Gheesling.(2001). Supplement 2000 (no. 44) to the Kauffmann-White scheme. Res. Microbiol.152:907–909.
11. Reeves, P. R. and Wang, L. (2002). Genomic organization of LPS-specific

loci. Curr. Top. Microbiol. Immunol. 264:109–135.
12. Samuel, G. and Reeves, P. R. (2003). Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. Carbohydr. Res. 338:2503–2519.
13. Collee, J. G.; Fraser, A.G.; Marmion, B.P. and Simmons, A.S. (1996). Practical medical microbiology .14th[ed]. Churchill living stone, N.Y.
14. Luk, J. M. C.; Kongmuang, U.; Reeves, P. R. and Lindberg't, A. A. (1993). Selective Amplification of Abequose and Paratose Synthase Genes (rfb) by Polymerase Chain Reaction for Identification of *Salmonella* Major Serogroups (A, B, C2, and D). J. Clin Microbiol .31: 2118-2123.
15. Niazi, A.D.(2000). Statical Analysis in Medical Research . Republic of Iraq.Al-Nehrein university.P148.
16. McDonough, P. L.; Shin, S. J. and Timoney, J. F. (1986). *Salmonella* serotypes from animals in New York State, 1978–1983. Cornell Vet. 76:30–37.
17. Al–Karawiy, H.A.M(2008). Isolation and identification of *Salomonella typhimurium* and detection of gene encoded type -1- fimbraiby using polymerase chain reaction. M.Sc. Thesis , College of Veterinary Medicine , University of Basrah.
18. Al – Janabi, J.K. (2001). Characteristics of *Salmonellae* isolated from children with diarrhea In Al-Diwaniya City. M.Sc. Thesis, College of Education, Al–Qadisiya Univer-sity.
19. Al – Janabi, J.K. (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 serotyping. J. Clin. Microbiol. 46:685–688.
20. Dijk, S. V.; Bruins, M. J.; Gijs, J. H. and 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.
21. Dusch, H. and Altwegg, M. (1995). Evaluation of five new plating media for isolation of *Salmonella* species. J. Clin. Microbiol. 33:802–804.
22. Perez, J. M.; Cavalli, P.; Roure, C.; Renac, R.; Gille, Y. and Freydiere, A. M.(2003). Comparison of Four Chromogenic Media and Hektoen Agar for Detection and Presumptive Identification of *Salmonella* Strains in Human Stools. J. Clin. Microbiol.41 (3): 1130–1134.
23. Kim, S.; Frye, J. G.; Hu, J.; Fedorka-Cray, P. J.; Gautom, R. and Boyle, D. S.(2006). Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. J. Clin. Microbiol. 44:3608–3615.
24. Schrader, K.; Fernandez-Castro, A.; Cheung, W. K.; Crandall, C. M. and Abbott, S.(2008). Evaluation of commercial antisera for *Salmonel.*