Conventional and Molecular Typing of *Salmonella enterica* serotype Typhi Locally Isolated In Baghdad

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Received 20, December, 2010
Accepted 11, March, 2012

Abstract:

Phenotypic And genotypic characteristics of *Salmonella enterica* serotype Typhi have been determined for 29 isolates, from Baghdad in 2007. Conventional typing methods were performed by biochemical tests, and antimicrobial susceptibility test. Molecular typing performed by analysis plasmid DNA beside using the Random Amplified Polymorphic DNA (RAPD-PCR). For the latter, two universal primers that have selected for the high discriminatory power were used for RAPD analysis. All isolates were belong one biotype according to the differention by their ability to decarboxylat lysine, 29(100%) were lysine (+). All the isolates were susceptible to the Antibiotics used. However, all the strains free of plasmids. RAPD was capable of grouping the strains in 6 genotypic patterns using primer 784, in 4 genotypic patterns using primer 787. Conventional phenotypic typing methods, as well as the DNA plasmid analysis, presented non significant discriminatory power; however, RAPD-PCR analysis showed discriminatory power, reproducibility, easy interpretation and can be considered as a promising alternative typing method for *S.* Typhi.

Keywords: Typing; *Salmonella* Typhi; Random Amplified Polymorphic DNA-Polymerase Chain Reaction;

Introduction:

Salmonella enterica serotype Typhi is noteworthy in the etiology of outbreaks and sporadic cases of typhoid fever, which remains as an important public health problem, causing 16 to 17 million cases of the disease and about 600,000 deaths, annually, all over the world [1,2]. The investigation of the epidemiology of *S.* Typhi relevant, mainly in those areas where typhoid fever is endemic, resulting in a clear evaluation about the dissemination of strains and the establishment of prophylactic strategies. Molecular methods for characterization of *S.* Typhi strains have been developed and improved to complement conventional phenotypic -typing and allow the differentiation of non-related strains that belong to the same phage type, for example, multilocus enzyme electrophoresis [3], ribotyping [4] , and pulsed field gel electrophoresis PFGE [5,6]. However, these techniques were all time consuming and technically demanding. A PCR–based typing method, RAPD-PCR (Random Amplified Polymorphic DNA), has been described as a simple and rapid method able to offer detailed fingerprinting of the genomic composition of the organism [7,8] the success of this method is due to the fact that no prior sequence information about the target is needed and a single short 10-mer oligonucleotide primer can be used in the reaction. The amplification happens at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the DNA sequence variations may

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work as genetic markers that can be used in epidemiologic studies. Quintaes, *et al*. [9] have been demonstrated that RAPD-PCR reaction had the potential to provide a discriminatory, reproducible and easy to interpret method to type *Salmonella enteric* serovar Typhi strains. However, in order to use RAPD-PCR for differentiation between bacterial strains, the optimization of the reaction is imperative to eliminate most of the variations that are sometimes observed in duplicate DNA profiles [10]. Quintaes, *et al*. [11] reported and optimized of RAPD-PCR to *Salmonella enterica* serovar Typhi DNA using 10 mer oligonucleotide primer and demonstrate the effects in the fingerprint pattern caused by varying the target DNA, MgCl2 and *Taq* DNA polymerase enzyme concentrations and the thermal cycling profile, also evaluate a total DNA extraction methodology, observing its time consumption and the stability of the resulting genetic material. In the present study the conventional system for typing *Salmonella enterica* serotype Typhi (biotyping, antimicrobial susceptibility) was evaluated and its performance compared to molecular typing methods (plasmid and RAPD-PCR analysis).

Materials And Methods:

Bacterial isolates

Salmonella Typhi isolates were obtained from blood samples (Table 1) and maintained in stock agar culture [12] at Central Public Health Laboratory(CPHL) Department of Bacteriology/ Enteric Diseases. The isolates were preliminarily grown in Brain Heart Infusion (Difco) for 24h at 37 ºC Standard laboratory methods to isolate *Salmonella* ser. Typhi from blood samples were followed Bopp, *et al*.[13]. The conventional laboratory procedure involved culturing on Kliglar's iron agar for fermentation and H_2S production, Urea agar for detection of urease , Simmon's citrate agar for detection of citrate utilization, semi solid mannitol for fermentation of mannitol and motility, peptone water for indol production and others ,the isolates were confirmed as *S.* Typhi by the biochemical characteristics and serotyped based on the Antigenic formulas of the *Salmonella* serovars Kuffman and White scheme was followed for serological confirmation and typing of *Salmonella* ser. Typhi by using Anti-Salmonella sera contain Salmonella: polyvalent O, polyvalent H Specific O-group: 9.12(D) and Vi Specific H: d prepared by Biomerieux laboratory. One drop of agglutinating sera of Salmonella Typhi was placed on a clean glass slide. One colony of test strain was picked up with a loop from a MacConkey's agar plate. The bacterial culture and agglutinating serum were mixed slowly with a sterile stick. When fully mixed the slide was rotated for 5-10 second. The agglutination was watched by naked eye. positive and negative controls were tested in similar way on the same slide. [13]. The bacteria were further investigated for biotyping. According to the classification proposed by The API 20E system which contains 20 different biochemical reaction including 10 enzymatic reactions, 10 fermentation oxidation reactions and an oxidas test was used. Susceptibilities to antimicrobial agents were determined by using the high-potency disk diffusion method [14]. The included antibiotics were ampicillin (10 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg) and trimethoprim-sulfamethoxazole (25 µg). Plasmid extraction All *S.* Typhi isolates were screened for plasmid content by the alkaline method Birnboim and Doly.,

1979 [15]. Electrophoresis of plasmid DNA was performed in 1X TBE at 7v/cm for 1.5 h on 0.8% agarose gels, afterwards stained with ethidium bromide. Electrophoretic separation of plasmid species by molecular weight and subsequent size estimations using Lambda DNA/*Hind*III Markers (Promega, USA).

RAPD- PCR

The RAPD analysis was performed by Total DNA Extraction which was extracted from bacterial culture of *S.* Typhi using Wizard Genomic DNA Purification Kit, Promega (USA). The DNA was quantified, after electrophoresis in a 1% agarose gel. The RAPD-PCR Amplification was performed using two different primers according to the protocol of Quintaes *et al*. [9] . The sequence of primers were used, Primer 784 - 5'GCG GAA ATA G 3' and primer 787 - 5'AAC GCG CAA C 3' .The reaction was prepared using 50 µl per-tube, containing 20 ng DNA of each isolates, 1 unit of *Taq* DNA polymerase enzyme (promega/USA), 1x PCR buffer, 2.0 mM $MgCl_2$, 200 µM of each dNTP, and 20 Pmol of primer. It was carried out in a thermal cycler programmed for 30 cycles composed of one step of denaturation for 1 min at 94 °C, one step of annealing for 1 min at 36 °C followed by one step of synthesis for 2 min at 72 °C. Amplification products were run on 1.5%agarose gel electrophoresis followed by staining in ethidium bromide, and visualized by UV transilluminator. A negative control was included in each PCR run with no target DNA. The ladder 1 kbp (Pomega/USA) were employed as molecular markers for bands weight.

Table(1): Phenotypic and genotypic characteristic of *Salmonella* serotype Typhi isolated in Baghdad

isolates No.	Biotyping	Antibiogram	Plasmid	RAPD-PCR primer784	RAPD-PCR prime 787
ST1	I	A1	-	R5	D1
ST 2	I	A1	-	R2	D1
ST 3	I	A1	-	R2	D1
ST 4	I	A1	-	R2	D2
ST 5	I	A1	-	R5	D2
ST 6	I	A1	-	R5	D1
ST 7	I	A1	-	R5	D1
ST 8	I	A1	-	R2	D1
ST 9	I	A1	-	R2	D1
ST 10	I	A1	-	R5	D1
ST 11	I	A1	-	R4	D2
ST 12	I	A1	-	R4	D3
ST 13	I	A1	-	R4	D1
ST 14	I	A1	-	R2	D4
ST 15	I	A1	-	R2	D4
ST 16	I	A1	-	R2	D4
ST 17	I	A1	-	R2	D1
ST 18	I	A1	-	R5	D1
ST 19	I	A1	-	R5	D1
ST 20	I	A1	-	R2	D1
ST 21	I	A1	-	R1	D4
ST 22	I	A1	-	R6	D4
ST 23	I	A1	-	R2	D4
ST 24	I	A1	-	R3	D1
ST 25	I	A1	-	R3	D1
ST 26	I	A1	-	R2	D3
ST 27	I	A1	-	R1	D3
ST 28	I	A1	-	R4	D1
ST 29	I	A1	-	R4	D1

ST: *Salmonella* serotype Typhi A: Antibiogram pattern R: RAPD-PCR Primer 784 pattern D: RAPD-PCR Primer 787 pattern

Results :

Based on biochemical findings one biotypes were observed (Table 1). They were differentiated by their ability to decarboxylat lysine. 29 isolates (100 %) were lysine (+). Antimicrobial sensitivity test of all the twenty nine isolates were done. All the isolates were uniformly susceptible to the antibiotics used. Depending on the antibiotic sensitivity findings one antibiogram patterns were obtained. Plasmid DNA extraction from the isolates showed that all the isolates 29(100%) did not show any plasmid even analysis. Genetic differences among *S.* Typhi isolates were assessed by genomic fingerprinting obtained by RAPD-PCR using two different oligonucleutides primer. The amplifications with primer 784 divided the 29 analyzed strains into 6 distinct RAPD profiles (Fig. 1), revealing fragments ranging from 400 to 2500 bp. Fragments of 400 bp are identical for all patterns except for pattern R3 where the 400bp band is absent. The profile R4 was prevalent in 41.37% of the strains (Table 1). Primer 787 divided the strains into 4 profiles (Fig. 2), showing less polymorphic bands patterns with fragments ranging from 400 to 2500 bp. Fragments of 600 bp are identical for all patterns .The profile D1 was dominating (58.62%) (Table 1) .When the combination of amplification products patterns, generated by two primers were used to subtype *S.* Typhi strains, we found out a high diversity and it was possible to divide them into 10 distinct types.

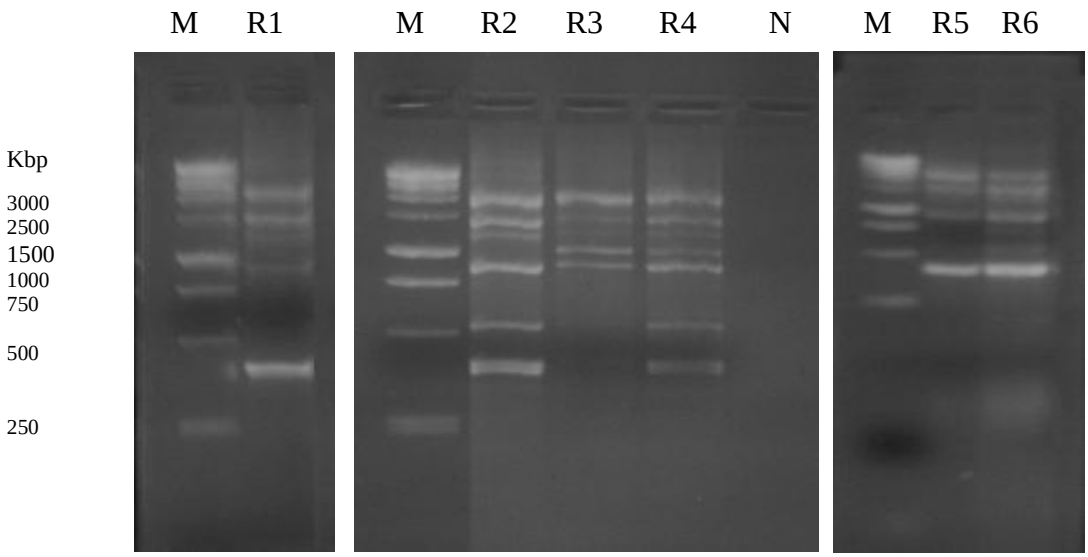

Fig 1.Representative profiles generated by RAPD-PCR using primer 784 lane:M. Marker 1 kbp(Promega,USA) :1.RAPD profile R1: 2.RAPD profile R2: 3.RAPD profile R3: 4.RAPD profile R4: 5. RAPD profile R5: 6. RAPD profile R6. lane:N. negative control PCR products were separated on 1.5% agaros gel, 7 v/cm, one hour electrophoresis.

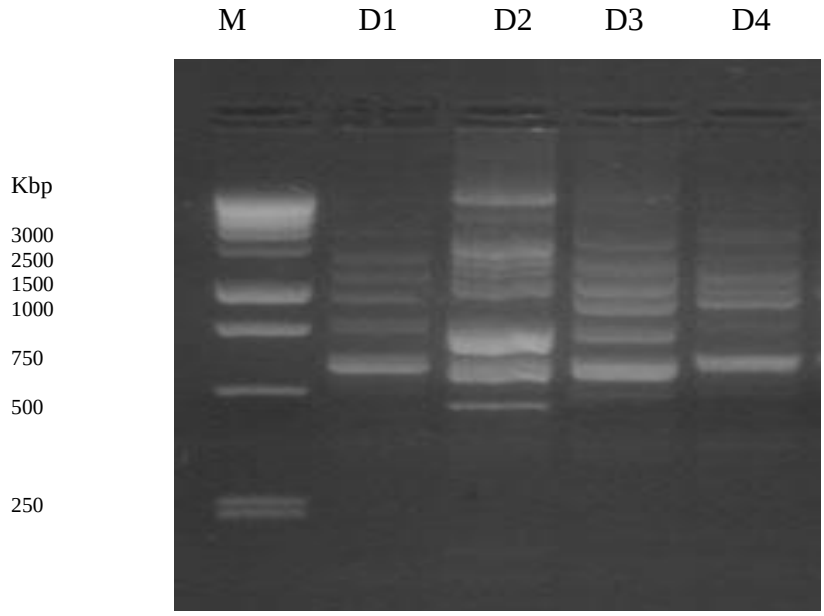

Fig 2.Representative profiles generated by RAPD-PCR using primer 787. lanes:M. Marker 1 kbp(Promega,USA) :1.RAPD profile D1: 2.RAPD profile D2: 3.RAPD profile D3: 4.RAPD profile D4. Products were separated on 1.5% agaros gel, 7 v/cm, one hour electrophoresis.

Discussion:

The presence of *S.* Typhi in Iraq areas as a recurrent pathogen and as the cause of typhoid fever worldwide has determined the need to monitor the epidemic spread of this microorganism. The Kauffmann-White serotyping scheme is the traditional method for primary characterization of *Salmonellae* in the diagnostic setting [15]. While valuable for diagnosis and epidemiology, it is considered to be inappropriate for phylogenetic purposes [16]. Thus, it is necessary to explore a range of other techniques to establish genetic relationships among *Salmonella* serotypes [17] In this study, Biotyping, relying on the 20 biochemical reactions in API 20E system showed only one pattern of biochemical reactions (Table 1). Thus biotyping revealed a limited ability to differentiate between strains within a species and so has a poor discriminatory power. These results agreed with other researchers [9]. Antibiogram showed susceptibility to all antibiotics used (Table 1). This result is consistent with others researchers[9,18]. Based on antibiogram findings epidemiological analysis revealed that all isolates were indistinguishable, thus antibiotic susceptibility test was not reliable as an epidemiological marker for discrimination of *S.* Typhi in this study.Plasmid profiles proved not to be a good epidemiological marker for *S.* Typhi when analysing strains. The analysis of *S.* Typhi plasmids would be more suitable to characterize their temporal occurrence in an endemic setting and their possible association with the typhoid fever severity [19] as well as for analysis of outbreaks of nosocomial infections. Plasmid profiles are not very useful for subtyping *S.* Typhi since less than 10% of the strains, in general, harbor plasmids [20]. Indeed, we found that all of the strains were free of plasmid. This could be explained as a result of plasmid instability in *S.* Typhi, rather than an inherent barrier to the entry or establishment of foreign plasmid DNA. To interpret fragment patterns generated by RAPD-PCR, we should

understand that the occurrence of random genetic events, including point mutation and insertions and deletions of DNA, can alter the RAPD fingerprinting patterns [9,21]. For this reason, we presumed the differentiating bands in the profiles could be due to one or more genetic events (Fig. 1 and 2). The discriminatory power of RAPD was tested by considering the number of profiles generated both with each primer separately, and combining the results obtained with the two primers (Table 1). When the RAPD profiles were analyzed considering the temporal occurrence, the prevalence of profiles R4 and D1 was evident. The traditional phenotypic typing methods used antibiogram typing and biotyping as well as plasmid profile, have low discriminatory power, while RAPD-PCR proved capable of discriminating between *S.* Typhi strains [22] RAPD-PCR assays are simpler, faster, more convenient and easier to perform low cost and potential to generate polymorphisms than most other molecular typing methods [21] .Our results suggest that there is a considerable possibility for increasing the efficiency of the RAPD-PCR reaction if a precise standardization protocol is determined. We have proposed a model that can be used as a support for typing strains of *S.* Typhi.

AcknowledgementsWe thanks. Ali H. AL-Wardy Director of Enteric Disease/Departmentofacteriology/CPH L/Baghdad for providing *Salmonella enteric* serotype Typhi isolates from Baghdad.

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دراسةالتنميط الجيني و التنميط المظهري لبكتريـــــا *Salmonella enterica serotype Typhi* في بغـــداد

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الخلاصـــة:

اجريت هذه الدراسة لايجاد مميزات التنميط المظهري و التنميط الجيني لـ 29 عزلة لبكتريا *Salmonella enterica* serotype Typhi جمعت في عام 2007 . شمل التنميط المظهري:التنميط الحيوي (Biotype) والتنميط للمضادات الحياتية .التنميط الجيني وقد شمل:المحتوى البلازميدي (plasmid content) و استخدام احدى مؤشرات الدنا (DNA Markers) المعتمدة على الـ PCR وهي مؤشرات التفاعل التضاعفي العشوائي المتعدد الاشكال لسلسلة الدنا او الـ Random Amplified Polymorphic DNA (RAPD) حيث اختير لهذا الغرض بادئان (primer) لتحديد الحزم المتباينة Polymorphic bands. أظهرت الفحوص الكيموحياتية للسالمونيلا التايفي29 (100 %) تحت نمط واحد باستعمال نظام API-20E اعتمادا على قابلية البكتريا لاختزال مجمو عة الكربوكسيلية لـــ لايسين و كان جميعها (+) و اعتمادا على حساسية العزلات للمضادات الحياتية، وجد أن 29(100%) عزلة كانت حساسة لجميع مضادات الحياة المستعملة .كذلك عند استخدم الترحيل الكهربائي لل DNA البلازميدي حيث أظهرت جميع العزلات المدروسة عدم امتلاكها للبلازميدات , عند إجراء التجارب الأولية لإيجاد الظروف المثلى لتفاعلات الـ PCR باستخدام دنا القالب والبادئات تم الكشف عن التباينات بين القطع المتضاعفة لكل عزلة (اعدادها واحجامها الجزيئية) عند ترحيل نواتج التضاعف للعينات على هلام الاكاروز بعد تصبيغها ببروميد الاثيديوم. اعطت النتائج RAPD-PCR عند استخدام لبادئ 784 ستة أنماط جينية مختلفة و عند استخدام البادئ787 اظهرت النتائج أربعة أنماط جينية مختلفة . لم يظهرالتنميط المظهري و كذلك تحليل المحتوى البلازميدي (plasmid profile) إمكانية التفريق بين العزلات لغرض المقارنة وتحديد الانماط بين العزلات المدروسة. بينما أظهرت طريقة RAPD -PCR وجود القدرة التمييزية لتحديد الانماط الجينية بين العزلات المدروسة وبهذا اثبتت هذه الطريقة فاعليتها كوسيلة تنميط وبائية مقارنة بالطرق الاخرى مثل التنميط الحيوي (Biotype) والتنميط للمضادات الحياتية و طريقة الكشف على المحتوى البلازميدي .