Multiplex PCR for Identification of *Salmonella enterica* serovars Typhi and Paratyphi A by Selective Amplification of *tyv, prt, viaB, fliC-d* and *fliC-a* Genes

استخدام تقنية تفاعل التضاعف المتعدد لسلسلة الدنا لكشف بكتريا *Salmonella enterica* serovars Typhi و Paratyphi A بالتحري عن الجينات *tyv, prt, viaB, fliC-d, fliC-a*

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Abstract

***Salmonellosis* is responsible for large number of infections in both human and animals. *Salmonella enterica* serovar Typhi is a causative agent of typhoid fever and *Salmonella enterica* serovar Paratyphi A is a causative agent of paratyphoid fever. Conventional methods of isolation of *Salmonella* strains take 4-6 days to complete and are therefore laborious and require substiantial manpower. Therefore development of a PCR assay that can target multiple genes for rapid detection of *S*. Typhi and *S*.Paratyphi A. Methods: Synthetic primers for O, H, and Vi antigen genes, *tyv* , *prt* , *fliC-d, fliC-a*, and *viaB*, were used for the rapid identification of *S*. Typhi and Paratyphi A with Multiplex PCR. Results: All the clinical isolates examined were accurately identified by this PCR technique, that differentiated *S*. Typhi and Paratyphi A, based on size and number of amplified fragments. *S. enterica* serovar Typhi, yielded four bands of *tyv(tyvelose epimerase gene, 615bp),prt (paratose gene, 258bp),flic-d(phage-1 flagellin gene for d- antigen 750bp) and viaB (vi antigen gene, 439bp); S.enterica* serovar Paratyphi A yielded only two bands *prt (paratose gene, 258bp)* and *flic-a (phage-1 flagellin gene for a- antigen 329bp).* Conclusion: These data indicate that multiplex PCR is a potentially valuable tool for rapid diagnosis of *Salmonella enterica* serovar Typhi and Paratyphi A from clinical isolates.**

Key words: *Salmonella enterica* serovar Typhi and Paratyphi A, Multiplex PCR

المستخلص

السالمونيلا تايفي هو العامل المسبب لحمى التيفوئيد والسالمونيلا باراتايفي هو العامل المسبب لحمى الباراتايفي . تم تشخيص و تفرقة هذه العزلات على المستوى الجزيئي لغرض تقليل الوقت و الكلفة في تشخيص لحمى التايفوئيد و الباراتايفي, باستخدام تقنية تفاعل التضاعف المتعدد لسلسلة الدنا (Multiplex-PCR) و الذي سيزيد من امكانية التشخيص المتكامل السريع لهذه البكتريا حيث تم التحري عن وجود او غياب خمسة أنواع من جينات هذه البكتريا (*tvy , prt, fliC-d, flic-a , ViaB*). طريقة العمل : صممت بادئات تركيبية لمستضدات O و H وvi و المشفرة لجينات ، *tyv*، *prt*، *flic-d*، *flic-a* و *viaB*، و قد أستخدمت هذه البادئات للكشف السريع عن السالمونيلا تايفي مع السالمونيلا باراتايفي باستخدام تقنية تفاعل التضاعف المتعدد لسلسلة الدنا (Multiplex-PCR). النتائج: أظهرت النتائج دقة البادئات في الكشف عن الجينات المدروسة في جميع العزلات السريريةباستخدام هذه التقنية مما تمكنا من التفريق بين السالمونيلا تايفي والسالمونيلا باراتايفي، استنادا إلى حجم وعدد من الحزم المضخمة. فبالنسبة للسالمونيلا تايفي، أظهرت أربعة حزم من *tyv* (جين *tyvelose epimerase*، 615bp) ، *prte* (جين*paratose*، 258bp)، *flic-d* (بالعاثية 1-جين flagellin المستضد 750bp) و *viaB* (*vi* مستضد الجينات، 439bp) ; و بالنسة للسالمونيلا باراتايفي أظهرت فقط حزمتين (*prt* جين *paratose*، 258bp)و *flic-a* (بالعاثية 1-جين *flagellin* عن مستضد 329bp). الاستنتاج: هذه البيانات تشير إلى أن تقنية تفاعل التضاعف المتعدد لسلسلة الدنا Multiplex-PCR) هي تقنية ذات قيمة عالية للتشخيص السريع لبكتريا السالمونيلا تايفي والسالمونيلا باراتايفي من العزلات السريرية.

الكلمات المفتاحية : التيفوئيد والسالمونيلا باراتايفي، البراتايفي A، تفاعل التضاعف المتعدد لسلسلة الدنا

Introduction

Salmonella enterica serovar Typhi and *Salmonella enterica* serovar Paratyphi A are two infectious agents responsible for enteric fever, which remains endemic in many parts of the world in which sanitary conditions remain poor and the water supply is not treated. Typhoid fever was generally considered as a more critical illness than paratyphoid fever but is generally being superseded by paratyphoid fever in the past decade [1].The clinical syndrome caused by paratyphoid fever overlaps with other febrile illnesses and cannot be distinguished from typhoid fever. It also results in fatal

infection among adults and children, if untreated causing bacteraemia and inflammatory destruction of the intestine and other organs [2]

In the year 2000 it was estimated that typhoid fever caused 21.7 million illnesses and 217,000 deaths, and paratyphoid fever caused an estimated 5.4 million illnesses worldwide . Infants, children, and adolescents in south-central and Southeastern Asia experience the greatest burden of illness. Typhoid and paratyphoid fever most often present as clinically similar acute febrile illnesses, and accurate diagnosis relies on laboratory confirmation [3].

The diagnosis of typhoid fever or paratyphoid fever is made by ordinary culture methods and biochemical tests. The classic diagnosis method for typhoid fever or paratyphoid fever requires at least 4 or 5 days for positive results. A rapid, alternative method is needed for the diagnosis of typhoid fever or paratyphoid fever[4]. Molecular biology based techniques including PCR assays have been reported for the rapid, specific, and sensitive detection of microorganisms in different clinical samples [5].

Some researchers have already reported serovar Typhi detection methods with PCR that use the *fliC-d* gene [6], the Vi capsular antigen gene [7], and the 16S rRNA gene [8]. As only one gene was targeted for the identification of serovar Typhi in these methods, strains of *Salmonella* serovars other than serovar Typhi were detected in some cases . Others have already used(Vi antigen gene , H antigen genes , and O antigen synthesis genes), for rapid detection of typhoid fever and paratyphoid fever based on a multiplex PCR technique that detected the Vi antigen gene (*viaB*), H antigen genes (*fliC-d* and *fliC-a*), and O antigen synthesis genes (*tyv* and *prt*). [2,4,9,10]. The gene *prt* encodes CDP-paratose synthase, which converts CDP-4-keto-3,6-dideoxyglucose to CDP-paratose. The gene *prt* is present in both serovars Typhi and Paratyphi A. The gene *tyv* encodes CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose. The *tyv* gene is present in both serovars Typhi and Paratyphi A, but the *tyv* gene of serovar Paratyphi A does not produce active CDP tyvelose epimerase due to the 1-bp deletion which causes the frameshift mutation and converts codon 4 of *tyv* to a stop codon [2].they used this deleted region for the design of primer *tyv-s* to specifically detect the *tyv* gene of serovar Typhi but not of serovar Paratyphi A. The *fliC-d* and *viaB* genes are present in serovar Typhi, and the *fliC-a* gene is present in serovar Paratyphi A.

Therefore, the aim of this study was genetically identification of clinical isolates of *Salmonella enteric* serovar Typhi and *Salmonella enterica* serovar Paratyphi A through multiplex PCR technique.

Materials and methods

Bacterial strains: The study included six isolates of *Salmonella enteric* serovar Typhi and three isolates of *Salmonella enterica* serovar Paratyphi A obtained in 2007 from the Central Public Health Laboratory (CPHL)- Enteric Department in Baghdad and diagnosed as *Salmonella* using conventional methods by biochemical tests, Kuffman and white scheme was followed for serological confirmation and typing using Anti-Salmonella sera (Biomerieux). Different isolates were used in this study provided from CPHL for the specificity test required for Multiplex-PCR, included *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Proteus mirablis*.

DNA extraction: To recover DNA for PCR amplification, the procedure carried out using Wizard Genomic DNA Purification Kit, Promega (USA). 2 ml of overnight LB broth bacteria culture (OD600=2) centrifugated at 12000 rpm for 2 minut.The cells were re-suspended by 600µl of Nuclei lysis solution.The lysate was incubated at 80°C for 5 minutes to lyse cells, and then cooled to room temperature.3µl of RNase solution was added to the lysate, then the tube was inverted 2 -5 times to mix and incubated at 37°C for 15-60 min. 200µl of Protein precipitation solution was added to the treated cell lysate, then mixed by vortex for 20 seconds and incubated on ice for 5 minute. Centrifuged at 14.000 rpm for 3minutes, the upper layer was taken to 1.5 ml microcentrifuge tube. 600µl of Isopropanol was added, and then gently mixed by inversion until the thread-like strands of DNA appear. Centrifuged at 14.000 rpm for 2 minutes, the upper layer was removed. Drained the tube on clean absorbent paper. 600µl of 70% ethanol was added. The tube was inverted several times to wash the DNA pellet.Centrifuged at 14.000 rpm for 2minutes, the upper layer was removed.The tube was drained on absorbent paper and allowed to air-dry for 10 – 15 minutes.100µl of DNA rehydration solution (TE buffer) was added and DNA was stored at – 20°C.

PCR primer, DNA amplification and detection: The multiplex PCR was performed using five primers-pairs ,the primers tyv-s and tyv-as for detection of the tyvelose epimerase gene (*tyv*, previously called *rfbE*). The primers parat-s and parat-as were designed for detection of a paratose synthase gene (*prt*, previously called *rfbS*), the primers fliCcom-s and fliCd-as for detection of the *fliC-d* gene (phase-1 flagellin gene for d antigen [H:d]) of *Salmonella* serovar Typhi. and the primers fliCcom- s and fliCa-as were designed for detection of a *fliC-a* gene (phase-1 flagellin; H:a) of *Salmonella* serovar Paratyphi A. The primers vi-s and vi-as for the *viaB* gene[2]. This study consistent with the findings of a previous report [2, 7,11]. The primer sequences used in this study are listed in Table (1).

Table (1): Primers for multiplex PCR amplification of *Salmonella* serovar Typhi and Paratyphi Hirose *et al*., (2002)

Gene	Primer (oligonucleotide sequence)	Length (bp)	Amplified Fragment size(bp)
tyv (rfbE)	tyv-s (5-GAG GAA GGG AAA TGA AGC TTT T-3)	22	615
	tyv-as (5-TAG CAA ACT GTC TCC CAC CAT AC-3)	23	
prt (rfbS)	parat-s (5-CTT GCT ATG GAA GAC ATA ACG AAC C-3_)	25	258
	parat-as, (5-CGT CTC CAT CAA AAG CTC CAT AGA-3)	24	
viaB	vi-s (5-GTT ATT TCA GCA TAA GGA G-3_)	19	439
	vi-as (5-CTT CCA TAC CAC TTT CCG-3)	18	
fliC	fliCcom-s (5-AAT CAA CAA CAA CCT GCA GCG-3)	21	
	fliCd-as (5-GCA TAG CCA CCA TCA ATA ACC-3)	21	
	fliCa-as (5-TAG TGC TTA ATG TAG CCG AAG G-3)	22	
	fliCcom-s –fliCd-as		750
	fliCcom-s –fliCa-as		329

The PCR was carried out with a 50 µl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 1 U of *Taq* DNA polymerase (Promega,USA.), 0.2 mM dNTPs, a 0.1 µM concentration (each) of primers (tyv-s, tyv-as, parat-s, fliCcom-s, fliCd-as, and fliCa-as, a 0.2 µM concentration (each) of primers (parat-as, vi-s, and vi-as), and 5 µl of the DNA sample.

The PCR was carried out under the following conditions: 25 cycles with heat denaturation at 95°C for 30 s, primer annealing at 55°C for 60 s, and DNA extension at 72°C for 90s by a DNA thermal cycler (Thermo, USA). The amplified DNA was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

Results

The multiplex PCR using five sets of primer pairs, which were targeted for the *prt, tyv, viaB*, *fliC-d*, and *fliC-a* genes, correctly identified *Salmonella* serovars Typhi and Paratyphi A and differentiated the two serovars by the combinations of the different-size bands produced: four positive bands, which consist of *prt, tyv, viaB,* and *fliC-d* PCR products, in serovar Typhi and two positive bands, which consist of *prt* and *fliC-a* PCR products, in serovar Paratyphi A Fig. (1). As expected, the *prt* primers in this study reacted with both serovars Typhi and Paratyphi A, yielding PCR products of the same size. The presence in both serovars Typhi and Paratyphi A of the *prt* gene was consistent with the findings of a previous report [8]. The primer pairs for *fliC-d* and *fliC-a* specifically detected the *fliC-d* and *fliC-a* genes, respectively, for the *Salmonella* serovars, and were able to distinguish *fliC-d* and *fliC a* genes from other *Salmonella* serovar *fliC* genes. Primers specific for the Vi antigen gene also reacted with the chromosomal DNAs of serovars typhi only.

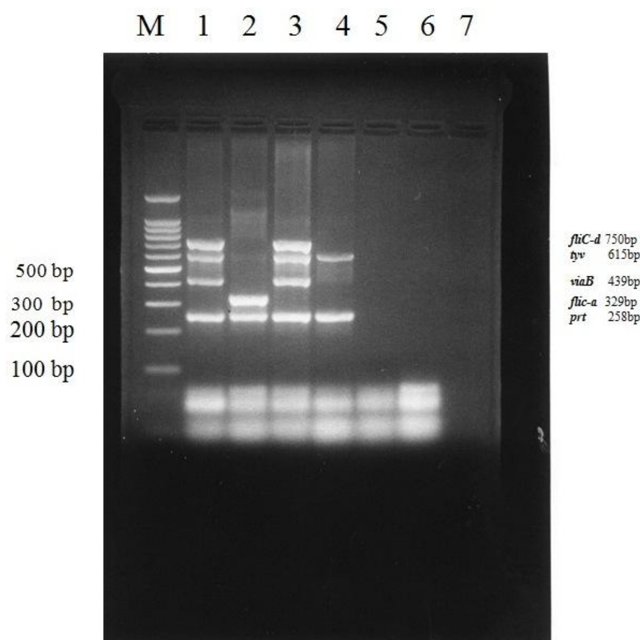

Fig. (1): Multiplex – PCR products of *tyv*, *prt*, *viaB*, *fliC-d* and flic-a genes for *Salmonella* Typhi ,Paratyphi A and different isolates. Lanes: M.100bp DNA Ladder, 1,3. *Salmonella* Typhi, 2. *Salmonella* ParatyphiA, 4. *Salmonella* EnteritidiS 5. *Salmonella* Typhimurium, 6. *Proteus mirabilis,*7. Negative control. PCR products were separated on 2% agarose gel electrophoresis.

To examine possible cross-reactions (non specific amplification) of the selected *viaB, prt, tyv*, *fliC-d*, and *fliC*-a, DNA from major enteric pathogens were tested by the multiplex-PCR assay. Using five sets of primers targeting these genes. These were; *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Proteus mirabilis*. The product's size is illustrated in Fig.(1) and summarized results of amplification are shown in Table (2).

Table (2): Bacterial isolates used to evaluate the specificities of multiplex-PCRs and the Multiplex-PCR results

	PCR result of genes amplification				
Bacterium	***fliC-d***	***Flic-a***	***tyv***	***viaB***	***prt***
***Salmonella* Typhi**	+	-	+	+	+
***Salmonella* Paratyphi A**	-	+	-	-	+
***Salmonella* Enteritidis**	-	-	+	-	+
***Salmonella* Typhimurium**	-	-	-	-	-
Proteus mirabilis	-	-	-	-	-

\+ **PCR positives.** – **PCR negative**

Discussions

Rapid detection and identification of pathogens is crucial for effective disease control. Polymerase chain reaction (PCR) provides a rapid means to monitor specific microorganisms in a variety of samples. Several amplification methods such as Uniplex PCR, Nested PCR, and Real time PCR have been used for detection of bacterial pathogens such as *Salmonella* species [12]. Most of the researchers who have already applied conventional and Real time PCR for detection of *Salmonella enterica* have used often only one gene of this organism in their studies. The Downloaded serovars other than Typhi have been detected in some cases due to cross reaction of single gene directed PCR [13]. Others have already used *fliC-d*, the *Vi* capsular antigen, and the *prt* genes for rapid detection by PCR [14]. Our data indicate that the Multiplex PCR test developed in this study using five gene primers was as sensitive as a standard culture method in detecting and differentiated *Salmonella* Typhi *and salmonella* Paratyphi A. Multiplex PCR method which involves in amplification of *viaB*, *flic-d, flic-a, prt* and *tyv* genes of

Salmonella spp. The *prt* gene encodes CDP paratose synthase which converts CDP-4-Keto-3,6-dideoxy to CDP-paratose. Is present in *Salmonella* ser. Typhi, *Salmonella* ser. Paratyphi A, *Salmonella* Enteritidise strains but not amplified with *Salmonella* ser. Typhimurium ,and *Proteus mirabilis*. The *tyv-s*,*tyv-as* gene encodes CDPtyvelose epimerase that converts CDP-paratose to CDP-tyvelose. Is present in *Salmonella* Typhi and *Salmonella* Enteritidise strains. The results indicated that *Salmonella* Typhi and *Salmonella* Enteritidise have high similarity percentage in O-antigen genes but without amplification the Vi antigen with *Salmonella* Enteritidise. The PCR primers specific for the Vi antigen gene reacted with the chromosomal DNAs of serovars Typhi [7]. The primer pairs for *fliC-d* and *fliC-a* specifically detected the *fliC-d* and *fliC-a* genes, respectively, for the *Salmonella* serovars, and were able to distinguish *fliC-d* and *fliC-a* genes from other *Salmonella* serovar *fliC* genes[2,8].

Detection of both *prt* and *fliC-a* correctly identified serovar Paratyphi A. Detection of the combination of *viaB, tyv*, *prt*, *fliC-d and flic-a* correctly identified serovar Typhi and Paratyphi A. The results of the specificity test obtained in this experiment were 100% in consistency with the works of other investigators [15, 2] (Fig.(1) and Table (2).

PCR based methods dramatically reduce the time required to detect *Salmonella* in samples, in comparison with the standard culture methods. The developed methods also offer the potential to provide more information if extended to include Multiplex- PCR detection with more than one primer set, allowing strain-identification capacities equal to, or better than phage signature data. The rapid availability of such detailed information is likely to have significant value in epidemiological and outbreak investigations[16].

In conclusion, the primers used in this study have been successfully amplified the targeting genes in purified colonies of *S. enterica* serovar Typhi and Paratyphi A by multiplex PCR and planning to use this technique in future for direct detection and identification of these serovars in clinical samples.

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