Detection of Some *Salmonella*EnteritidisVirulence Genes by Multiplex-PCR Assay Using Two Different DNA Extraction Methods

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(Received 28/1/2013, Accepted 14/3/2013)

الخلاصة

الهدف من هذه الدراسة هو تحديد بعض مورثات الفوعة الخاصة بجرثومة السالمونيلا باستخدام طريقة تفاعل البلمرة المتسلسل والمتعدد وبواسطة مجموعة من البوادئ الخاصة بالمورثات (*InvA*, S*ipB, SpiA, CdtB, PefA*) وباستخدام طريقتين مختلفتين لاستخلاص المادة الوراثية.

خمسة عشر عزلة من جرثومة السالمونيلا عزلت من عينات براز من حالات مرضية، نميت جرثومة السالمونيلا على الاوساط التقليدية ومن ثم نقيت على وسط (HiChrome Rajhans) ووسط (XLD) كاوساط انتقائية، تم اجراء التفاعلات الكيماحوية والمصلية لغرض تاكيد التشيخص ومن ثم اجري التصنيف المصلي في مختبر الصحة المركزي وباستخدام المستضدات (O)و(H). استخلاص المادة الوراثية تم باستخدام طريقة الغلي وطريقة الاستخلاص بالمحاليل الملحية وباستخدام عدة تجارية (Wizard® Genomic DNA purification Kit). النتائج اظهرت ان جميع مورثات الدراسة (المرتبطة بالكروموسوم او البلازميد) كانت موجودة في جميع عزلات السالمونيلا انتريدس التي شملتها الدراسة عدا المورثة (*cdtB*) واالتي يعتقد انها مقتصرة على بعض عزلات السالمونيلا تايفي والسالمونيلا باراتايفي ، كما ان وجود المورثة (*pefA*) يرتبط ببعض العزلات المستوطنة والمتكيفة للمضيف. اظهرت الطريقتين المستخدمتين لاستخلاص المادة الوراثية (طريقة الغلي وطريقة المحاليل الملحية) اظهرتا نتائج جيدة باستخدام تفاعل البلمرة المتسلسل والمتعدد.

ان هذه الدراسة تؤكد نتائج الدراسات السابقة من ان مورثات (SPIs) واسعة الانتشار بين عزلات السالمونيلا المرضية ومرتبطة بفوعة هذا الجرثوم كما ان طريقة الغلي في استخلاص المادة الوراثية جيدة وسهلة وكفؤة للاستخدام بتفاعل البلمرة المتسلسل والمتعدد.

Abstract

The aim of this study was screeningsome *Salmonella* genus-specific virulence genes by multiplex-PCR technique using a group of primers targeting *InvA*,S*ipB,SpiA,CdtB,PefA* genes using two different DNA extraction methods. A total of fifteen Salmonella isolates from patient's stool samples were collected. Suspected colonies onHiChromeRajhans (HCR) Medium and XLD medium were selected, and biochemical and serological tests were then performed for identification of *Salmonella*. Identification of *Salmonella* serotypes was done in Central Public Health Laboratory (CPHL) with O and H antisera. Extraction of bacterial genome was down by boiling method and salting out method using commercial kit (Wizard® Genomic DNA purification Kit). The results showed that all screened genes (chromosomally and plasmid-mediated) were found in all tested *Salmonella*Enteritidis strains except *cdtB* gene which is thought to be limited only to certain *Salmonella*Typhi and*S.*Paratyphi A strains. Moreover,the presence of *pefA* gene could be depending on host-adapted serovars. Boiling extraction method and commercial kit both gave a good result in multiplex-PCR technique. In conclusion, the results from this study of occurrence of SPIs genes support previous studies suggesting that these virulence genes are widely distributed among *Salmonella* and required for full *Salmonella* virulence, in addition, the boiling method was good, easy and specific method for multiplex-PCR technique.

Keywords: Salmonella virulence genes, multiplex-PCR, Boiling method.

Introduction

The World Health Organization (WHO) reported that about two million people die annually, from diarrheal diseases worldwide[1]. *Salmonella* are a leading cause of foodborne human gastroenteritis and bacteremia worldwide in both developed and developing countries[2,3]. The infection is associated with consumption of contaminated raw meats, poultry and poultry products, and dairy products[4].

Although, more than 2500 *Salmonella enterica*serovars are exist[5], a small number of these serovars are associated with human foodborne salmonellosis[6,7]. *Salmonella enterica*subspecies *enterica*serovarEnteritidis is a major causeof food-borne illness in animals and humanworldwide[8].

Salmonella have evolved several virulence and antimicrobial resistance mechanisms that allow for continued challenges to our public health infrastructure[4]. Many Salmonella contain pathogenicity islands scattered throughout their genomes that encode factors essential for bacterial adhesion, invasion, survival and infection. Among them some *Salmonella* virulence genes like *invA, sipB*and*spiA* that located on chromosome of *Salmonella*, are associated with type III secretion systems (TTSSs), complex structures of more than 20 proteins that are used in protein delivery[4].The *invA*gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for invasion of deeper tissue[9].

Additionally, the *invA*gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application[10]. This gene is recognized as an international standard for detection of *Salmonella* genus[11].

Plasmids are also known to harbor virulence factors that contribute to *Salmonella* pathogenicity. Several serotypes of medical importance, including Typhimurium, Enteritidis, Newport, Dublin, and Choleraesuis, are known to harbor virulence plasmids containing genes that code for fimbriae, serum resistance, and other factors. Plasmid-encoded fimbriae (*Pef*) play a role in colonization[12]. *Pef* binds to the villous intestine[13,14]. *cdtB*, a putative toxin-encoding gene carried on human specific serovars of *Salmonella*.

Polymerase chain reaction (PCR) is the best known and most successfully implemented nucleic acid detection technology[15]. Polymerase ChainReaction assay represents a major advance in diagnostic methods in terms of speed and sensitivity. However, the method of template preparation is crucial in a PCR-based assay to provide pure DNA lacking inhibitory factors. Some factors to consider when choosing an optimal protocol include type of samples and the relative need for speed and sensitivity. Many methods were described previously and heating method one of them[16].

Therefore, the aim of the present study was to survey of five *Salmonella* virulence genes (*invA* gene which is genus-specific, *sipB,* and*spiA* that located on chromosome of *Salmonella*, they are associated with TTSSs, and two plasmid-mediated genes, *PefA* and *cdtB*) which are clinically important virulence factors in *Salmonella* infection, by only one multiplex-PCR reaction. In addition, we intended to evaluate the efficiency of boiling method in comparison with salting-out technique (available as commercial kit) by multiplex PCR reaction.

Material and methods

Bacterial isolates:

Fifteen *Salmonellaenterica* serotype Enteritidis had been isolated from diarrhea patients of all ages from Al-Hakeem General hospital, Al-Sadder Medical City and Al-Zahra for child and Maternity Teaching Hospital. One gram of fecal specimen was inoculated into Carry-Blair medium and forward to the laboratory within 4h in ice box. The stools samples enriched in selenite-F broth for 18 h at 37°C , followed by HiChromeRajhans (HCR) Medium, modified (salmonella Agar Modified,M1634) and Xylose-lysine Desoxycholate (XLD) agar plate. The plates incubated at 37°C for 24h, then inspected for positive cultures[17].

The suspected colonies were screened by testing in triple-sugar-iron (TSI) agar, urea agar (UA), L-ysine Iron Agar (LIA), and Simon citrate (SC) test. The presumptively positive *Salmonella* isolates then identified by using HiMotility™ Biochemical kit (HiMedia, India). All isolates were examined for positive agglutination with polyvalent O antisera by using HiSalmonella™ Latex Test kit (HiMedia, India)[2,18].

Serotyping: All *Salmonella enterica* isolates were sent to Central Public Health Laboratory (CPHL) for serotyping with O and H antisera, Organisms were stored frozen at −20 ^{o}C in brain heart infusion broth (Oxoid, UK) containing 15% glycerol until use[19].

DNA extraction:

*Salmonella*Enteritidis isolates were grown at 37°C either in 5ml Brain-Heart Infusion broth (Oxoid) with shaking for 18-24 hour or on Brain-heart infusion agar, two methods of DNA template extraction were used: Commercial salting-out method was done using Wizard® Genomic DNA purification Kit (Promega, USA) according to manufacturer's instructions. Also, boiling method was carried as follows: three single well isolated colonies had been picked up with sterile loop and suspended in 150µl double distilled water, vortex for 10 seconds, then boiled at 95°C in a thermocycler for 15 minutes and immediately cooled on ice for 5 min, centrifuged at 16000 rpm for 5 min. The supernatant was aspirated into new sterile eppendorf tubes and stored at -20°C for four months until used for Multiplex PCR.

Multiplex PCR:

A multiplex PCR assay developed by Skyberg[20] was used with modification to investigate the presence of 5 genes associated with pathogenicity in *Salmonella* spp. Sterile distilled water was used in the place of DNA template in the negative control. All primers synthesized by (bioCorp, Canada), primers sequences and expected amplicon sizes and gene functions for each primer summarized in table 1. Amplification was performed in a 25µl reaction mixture that included 2.5µl of template DNA, 12.5µl of 2X KAPA2G Fast Multiplex PCR Mix (Kapabiosystems, South Africa), and 0.5µl of 10µM forward and reverse primers. Reaction mixtures were subjected to the following cycling protocol in a TECHNE TC-300 thermocycler (Bibby Scientific, UK): initial denaturation 3 min at 95°C, 30 cycles of 15 sec at 94°C, 30 sec at 66.5°C and 1 min at 72 °C, with a final cycle of 10 min at 72°C. PCR products obtained were subjected to horizontal gel electrophoresis in 1.5% agarose at 70V for 2h, and the size of the amplicons was determined by comparison with the KAPA Universal DNA Ladder (Kapabiosystems, South Africa). Finally, the gel was photographed using Vision-Gel documentation system (SCIE-PLAS, UK).

Table 1:Primers used for the amplification of selected virulence genes.

Gene	Gene Function	Primer sequence (5' to 3')	product size/bp	Reference
InvA	Host recognition/ invasion	F:CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R:AGTTTCTCCCCCTCTTCATGCGTTACCC	1070	21
SipB	killing of macrophages Entry into non phagocytic cells	F: GGACGCCGCCCGGGAAAAACTCTC R: ACACTCCCGTCGCCGCCTTCACAA	875	20
SpiA	Survival within macrophage	F:CCAGGGCTCGTTAGTGTATTGCGTGAGATG R:CGCGTAACAAAGAACCCGTAGTGATGGATT	550	22
CdtB	Host recognition/ invasion	F: ACAACTGTCGCATCTCGCCCCGTCATT R: CAATTTGCGTGGGTTCTGTAGGTGCGAGT	268	23
PefA	Host recognition/ invasion	F: GCGCCGCTCAGCCGAACCAG R: GCAGCAGAAGCCCAGGAAACAGTG	157	24

Results and discussion

Suspected *Salmonella* isolates identified by biochemical and latex agglutination tests were shown in figure 1.

We observed that the *invA* gene was found in 100% of tested strains. Rahn*et.al.*[25] reported that two strains of *Salmonella* serotype Litchfield and two of serotype Senftenberg were not detected when primer set 139-141 was used. However, it has been shown that the *invA*gene is essential for the invasion of epithelial cells by *Salmonella*; consequently, the apparent absence of the *invA*gene suggests that such strains are not invasive or use alternative invasion mechanisms[21,26].

All the virulence genes targeted in this study except *cdtB*, *pefA*have previously been shown to be required for full *Salmonella* virulence in a murine model[20]. Therefore, all the screened genes were found in the all *Salmonella*Enteritidis isolates except cdt*B* gene (figure 2). Researchers thought that the *cdtB* gene was limited only to certain *Salmonella*Typhi and *Salmonella*Paratyphi A strains,which are exclusively human serovars[23].

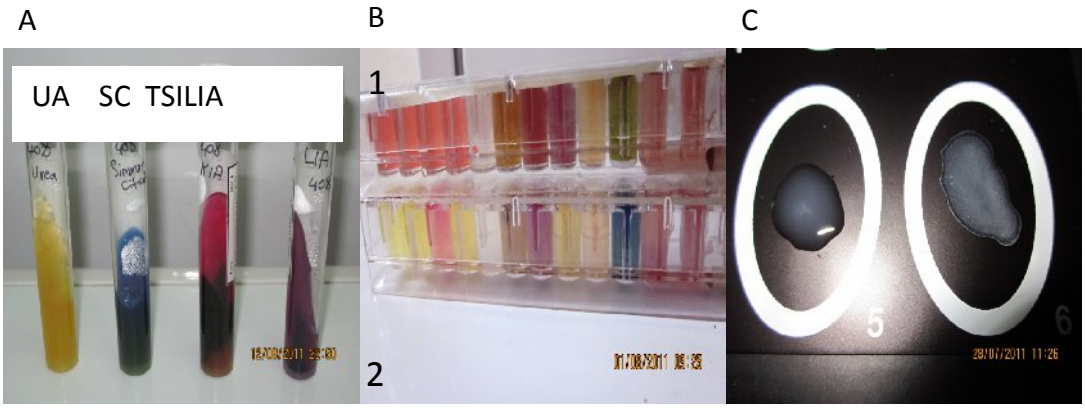
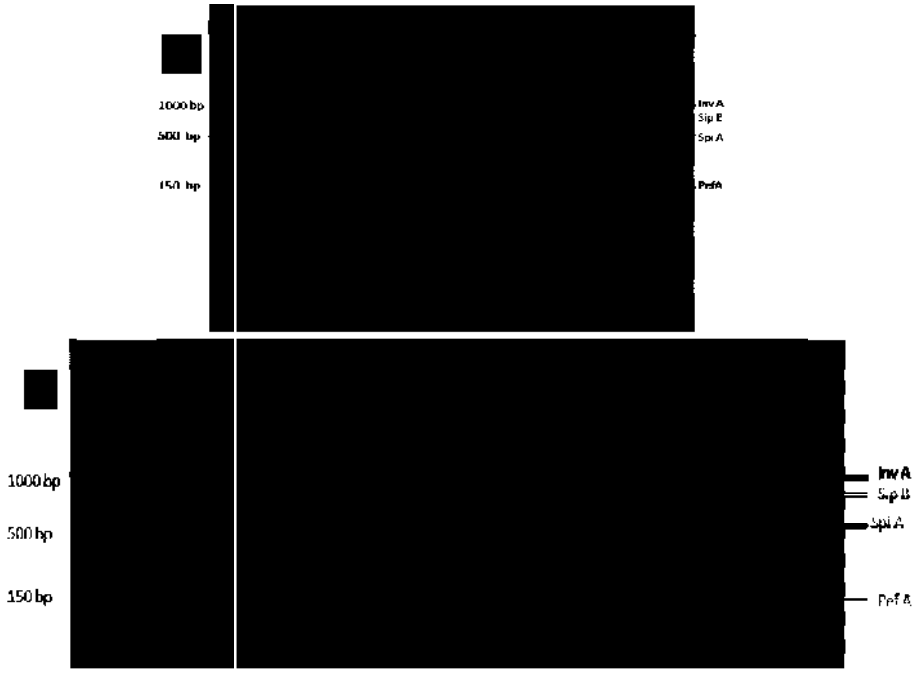


Figure 1: Identification of Salmonella isolates: A. screening by UA, SC, TSI, and LIA tests; B. HiMotility™ Biochemical kit, 1- Uninoculated kit, 2- Positive results; C. HiSalmonella™ Latex agglutination Test.

Present result was in agreement with such observation. Therefore, the absence of cdt*B* gene from all the tested strains of*Salmonella*Enteritidis that were collected from patients with diarrhea, apparently does not affect the diarrheagenic mechanism. As the mechanism of diarrhea appears to be due to proinflammatory cytokines production due to localization salmonella bacteria on the basolateral surface on epithelial cells in the mucosa of intestine.

Several researchers had been found no toxin-like genes in complete genome sequences of serovarsTyphimurium, Typhi, Dublin, and Choleraesuis[27]. However, others have been reported low prevalence of cdt*B* gene in *Salmonella* serotypes other than Typhi andParatyphi A, and they suggested that the occurrence of this gene is not restricted only to human serovars[20,28].

Present study has been found that all the strains of *Salmonella*Enteritidiscontainpef*A* gene. The *pefA*gene is located on a virulence plasmid rather than the bacterial chromosome[29]. Such plasmids can be serovar-specific[20]. In spite of that, several researchers have been found that not all isolates of plasmid-bearing serovars contain these plasmids[30]. Ndeandlogue[28], and Hughes *et.al.*[31] were observed Low prevalence (22·5% and 9.4%, respectively) for *pefA* in *Salmonella enterica*serovarsisolated from birds, so the presence or absence of this gene could be depend on host-adapted serovars.



A) Ethidium bromide-stained agarose gel of Multiplex-PCR results for virulence genes invA, sipB, spiA, cdtB, and pefA of 15 *Salmonella* Enteritidis isolates (lanes designated as 28, 7b, 24, 26, 25, 16, 27, 12, 20, 14,15, 6, 9, 13, and 21), using DNA template extracted by Promega kit. Lane(M) KAPA universal DNA ladder. B)Showing Multiplex-PCR results for five virulence genes among 15 *Salmonella* Enteritidis (lanes designated as 28, 7b, 24, 26, 25, 16, 27, 12, 20, 14,15, 6, 9, 13, and 21) isolates using DNA template extracted by boiling method. Lane(M) KAPA universal DNA ladder; Lane CN, *E. coli* ATCC 25922 as control negative.

The *invA*and*sipB*virulence-associated genes screened for are located on *Salmonella* pathogenicity island 1(SPI-1) while*spiA* gene on SPI-2[20,32], these genes associated with type III secretion systems[33,34]have been well-characterized for their role in both enteritis and systemic infection in mammalian models[31]. The results from this study of occurrence of SPIs genes support previous studies suggesting that these virulence genes are widely distributed among *Salmonella* and required for full *Salmonella* virulence.[20,28]

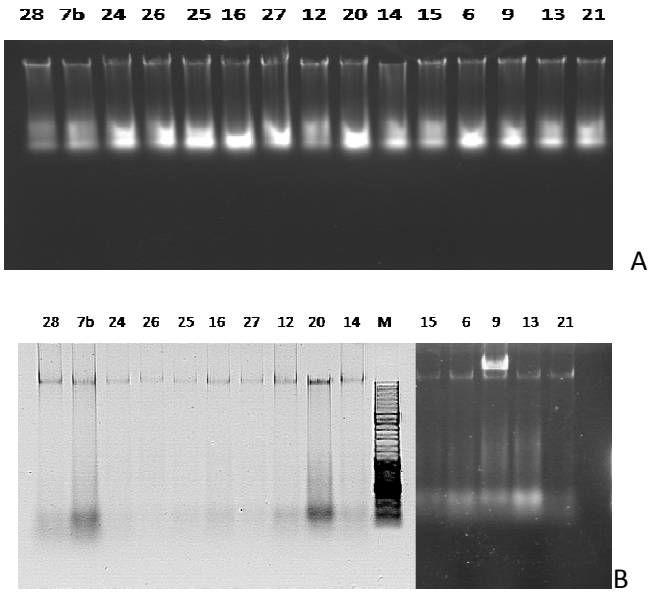


Figure 3:A) DNA profile of Fifteen *Salmonella Enteritidis* Isolates extracted by Wizard Genomic DNA purification Kit (promega) method. Lanes designated as 28,7b,24,26,25,16, 27,12,20,14,15,6,9,13 and 21 represent the DNA bands of *Salmonella*Enteritidis. B) DNA profile of Fifteen Salmonella Enteritidis Isolates extracted by boiling method.Lanes designated as 28, 7b, 24, 26, 25, 16, 27, 12, 20, 14, 15, 6, 9, 13, and 21 represent the DNA bands of Salmonella Enteritidis isolates. Lane (M) DNA molecular marker (100bp ladder).

Extraction DNA by boiling method gave a less quality extracted DNA (figure-3: B) in comparison with salting out method by kit (figure-3: A), but both techniques were gave good results with multiplex-PCR technique (figure-2). De Medici *et. al.*[35] was selected Boiling method as the preferred extraction method because it is the simplest and most rapid, for SYBR Green I real-time PCR. However, Freschi*et. al.*[36] found that the DNA samples prepared by boiling method were equivalent to those achieved by commercial kit (salting out), but can be preserved for short period. Present study showed that although the DNA samples prepared by boiling method were stored for 4 months, their efficiency in the amplification of targeted genes by multiplex PCR were very similar to those achieved by using the commercial kit (figure-2A &B). The superiority of our method may be due to preservation of DNA samples at −20 °C instead of 4°C in the previous studies.

We conclude that both methods of DNA extraction were satisfactory enough to support the amplification the target genes by multiplex-PCR technique, but boiling technique is cheaper, easy to down and sensitive enough for this procedure. Method presented here is rapid, simple, specific and sensitive, as Salmonella virulence genes can be detected directly from primarily culture with no complicated prior extraction of genomic DNA.

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