PLASMID FINGERPRINTING AND VIRULENCE GENE DETECTION AMONG INDIGENOUS STRAINS OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS

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Background: *Salmonella enterica* serovar Enteritidis is an important frequently reported zoonotic pathogen and a common cause of human gastroenteritis worldwide. The highly conserved Sero-specific plasmids (SSPs) and *Salmonella* plasmid virulence (*Spv*) genes have been shown to mediate extra-intestinal colonization and systemic infection. The objective of current study was to document the presence of SSPs and *Spv*B/*Spv*C genes prevailing in the indigenous population of serovar Enteritidis. **Methods**: A total of 48 epidemiologically unrelated strains of *Salmonella enteritidis* were included in the study. Preparation of plasmids DNA suitable for endonuclease digestion and separation of respective fragments by agarose gel electrophoresis followed previously described protocols. The plasmids of *Escherichia coli* V517, 1-kbp ladder, and λ DNA *Hin*dIII fragments served as DNA size standards. Transfer of DNA fragments from agarose gels to nitrocellulose membranes was achieved by capillary blot procedure. An ECL labeled 3.6 kbp *Hin*dIII fragment of plasmid PRQ 51 was used as probe for *Spv*B/*Spv*C gene detection. **Results**: Plasmid DNA fingerprinting revealed the presence of two different profiles of approximately 55 kbp and 90 kbp and were identified as virulence plasmids by DNA hybridization. The *Spv*B/*Spv*C genes were located on *Hin*dIII fragments of 3.6 kbp in each of the two types of virulence plasmids. **Conclusion**: The study confirms the presence of SSPs and *Spv*B/*Spv*C genes in indigenous strains of *S. enteritidis* isolated from Northern Punjab area of Pakistan and substantiate the previous data on such findings from other parts of the world.

Keywords: *Salmonella enteritidis*; virulence plasmids, *Spv*B/*Spv*C genes

INTRODUCTION

Food-borne diseases caused by zoonotic *Salmonella enterica* species represent an important public health problem associated with millions of cases and thousands of deaths worldwide each year.[1–3] Among more than 2,500 serotypes of the genus *Salmonella* described so far, the *Salmonella enterica* serotype Enteritidis (*S. enteritidis)* is the most common cause of human salmonellosis[4,5] causing meningitis and septicemia in immunocompetent adult[6] and brain abscess in sickle cell anemia patient.[7] The highly conserved Sero- specific plasmids (SSPs)[8] of *S. enteritidis* have been shown to mediate extra-intestinal colonization and systemic infection.[9] An important virulence factor common among predominant non-typhoid serovars including Enteritidis, is *Salmonella* plasmid virulence (S*pv*) operon which contains five genes (S*pvR,A,B,C,D*).[9–11] The S*pv* operon is associated with invasiveness and its removal has led to reduce virulence and loss of ability to adhere HeLa cells. *Salmonella* serovars carrying *Spv*B/*Spv*C genes on 3.6 kbp *Hin*dIII fragment of SSPs have LD_{50} <20 compared to LD_{50} $>10^6$ cells of S*pv* operon free strains.[8]

The findings on SSPs and *Spv*B/*Spv*C genes carried by *S. enteritidis* strains prevalent in Europe, USA, and some Asian countries are readily available[8–11] however; such data regarding strains prevalent in Pakistan has never been published. This study documents the first results on plasmid DNA fingerprinting and presence of the two most important genes (*Spv*B/*Spv*C) located on *Spv* operon among indigenous strains of *S. enteritidis.*

MATERIALS AND METHODS

Bacterial isolates and growth condition

A total of 48 epidemiologically unrelated strains of *S. enteritidis* isolated from different geographic locations of Pakistan, were included in the study. The presumptively positive *Salmonella* isolates were identified by using API 20E strips (bioMérieux, France). The somatic (O) and flagellar (H) antigens were characterized by slide agglutination with commercially available anti-sera (Behring Werke, Marburg, Germany) and the serotype was assigned according to the Kauffmann-White scheme. The isolates were cultured overnight in 5 ml Luria Broth at 37 °C, and glycerine stock cultures of confirmed strain were kept at -70 °C.

DNA Preparation

Preparation of plasmids DNA was carried out according to the methods of Kado and Liu.[12] Briefly, bacterial pellet was thoroughly suspended in 20 µl of Tris/EDTA, followed by lyses with 100 µl of lysis buffer, then 100 µl of phenol-chloroform (1:1 v/v) was added to extract the plasmid DNA. Eighty (80)

µl of the plasmid DNA was mixed with 16 µl of the bromophenol blue marker and 20 µl of the mixture was applied in each slot of the gel. The plasmids of *Escherichia coli* V517 served as size standards for the determination of plasmid sizes in the *S. enteritidis* isolates.[13] Plasmids suitable for endonuclease digestion were obtained by the method of Olsen.[14] Restriction analysis of plasmid DNA and separation of respective fragments by agarose gel electrophoresis followed previously described protocols.[15,16] The 1-kbp ladder and λ DNA-*Hin*dIII fragments (Gibco-BRL, Paisley, Scotland) served as DNA size standard. All centrifugation procedures were carried out at 13,000 rpm at room temperature in the Biofuge 13, Heracus, Rotor 3743, Sepatech, Osterode, Deutschland.

Southern blot hybridization and Gene probe

Transfer of DNA fragments from agarose gels to nitrocellulose membranes (Hybond N; Amersham-Buchler, Braunschweig, Germany), was achieved by capillary blot procedure.[15] A 3.6 kb *Hind*III fragment of plasmid PRQ 51 comprising of *Spv*B/*Spv*C genes, within the common region of virulence plasmid in *S. enterica,* serovars Enteriditis, Typhimurium, Dublin and Choleraesuis, served as a virulence gene probe.[16] The used probe was labeled by non-radioactive ECL system (Amersham-Buchler). Hybridization and signal detection were carried out strictly according to the manufacturer's recommendations with the solutions included in the ECL kit.[15]

RESULTS

Plasmid fingerprinting of *S. enteritidis* strains revealed the presence of two different plasmid profiles. Forty-five (93%) of the 48 isolates possessed a single large plasmid of approximately 55 kbp. The remaining three strains (7%) possessed a 90 kbp plasmid together with an additional plasmid of 2.1 kbp (Figure-1A). The 55 kbp and 90 kbp plasmids were identified as virulence plasmids by hybridization with a *Spv*B/*Spv*C virulence gene probe (Figure-1B). The digestion of the plasmid DNA with *Hin*dIII confirmed the structural homogeneity of all 55 kbp plasmids on one hand and that of the 90 kbp plasmids on the other hand. *Hin*dIII endonuclrase restriction of 55 kbp and 90 kbp virulence plasmids generated six DNA fragments of varying size with a common fragment of 3.6 kbp in each case (Figure-2A). Plasmid DNA hybridization experiments with virulence gene probe showed that SpvB/*Spv*C genes were located on 3.6 kbp *Hin*dIII fragments shared by both types of virulence plasmids (Figure-2B). The 3.6 kbp *Hin*dIII fragment of 90 kbp virulence plasmid hybridized strongly with *Spv*B/*Spv*C genes probe as depicted by signal detection with ECL system (Figure-2B, Slot-3).

DISCUSSION

DNA fingerprinting and hybridization techniques have proven to be very useful and reliable for the characterization and differentiation of strains of several *Salmonela* serovars including Eenteritidis,[17–19,] Typhimurium,[20] Dublin,[21] Virchow,[22] Heidelberg,[23] Berta,[24] Typhi,[25] and Paratyphi[26]. The current study demonstrates potential of these methods to fingerprint plasmid DNA and detect *Spv*B/*Spv*C genes located on the plasmids of indigenous *S. enteritidis* isolates. The results correspond closely to previously published data. Virulence plasmid occurrence rate of 100% was observed and analysis reveals 2 different profiles as determined by hybridization with *Spv*B/*Spv*C gene probe. Forty-five (93%) of the isolates possessed a single plasmid of 55 kbp. The remaining 3 (7%) isolates carried a 90 kbp plasmid along with an additional plasmid of approximately 2.1 kbp The high prevalence of *S. enteritidis* strains carrying 55 kbp plasmid alone or in combination with other plasmid have also been reported previously,[27,28] and probably represent sero-specific virulence plasmid[29]. The 55 kbp and 90 kbp plasmid were identified as virulence plasmid by hybridization with *Spv*B/*Spv*C gene probe. Moreover, all virulence plasmids (55 kbp and 90 kbp) proved to be identical and indistinguishable by their *Hin*dIII restriction pattern. The endonuclease *Hin*dIII has successfully been used previously to detect structural differences in intra-serovar virulence plasmids[28] but failed to discriminate within the serovar Enteritidis. Two plasmids of the same molecular size, but with different DNA sequences look identical in plasmid fingerprinting. The confidence of characterization can therefore be improved by digestion of plasmid with suitable digestion enzyme that cuts the DNA sequences at specific sites.[30] The virulence gene probe hybridized to 3.6 kbp *Hin*dIII fragments of all isolates, which had been reported to be an internal area of the *Spv* gene region in virulence plasmid of *S. enteritidis*[28], *S. typhimurium*[31], and *S. dublin*[32]. Plasmid fingerprinting might be a valuable tool for differentiation of *Salmonella* serovars and detection of virulence genes, however, the mobile character and appearance of single plasmid in a bacterial strain such as reported in current study may represent its limited use in epidemiological analysis.[33]

CONCLUSION

The current study confirms the spread of 55 kbp SSPs and presence of *Spv*B/*Spv*C genes on 3.6 kbp *Hin*dIII fragments of 55 kbp and 90 kbp virulence plasmids carried by *S. enteritidis* strains from Pakistan. The data generated through this study would further substantiate the already documented findings on plasmid fingerprinting and wide spread presence of *Spv*B/*Spv*C genes among serovar Enteritidis.

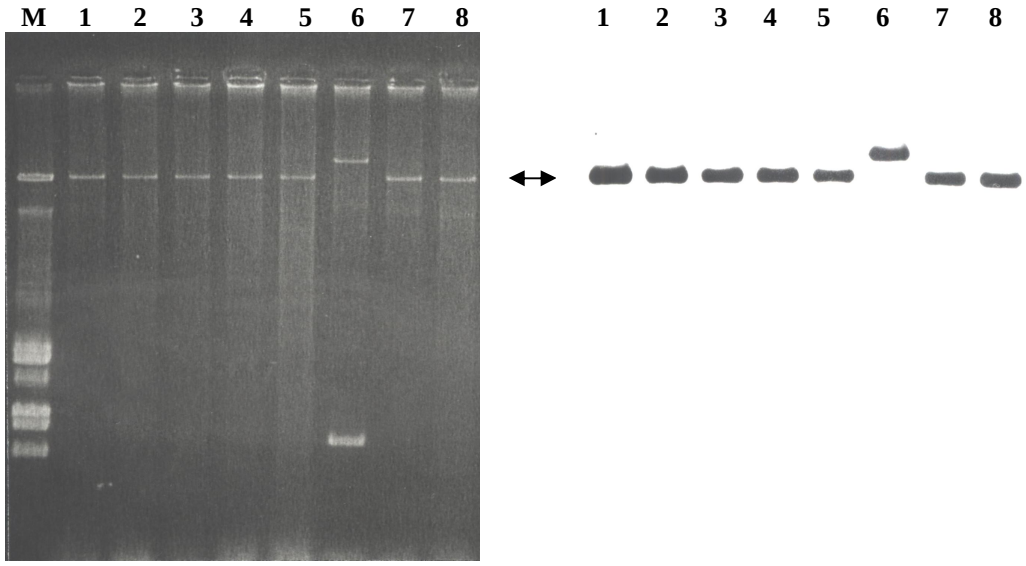

Figure-1A: Agarose gel electrophoresis of plasmid DNA detected in *S. enteritidis* isolates (Slot 1–8)
B: Corresponding hybridization pattern obtained with a *Spv*B/*Spv*C virulence gene probe (Slot 1-8)
M: V517 size reference plasmids (54.3, 7.3, 5.5, 3.9, 3.0, 2.7, and 2.0 kbp). The arrow indicates the hybridization plasmids of 55 kbp and 90 kbp

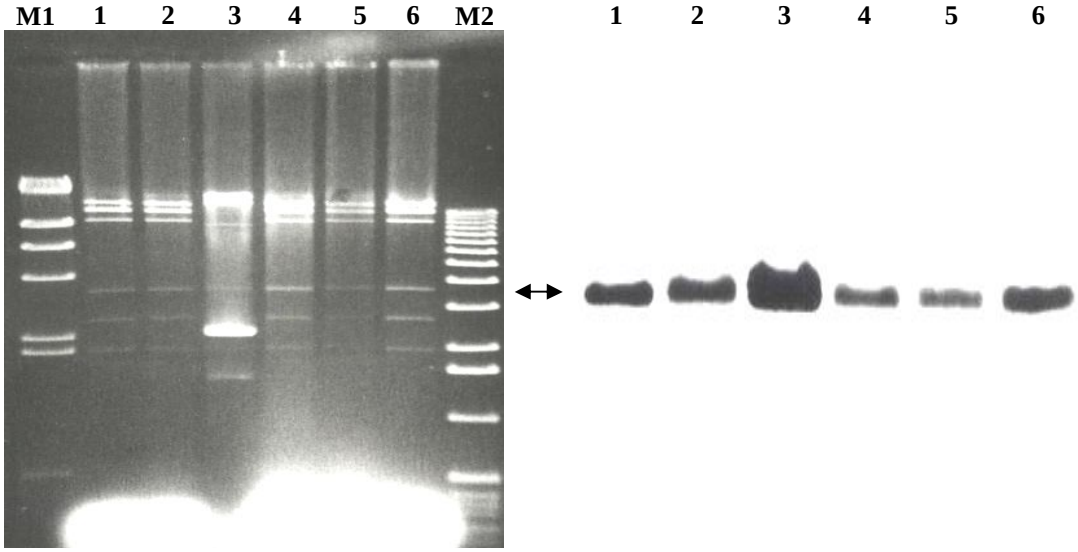

Figure-2A: *Hin*dIII restriction patterns of the 55 kbp (1, 2, 4, 5, and 6) and 90 kbp (3) virulence plasmids found in *S. enteritidis* isolates. B: Corresponding hybridization patterns obtained with *Spv*B/*Spv*C virulence gene probe. M1-DNA length standard (λ DNA *Hin*dIII fragments). M2-DNA length standard (1 kb ladder, Gibco-BRL). The arrow indicates the hybridizing fragments of 3.6 kbp.

ACKNOWLEDGEMENTS

This study was supported by academic exchange service of the Federal Republic of Germany. SU Sajid is the recipient of Deutscher Akademischer Austauschdienst (DAAD) award. We thank Kristina and Erika for excellent technical assistance.

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