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DETECTION OF VIRULENCE GENES IN RARE SEROVARS OF *SALMONELLA* ISOLATED FROM RIVER GANGES

Gunjan Shrivastav, Rajesh Kumar* and M.K. Saxena
Animal Biotechnology Centre, Department of Veterinary Physiology and Biochemistry
*Department of Veterinary Microbiology, College of Veterinary and Animal Science
G.B. Pant University of Agriculture & Technology, Pantnagar-263145 (India)

Abstract: Water pollution is a major problem in India. Ganga is the most holy river of the country and contributes significantly in Indian economy. Water of river ganga has been polluted seriously in last few years. In the present study few isolates of *Salmonella* which are not so common in India were isolated from river Ganga were evaluated for the presence of virulence contributing genes.
In present study 24 isolates were studied for presence of three major virulence contributing genes ie stn, vir and pef. Out of 24 isolates vir gene was present in 11 isolates, pef was present in 18 isolates and stn was present in 19 isolates. Five isolates were having all three genes. Most of these isolates have exhibited multiple drug resistance. Therefore, there presence in gangetic water can cause serious health problems.
Keywords: *Salmonella*, PCR, Virulence, River Ganges.

Introduction

From the glaciers of the Himalayas in the north, down the mountains, and across the plains to the Bay of Bengal, the Ganges river is one of the world's greatest rivers. Over 400 million people live along the Ganges river, and in spiritual and religious significance this river has no equal (Brook & Bhagat 2004). The river is revered as a goddess whose purity cleanses the sins of the faithful and aids the dead on their path toward heaven.

But while her spiritual purity has remained unchallenged for millennia, her physical purity has deteriorated as India's booming population imposes an ever-growing burden upon her. The majority of the Ganges' pollution is organic waste sewage, trash, food, and human and animal remains (Hamner et al., 2006). The result of this pollution is an array of water-borne diseases including cholera, hepatitis, typhoid, amoebic dysentery and non-typhoidal Salmonellosis.

Waterborne and food borne *Salmonella* accounts for heavy economic losses in livestock industry as well as that spent on disease in humans and its medical treatment. *Salmonella* has long been recognized as important zoonotic pathogen of global significance. In US, estimates

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of annual number of non-typhoidal *Salmonella* infections have ranged from 0.8 to 4.0 million with 1.4 million as an average (Voetsch et al., 2004). In Europe also, Salmonellosis is one of the most commonly reported zoonotic disease (Kangas et al., 2007).

Pathogenic process in salmonellosis are dictated by an array of factors that act in tandem and ultimately manifest in the typical symptoms of salmonellosis. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Some genes are known to be involved in adhesion and invasion viz., sef1, pef, spv3 or inv*;* others are associated in the actual manifestation of pathogenic processes viz., sop6, stn7, pip A, B, D8,*vir* gene. For the effective prevention and control of the diseases caused by *Salmonella* rapid and sensitive detection methods of virulence are needed. Nucleic acid based diagnostic techniques are being employed for the detection of pathogens and differentiation (Saxena *et al.*, 2006, Shivchandra *et al*., 2006, Sivashankara *et al*., 2001, Naggappa *et al*., 2007). PCR had been used for detection of virulence factors viz., *Slamonella* vir gene (Saxena *et al*. 2004, *Salmonella* enterotoxin (*stn*) (Rahman *et al.,* 1999) Salmonella Enteritidis fimbriae (*sef*) plasmid encoded fimbriae (*pef* A) genes (Rahman *et al.*, 2000) and invasion gene (*inv* A) (Chaudhari *et. al,* 2015). The present study was undertaken to observe the presence of virulence contributing genes ie *vir, stn* and *pef* gene in *Salmonella* isolates isolated from river ganga.

Materials and Methods

Twenty four isolates of *Salmonella* isolated from river ganges (Table I) were obtained from Animal Biotechnology Center. These isolated were retested by *Salmonella* Specific PCR and Biochemical tests.

Isolation of genomic DNA

Genomic DNA was isolated by C-Tab method. The bacteria were grown for 18 hrs in 25 ml Broth with constant shaking at 140 rpm in a shaker cum incubator. The bacterial cells were pelleted by centrifugation at 10000 rpm per minute for 4 minutes at 4^0C. The pellet was then resuspended in lysis buffer containing 2 ml TE (10mM Tris EDTA) ,400µl SDS (10% w/v) and 20µl of Proteinase K (20 mg /ml) mixed properly by rapid pipetting and kept at 37^0C for 4 hrs. Then 1ml of 5M NaCl and 400 µl of C-TAB (7.5% in 5 M NaCl) were added to the tubes. The tubes were then kept at 68^0C in water bath for 10 minutes. The mixture was taken in 1.5 ml microfuge tubes each tube containing 500µl of the mixture. Same volume of chloroform was then added in the tubes, mixed well and then the tubes were centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer was transferred to fresh microfuge tubes

and the same volume of Phenol: Chloroform (1:1) was added and centrifuged at 10,000 rpm. Finally the DNA was spooled with a micropipette tip, and dissolved in 100 µl of sterilized TE. The DNA was quantified by observing O.D. at 260 nm and O.D. at 280 nm. Concentration of DNA was calculated by using the following formula.

Concn. of DNA (µg /ml) = 50 X OD $_{260}$ X Dilution factor/1000

The final concentration of the DNA was adjusted to 40ng/µl and then stored in deep freezer at -20^{0}C

'*vir*' gene detection:

Two primers with following sequence were used for detection of virulence gene by PCR as described by **Rexach *et al.* (1994).**

Primer 1: 5' TTG TAG CTG CTT ATG GGG CGG 3'

Primer 2: 5' TGG AGA AAC GAC GCA CTG TAC TGC 3'

The method of **Rexach *et al.* (1994**) was adopted with some modification for the '*vir*' gene analysis of the isolates. The target gene was amplified in a programmable Thermal cycler. PCR was done with the following reaction mixture:-

Template DNA (40 ng/µl) - 5µl

dNTPs (200µM each) - 0.5µl

Primers (20 picomolar) - 0.5µl

Taq DA polymerase (3U/µl) - 0.5µl

Distilled water - q.s. 25 µl

The PCR mixture was subjected to following amplification cycle

Initial denaturation - 94^{0}C for 5 minutes

Denaturation - 94^{0}C for 1minute

Annealing - 60^{0}C for 30 seconds

Extension - 72^{0}C for 1 minute

Final extension - 72^{0}C for 5 minutes

30 amplification cycles were carried out and the amplified product was checked on 2.0% percent agarose gel. Each sample was amplified thrice to avoid artifacts. Molecular weight of amplicon was calculated by comparison with standard molecular marker.

Enterotoxin '*stn*' gene detection:

Two primers with following sequence were used for detection of '*stn*' gene by PCR as described by **Murugkar *et al*., (2003).**

Primer 1: 5' - TTG TGT CGC TAT CAC TGG CAA CC – 3'

Primer 2: 5' - ATT CGT AAC CCG CTC TCG TCC – 3'

PCR reaction mix was prepared as described above. Annealing temperature was kept at 59^0C for 30 seconds, remaining conditions were similar to vir gene. 30 amplification cycles were carried out and the amplified product was checked on 2.0% percent agarose gel as describe above.

Plasmid encoded fimbriae '*pef*A' gene detection:

Two primers with following sequence were used for detection of '*pef* A' gene by PCR as described by **Murugkar *et al*., (2003).**

Primer 1: 5' - TGT TTC CGG GCT TGT GCT 3'

Primer 2: 5' - CAG GGC ATT TGC TGA TTC TTC C 3'

PCR reaction mix was prepared as described above. Annealing temperature was kept at 56^0C for 30 seconds, remaining conditions were similar to vir gene. 30 amplification cycles were carried out and the amplified product was checked on 2.0% percent agarose gel as describe above.

Result and Discussion

vir gene located on *spv* region is considered as essential for systemic infection in mice and *vir* gene product also affect the severity of enteric disease. It is also known to regulate other virulence effectors proteins. Out of 24 isolates subjected to *vir* gene PCR 11 isolates showed positive results (Table I). In positive serovars a 472 bp amplicon was observed on 2.0% percent agarose gel (Fig: 1). The target segment for *vir* gene PCR is located in the spv region of plasmids of *Salmonella*. The *spv* regions play a major role in plasmid mediated virulence of *Salmonella*. Its absence has been linked to the loss of virulence and plasmid cured pathogenic strains regained their virulence by transformation of plasmids containing this region (Gulig *et al.,* 1993). Virulence plasmids are one of several *Salmonella* virulence determinants *inv*olved in survival and growth in host cells (Finlay et al.,1989). *Vir* gene is considered to be essential for systemic infection in the mouse (Rodriguez *et al*.,1997). It is reported that *spv* genes product affects some component of eukaryotic signaling pathway that allows the body to detect and respond appropriately to infection (Wray and Wray,2000).

First primers were designed by Mohan and Lax (1993) for detection of *Spv* gene by PCR and they reported an amplification of 500 bp amplicon in *Salmonella* from avian origin. Later on Rexach *et al.,* (1994) constructed modified primers from mkf gene of *S.* Typhimurium and mka region of *S*. Chloerasuis and reported a single product of 472 bp. Saxena *et al*., 2004

also reported similar findings. Out of 24 isolates subjected to *vir* gene PCR 11 isolates showed positive results.

pef region which is located on *Salmonella* plasmid is responsible for biosynthesis of fimbriae. Out of 24 isolates subjected to *pef* A gene PCR 18 isolates showed positive results (Table I). Positive serovars showed a 700 bp amplicon (Fig.2) Role of *pef* gene in virulence is studied by Baulmer *et al* (1996). *pef region* is found to be responsible for biosynthesis of fimbriae. They reported that *pef* fimbrial operon of *Salmonella* Typhimurium mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse. Rahman *et al*., (2000) used PCR for determining occurrence of pef virulence gene among different serovars of *Salmonella*. Murugkar *et al*., (2003) used *pef* gene as virulence gene target of PCR in *Salmonella* serovars isolated from man and animals in India. In the present study out of 24 isolates subjected to *pef* A gene PCR 18 isolates showed positive results. Positive serovars showed a 700 bp amplicon as described by Murugkar *et al*., (2003).

Salmonella enterotoxin coded by *stn* gene has been shown to cause fluid secretion in ligated loop model with adult rabbits. Even small amounts of toxin produced by *Salmonella* spp. could exert substantial effects on water and electrolyte during intestinal infection. PCR assay carried out for the detection of the stn gene in 24 *Salmonella* isolates had revealed that the gene was present in 19 isolates that was demonstrated by the presence of a 617 bp PCR product. (fig.3)(Table I). *Salmonella* enterotoxin coded by *stn* gene, which play an important role in strain virulence, is encoded by a gene designated as *stn* which is used as a virulence determinant in clinical strains of *Salmonella enterica* by Rahman *et al*.,(2000), Murugkar *et al*., (2003) and Soto *et al*.,(2006) using PCR. *Stn* alters vascular permeability in the skin, increases cyclic AMP levels and exert substantial effects on water and electrolyte during intestinal infection. In the present study PCR assay carried out for the detection of the stn gene in 24 *Salmonella* isolates had revealed that the gene was present in 19 isolates that was demonstrated by the presence of a 617 bp PCR product which is in compliance with Murugkar *et al*.,(2003). These results indicate that there are at least five isolates viz., G-12, G-24, G-32, G-39, G-55 which are positive for allthree genes responsible for virulence, enterotoxin production and fimbrae biosynthesis. These isolates were also possessing antibiotic resistance. As these isolates harboring virulent genes were isolated from Ganga water, it makes situation more worrisome.

Table I: Summary of findings of *vir*, *stn*, *pef* A genes PCR

S.NO.	Isolate No.	*vir*	*stn*	*pef A*	Serovar
1	G-4	-	+	+	*S*. Abuja
2	G-6	-	+	+	*S*. Abuja
3	G-7	-	-	+	*S*. Pontypridd
4	G-9	+	+	-	*S*. Abuja
5	G-12	+	+	+	*S*. Lagos
6	G-13	-	+	-	*S*. Lagos
7	G-15	+	+	-	*S*. Lagos
8	G-17	+	+	-	*S*. Chincol
9	G-19	-	+	+	*S*. Chincol
10	G-22	+	-	+	*S*. Abuja
11	G-24	+	+	+	*S*. Chincol
12	G-25	-	+	+	*S*. Zwickau
13	G-26	-	-	-	*S*. Goldenberg
14	G-27	-	+	+	*S*. Lagos
15	G-30	-	+	+	*S*. Pontypridd
16	G-31	+	-	+	*S*. Abuja
17	G-32	+	+	+	*S*. Abuja
18	G-33	-	+	+	*S*. Typhimurium
19	G-39	+	+	+	*S*. Oritamerin

G-40	-	+	+	*S.* Typhimurium
G-43	-	+	+	*S.* Oritamerin
G-45	+	-	-	*S.* Typhimurium
G-55	+	+	+	*S.* Typhimurium
G-2	-	+	+	*S.* Abuja

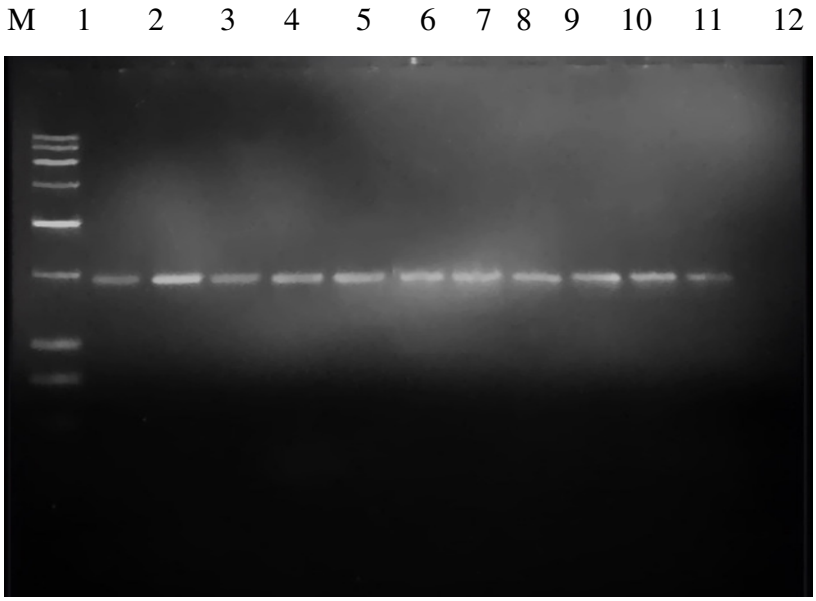

Fig.1 vir gene amplicons

M – Marker, Lane 1-12 – vir gene amplicon

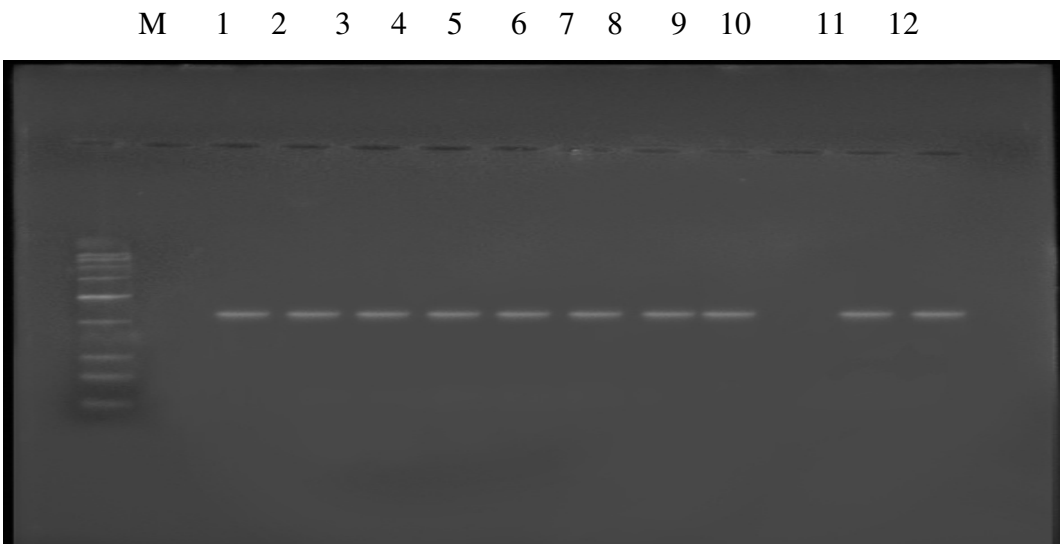

Fig -2

M – Marker, Lane 1-12 – pef gene amplicon

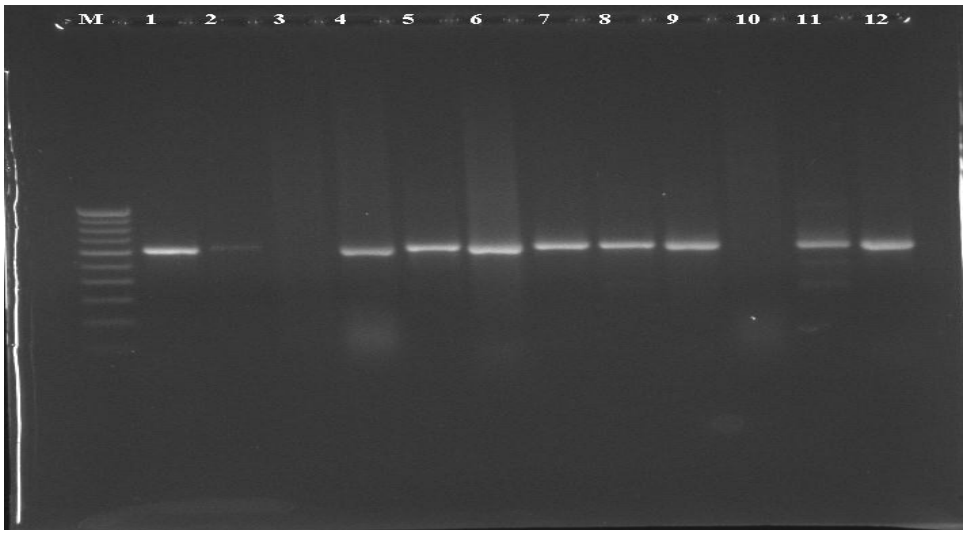

Fig -3

M – Marker, Lane 1-12 – stn gene amplicon

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