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Multiplex Pcr for Detection of Virulence Gene Profiles of Salmonella Isolates from Animals and Man

KEYWORDS: virulence gene, Salmonella, Multiplex PCR.

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ABSTRACT Salmonellosis is a leading cause of illness in animals and man worldwide. The present study was undertaken to isolate Salmonella from various sources including animals, birds and human and to study their virulence gene profiles based on multiplex PCR assays developed for the purpose. Out of a total of 453 samples examined, 30 (6.62%) were found to be positive for Salmonella. Among the 30 isolates recovered, the serovar Salmonella Enteritidis was found to be the most prevalent (53.33%). The other serovars recovered were Salmonella Typhi 6 (20.00%), Salmonella Typhimurium 4 (13.33%), Salmonella Weltevreden 2 (6.67%), and Salmonella Dublin and Salmonella Gallinarum 1 (3.33%) each. A multiplex PCR was designed for rapid detection of six important virulence genes of Salmonella, the chromosomal genes invA, agfA and stn in one set, and the plasmid encoded genes spvA, spv B and spv C in another. Isolates belonging to different serovars showed variable results in respect to possession of different virulence genes.

Introduction

Salmonellae are well recognized as potential pathogens that cause diseases in a wide range of mammals and birds and encode products that assist the organisms in expressing their virulence in the host cells. Some genes are known to be involved in adhesion and invasion, *viz., sef1, pef2, spv3* or *inv4*; others are associated with the survival in the host system- *mgtC5* or in the actual manifestation of pathogenic processes, *viz., sop6, stn7, pip A, B* and *D8*. Nucleic acid based diagnostic techniques are being employed for the detection of various gene-encoded virulence factors, *viz. Salmonella* enterotoxin (*stn*), *Salmonella* Enteritidis fimbriae (*sef*) and plasmid encoded fimbriae (*pef*) genes (Widjojoatmodjo *et al.*, 1992). 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 (Jamshidi *et al.*, 2008). Strains of *S. enterica* serovar Enteritidis often carry serovar-associated plasmids which encode a virulence operon consisting of five genes *spvR, spvA, spvB, spvC* and *spvD* (Aaboet *al.*, 2000). The *spv* genes play a role in the virulence of the host strain (Chu *et al.*, 1999). The present study was undertaken with a view to standardize a multiplex PCR (mPCR) protocol for detection of six important chromosomal and plasmid genes associated with virulence of common *Salmonella* serovars involved in clinical infections in man and animals.

Materials and Methods

Faecal /rectal swabs and tissue samples were collected from various species of animals and man from places in and around Guwahati. In addition, samples from free living birds were collected from different locations around the city. Rappaport Vassiliadis (RV) broth was used as an enrichment broth for primary isolation of *Salmonella* and Brilliant Green Agar (BGA) was used as a selective medium for subculture. For purification of suspected *Salmonella* colonies, Mac Conkey's Lactose Agar (MLA) and Xylose lysine deoxycholate agar (XLD) were used. Characterization and preliminary identification of suspected *Salmonella* cultures were made by standard methods and the confirmed isolates were sent for serotyping at the National Institute of Cholera and Enteric Diseases (NICED), Kolkata.

For preparation of DNA template, the bacterial isolates were grown in LB broth for overnight at 37°C and boiled at 100°C for 20 minutes. After boiling, the cell suspensions were snap-cooled on ice and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was then collected without disturbing the sediment and was stored at -20°C (Titballet al., 1989). The isolates were further confirmed as Salmonella by detecting Salmonella-specific gene STM0171 by simplex PCR using specific primers (Forward - GACCCCGGATTTTTTGAGAA), Reverse - (ACCACGGAGAGACAGTTCAGAT) as reported by Borah (2012). All the standard strains and field isolates of Salmonella were screened for the presence of six important virulence genes, viz.stn, agfA, invA, spv A, spvB and spvC by PCR using specific primers (Table 1).

For the present study, specific primers for invA and spvB

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were designed against the corresponding gene sequences downloaded from NCBI using Primer 3 software. Altogether six virulence genes were screened, viz. stn, agfA, invA, spv A, B and C. Multiplex grouping was done considering the product size, annealing temperature and genomic location of the genes. Two groups were formed comprising of three genes in each group. The PCR conditions suitable for amplification of the genes were standardized as follows: denaturation at 94° C for 15 sec, primer annealing at 58° C (Group I) and 55° C (Group II) for 30 sec, extension at 72° C for 25 sec and final extension at 72° C for 5 min. Control reaction mixtures containing no template or genomic DNA from Salmonella Typhimurium, S.Typhi or S. Enteritidis were also included in each of the sample run. The mPCR reaction mixture comprised of 25 µL consisting of (2X) PCR master mix 12.5 µL (Thermo Scientific), 20 pmol of each primer and 1 µL (10 ng) of template. The PCR products were analyzed in 1.5% agarose gel (Sigma) stained with ethidium bromide (0.5 µg/ml) and then visualized using a Gel Documentation system (BioRad). A 100 bp DNA ladder (Thermo Scientific) was run simultaneously in each gel run.

Results and discussion

In the present investigation, a total of 453 samples collected from different sources were examined, of which 30 (6.62%) were found to be positive for *Salmonella*. On serotyping, 16 (53.33%) isolates were found to belong to *Salmonella enterica* serovar Enteritidis, 6 (20.00%) isolates to serovar Typhi, 4 (13.33%) to serovar Typhimurium, 2 (6.67%) of serovar Weltevreden, and 1 (3.33%) each to serovars Dublin and Gallinarum. *Salmonella* Enteritidis was the commonest serotype world-wide, followed by *S*. Typhimurium (Galanis *et al*., 2006). This was in close agreement with the present findings. In India among the typhoidal serovars, the most common serovars from humans are *Salmonella* Typhi (73%) and *S*. Paratyphi A (24%) and among non-typhoidal serovars, *S*. Worthington (28.2%) and *S*. Typhimurium (22.5%) are more frequently isolated. In animals, *S*. Typhimurium is the commonest serovar followed by *S*. Weltevreden (Kumar *et al*., 2009). This was also in partial agreement with the present findings.

All the 30 *Salmonella* isolates were subjected to multiplex PCR assay for detection of the specific virulence genes (Figures 1 & 2). The virulence genes *invA* and *stn* were detected in all (100%) the *Salmonella* isolates examined. The *agfA* gene was present in 13 (43.33%) of the 30 isolates, *spvA* in 5 (16.67%), *spvB* in 12 (40%) and *spvC* in 7 (23.33%) of the 30 *Salmonella* isolates screened (Table 2).

The only isolate of serovar Enteritidis from human and three other isolates of the same serovar from birds revealed the presence of all the virulence genes under study. All the six isolates of serovar Typhi isolated from diarrhoeic stool samples of human exhibited presence of *invA* and *stn* genes, while 3 (50%) isolates of this serovar showed presence of *agfA* gene. None of the isolates, however, showed presence of genes under the *spv* cluster. The *agfA* gene was also found to be absent in the two *S*. Typhimurium isolates from cattle and the two Weltevreden isolates from birds. All the other isolates belonging to serovars Typhimurium, Gallinarum and Dublin showed the presence of *invA* and *stn* genes as well as one or the other member of the *spv* gene cluster.

The *invA* (invasive) gene of *Salmonella* contains sequences unique to this genus (Jamshidi *et al*., 2008) which is typical to *Salmonella* isolates. This gene was detected in the present study in all isolates tested, which was in agreement with Salehi *et al*. (2005), Bacci *et al*. (2006) and Shanmugasamy *et al*. (2011). The *stn* gene that encodes for enterotoxin was also found in all the 30 isolates screened, which was in agreement with Murugkar *et al*. (2003). In the present study, *spvA* gene was found to be present in 5 (16.67%), *spvB* in 12 (40%) and *spvC* in 7 (23.33%) of the 30 *Salmonella* isolates screened. Among a subset of 18 strains of the SARB (*Salmonella* Reference collection B) collection examined (Boyd *et al*., 1993), the *spv* region was found to be present in isolates of serovar Choleraesuis, Dublin, Enteriditis, Paratyphi C, Pullorum, and Typhimurium (Baumler *et al*., 1997). The function of *spvA, B, C* and *D* gene products has remain undetermined, but it has been shown that the presence of the virulence plasmid increases the growth rate of *Salmonella* in mice (Gulig and Doyle, 1993). In the present study, the *agfA* gene was found to be present in 13 (43.33%) of the 30 isolates. The *agfA* gene was considered to be a structural gene for the *sef17* fimbriae which mediate fibronectin binding and bacterial agglutination (Doran *et al*., 1993) thereby facilitate colonization.

Conclusion

Among the isolates recovered from different sources, *S*. Enteritidis (53.33%) was the most frequent serovar, followed by *S*. Typhi (20.00%) and *S*. Typhimurium (13.33%). A Multiplex PCR assay was developed for detection of six important virulence genes of *Salmonella*, *viz. invA*, *stn*, *agfA*, *spvA*, *spvB* and *spvC*. All the 30 *Salmonella* isolates showed presence of *invA and stn* genes suggesting the possibility of using these genes for identification of *Salmonella*. Presence of *agfA, spvA, spvB* and *spvC* genes was detected in varied percentages. Further studies may be undertaken in future involving larger samples size and more number of field isolates to investigate about the distribution of these and other important virulence genes among various clinical serovars of *Salmonella*. Such works may also include study on expression of the virulence genes in different growth conditions of the bacteria.

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Table No. 1: Primer sequences used for detection of virulence genes of *Salmonella*

Gene	Primer Sequence	Product Size	Primer Concentration (pMol/µl)	Reference
agfA	Forward –TCCGGCCCGGACTCAACG Reverse- CAGCGCGGCGTTATACCG	261 bp	20	Doran *et al*. (1993)
Stn	Forward- TTGTGTCGCTATCACTGGCAACC Reverse – ATTCGTAACCCGCTCTCGTCC	618 bp	20	Murugkar *et al*. (2003)
invA	Forward – GAACTGACTACGTAGACGCTC Reverse - ACCACGCTCTTTCGTCTGGT	943 bp	20	Designed
spvA	Forward- GTCAGACCCGTAAACAGT Reverse- GCACGCAGAGTACCCGCA	641 bp	10	Guerra *et al*. (2001)
spvB	Reverse- GAGGGATTAAGTTCACTTCC Forward- CATCTCCGAGTATTCTACCT	223 bp	10	Designed
spvC	Forward- ACTCCTTGCACAACCAAATGCGGA Reverse- TGTCTTCTGCATTTCGCCACCATCA	566 bp	10	Guerra *et al*. (2001)

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Table No. 2: Detection of virulence genes in *Salmonella* isolates by multiplex PCR

Serotypes	No. of isolates	Health Status	Source	Detection of virulence genes					
				Multiplex group I			Multiplex group II		
				spv	*invA*	*agfA*	*spvA*	*spvB*	*spvC*
S. Enteritidis	16	Apparently healthy	Human (1)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
			Bird(15)	15 (100)	15 (100)	6 (40)	3 (20)	7 (46.67)	4 (26.67)
S. Typhi	6	Diarrhoea	Human(6)	6(100)	6(100)	3(50)	0	0	0
S. Typhimurium	4	- Do -	Bird(2)	2(100)	2(100)	1(50)	0	1(50)	1(50)
		- Do -	Cattle (2)	2(100)	2(100)	0	0	0	0
S. Saintpaul	2	- Do -	Bird (2)	2 (100)	2 (100)	0	1 (50)	1 (50)	1 (50)
S. Oslo	1	- Do -	Bird(1)	1(100)	1(100)	1(100)	0	1(100)	0
S. Dublin	1	- Do -	Cattle (1)	1(100)	1(100)	1(100)	0	1(100)	0
Total	30			30 (100)	30 (100)	13 (43.33)	5 (16.67)	12 (40.00)	7 (23.33)

*Figures in parentheses indicates percentages

Figure 1: Screening of *Salmonella* isolates by Multiplex PCR (Group I)

Lane 1 to 4 – Field isolates; Lane 5- Positive control; Lane 6-Negative Control; Lane 7- 100 bp Ladder

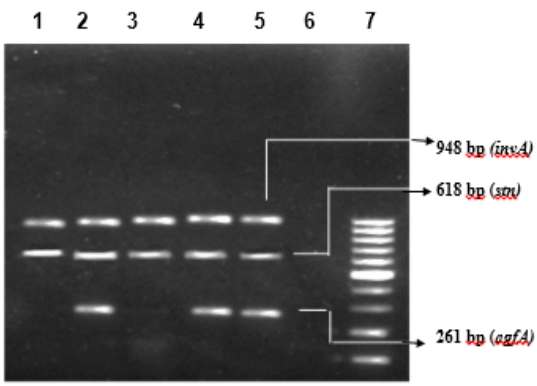
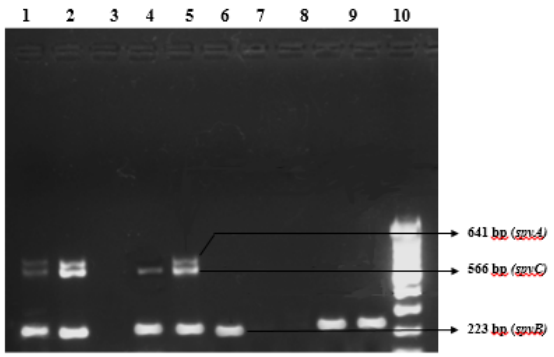


Figure 2: Screening of *Salmonella* isolates by Multiplex PCR (Group II)

Lane 1 to 8 – Field isolates; Lane 8- Positive control; Lane 9-Negative Control; Lane 10- 100 bp Ladder



REFERENCE

Aabo, S., Brown, D. J. & Olsen, E. J. (2000). Virulence characterization of a strain of Salmonella enterica subspecies Houten with chromosomal integrated Salmonella plasmid virulence (spv) genes. Research in Microbiology, 151, 183-189. | Bacci, C., Paris, A., Salsi A. & Brindani, F. (2006). Genotypic and phenotypic virulence features in Salmonella enterica strains isolated from meat. Ann. Fac. Medic. Vet. Di parma., 26, 165-174. | Baumler, A. J., Gilde, A. J., Tsolis, R. M., van der Velden, A. W. M., Ahmer, B. M. M. & Heffron, F. (1997). Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operons during evolution of Salmonella serovars. Journal of Bacteriology, 179, 317–322. | Borah, P. P. (2012). Identification of common clinical serovars of Salmonella by multiplex PCR. Thesis, Assam Agric. Univ., Guwahati, India. | Boyd, E. F., Wang, F. S., Beltran, P., Plock, S. A. & Nelson, K. (1993). Salmonella reference collection B (SARB): strains of 37 serovars of subspecies I. Journal of General Microbiology, 139, 1125–1132. | Chu, C., Hong, S. F., Tsai, C., Lin, W. S., Liu, T. P. & Ou, J. (1999). Comparative physical and genetic maps of the virulence plasmids of Salmonella enterica serovar Typhimurium, Enteritidis, Choleraesuis and Dublin. Infection and Immunity, 99, 2611- 2614. | Doran, J. L., Collinson, S. K., Burian, J., Sarlos, G., Todd, E. C. D., Munro, C. K., Kay, C. M., Banser, P. A., Peterkin, P. I. & Kay, W. W. (1993). DNA-based diagnostic tests for Salmonella species targeting agfA, the structural gene for thin, aggregative fimbriae. Journal of Clinical Microbiology, 31, 2263–2273. | Galanis, E., Lo Fo Wong, D. M. A., Patrick, M. E., Binsztein, N., Cieslik, A. & Chalermchikit, T. (2006). Web-based surveillance and global Salmonella distribution, 2000-2002. Emerging Infectious Disease Journal, 12, 381-388. | Guerra, B., Soto, S. M., Arguelles, J. M. & Mendoza, M. C. (2001). Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent Salmonella enterica serotype [4,5,12:i:−]. Antimicrob. Agents Chemother., 45, 1305-1308. | Gulig, P. A. & Doyle, T. J. (1993). The Salmonella Typhimurium virulence plasmid increases the growth rate of salmonellae in mice. Infection and Immunity, 61, 504–511. | Jamshidi, A., Bassami, M. R. & Afshari-Nic, S. (2008). Identification of Salmonella serovars Typhimurium by a multiplex PCR-Based assay from Poultry carcasses in Mashhad-Iran. International Journal of Veterinary Research, 3, 43-48. | Kumar, Y., Sharma, A., Sehgal, R. & Kumar, S. (2009). Distribution trends of Salmonella serovars in India (2001–2005). Transactions of the Royal Society of Tropical Medicine and Hygiene, 103, 390—394 | Murugkar, H.V., Rahman, H. & Dutta, P. K. (2003). Distribution of virulence genes in Salmonella serovars isolated from man & animals. Indian Journal of Medical Research, 117, 66-70. | Salehi, T. Z., Mahzounieh, M. & Saeedzadeh, A. (2005). Detection of invA Gene in isolated Salmonella from broilers by PCR Method. International Journal of Poultry Science, 4, 557-559. | Shanmugasamy, M., Velayutham T. & Rajeswar J. (2001). InvA gene specific PCR for detection of Salmonella from broilers. Veterinary World, 4, 562-564. | Titball, R. W., Hunter, S. E. C., Martin, K. L., Morris, A. D., Shuttle Worth, A. D., Rubdug, T., Anderson, D. W. & Kelly, D. C. (1989). Molecular cloning and nucleotide sequence of alpha toxin of Clostridium perfringens. Infection and Immunity, 57, 367-376. | Widjojoatmodjo, N. M.; Fluit, A. C.; Torensma, R.; Verdonk, G. P. & Verhoef, J. (1992). The magnetic immune polymerase chain reaction assay for direct detection of Salmonella in faecal sample. Journal of Clinical Microbiology, 30, 3195-3199. |