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Plasmid profiling and comparison of the plasmid profiles with virulence of Indian *Salmonella* Typhimurium strains of varied origin

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ABSTRACT

Among 2,668 *Salmonella enterica* serovars *Salmonella* Typhimurium is the most common serovar and discrimination of isolates within this serovar is necessary for the effective epidemiological surveillance and control. Plasmid profiling is an efficient tool for discrimination of bacterial isolates. Hence, 22 strains of *S.* Typhimurium isolated from different parts of India were analyzed by plasmid profiling to check its differentiation power and to correlate the results with virulence. Nine different sized bands were detected after plasmid isolation with 90.0 kb band shared by most isolates. At least one plasmid was present in all isolates. Based on plasmid profiles 22 isolates were classified into 10 groups with a discriminating power of 0.89. The maximum dissimilarity distance was 0.96496 units. No remarkable correlation between plasmid pattern and host origin could be established pointing out the potential hazard of interspecies sharing. However, comparison of plasmid profiles with virulence revealed the absence of 90.0 kb plasmid in non virulent isolates. The present study thus confirmed the potential discriminatory power of plasmid profiling along with extraordinarily high incidence of plasmids among Indian *S.* Typhimurium isolates. Additionally it indicated the presence of 90 kb plasmid to be used as a virulence marker of this serovar.

Key words: Heterogeneity, Plasmid profile, *Salmonella* Typhimurium, Virulent plasmid

Among various food-borne pathogens salmonella is one of the most significant candidates causing numerous food-borne outbreaks (Bhowmick *et al*. 2012). Among 2,668 *Salmonella* Enterica serovars (Popoff *et al*. 2004), *Salmonella* Typhimurium is the most common (Abbassi-Ghozzi *et al*. 2012). Discrimination of isolates between and within salmonella serotypes is necessary for the effective epidemiological surveillance and control (El-Sebay *et al*. 2012). Conventional approaches used for these purposes are lengthy, laborious and costly (Gallegos-Robles *et al*. 2008) and usually do not give differences beyond species or serotype levels (Nhidza *et al*. 2012). Thus standardization of easy, economical and quicker molecular methods to discriminate between *S*. Typhimurium isolates is necessary.

Since the report on prevalence of plasmids in salmonella by Taylor *et al*. (1982), there have been many reports that plasmid profiling is an efficient tool in the discrimination of various bacterial isolates (Halawani and Shohayeb 2008). Compared to other molecular techniques, this has extra advantages such as rapidity, high reproducibility, relative simplicity and requirement of only basic apparatus etc (Liebana *et al*. 2001). Moreover, plasmids in many salmonella control various medically imperative properties (Rychlik *et al*. 2006). In India salmonellosis is hyper-endemic (Nagappa *et al*. 2007) and more than 235 serovars were reported (Singh 2005). Though there are several reports on the prevalence of salmonella from India, information on genetic diversity is limited (Bhowmick *et al.* 2012). Keeping these facts in view, the objective of the present study was to check the differentiation power of plasmid profiling among Indian *S.* Typhimurium isolates and to find out any correlation between plasmid profiles with virulence of this serovar.

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MATERIALS AND METHODS

Bacterial cultures

S. Typhimurium isolates (22) of varied origin (Table 1) were obtained from National Salmonella Centre, Indian Veterinary Research Institute (IVRI), Izatnagar. After sub culturing of each isolate on Mac-Conkey agar, colonies were characterized morphologically using Gram's stain and serologically with poly 'O' sera (obtained from Division of Biological Products, IVRI) based on Kauffmann-White

Table 1. Host and epidemiological sources of *S.* Typhimurium isolates

Host	Culture code (source)
Poultry	E2394[a] and E2393[a] (Chick), E2375 [a](Broiler), E2622[a] (Liver), E4638[b] (Egg), E4227[b] (Poultry), E4841[c] & E4809[c] (Eggs), E4885[d] and E4896[d] (Chicken), E4256[b] (Chicken Pizza)
Bovine	E2416[b] (Liver), E5158[b], E4935[b] and E4946[b] (Faeces), E4242[e]
Human	E4938[b] (Stool)
Feed	E4231[b] and E4658[b]
Water	E4659[b], E4490[c]
Corriander leaves	E2677[c]

Place of isolation: [a]Pune; [b]Bareilly; [c]Pantnagar; [d]Mumbai; [e]Babugarh.

scheme (Kauffmann 1964). Molecular confirmation was done using salmonella specific *fimA* (Cohen *et al*. 1996) and Typhimurium specific *STM4497 fr3* primers (Kim *et al*. 2006). After characterization glycerol stock were made and kept at –20°C.

Experimental animals

Clinically healthy Swiss albino mice and chicks of either sex were procured from the Laboratory Animal Resources (LAR) Section, IVRI and Central Avian Research Institute, Bareilly, Uttar Pradesh respectively and maintained under standard conditions of nutrition and management. The animal ethics committee of the IVRI, Deemed University approved the study.

Plasmid profile analysis

The method of Singh *et al*. (2010) was followed for plasmid profiling. Plasmids were isolated from 10 ml overnight LB culture of all isolates using alkali lysis method (Birnboim and Dolly 1979) and the pellet was resuspended in 50 µl of sterile nuclease free water and stored at –20°C. 10 µl of isolated plasmid from each isolate was run in 0.7% agarose gel and photographed under UV illuminator. Lambda DNA/*Eco*RI + *Hind*III marker and 100 bp plus marker were run simultaneously to determine the molecular size. Gel images were then analyzed using GelQuest-DNA fingerprint analysis software. In this, each band on the gel were scored as discrete variables, using ''1'' to indicate presence and ''0'' to indicate the absence. This binary data was directly used to calculate distance matrices thus to construct phylogenetic relationships by neighbor-joining method. The numerical index of discrimination (D) was calculated using Simpson's index of diversity (Hunter and Gaston 1988).

Pathogenicity testing of S. *Typhimurium isolates and comparative analysis of plasmid profiling with pathogenicity*

Work on the pathogenicity of *S*. Typhimurium was done in mice or chicks by intraperitoneal injection of bacteria (Chapes and Beharka 1998, Dieye *et al.* 2009, Benincasa *et al.* 2010, Mair *et al.* 2011). Hence pathogenicity of *S.* Typhimurium was tested in mice and chicks by intraperitoneal route of injection. For this, 0.1 ml of 10^{-1} dilution and 1 ml of 18 hr old broth culture (1.47×10^8 CFU/ml) of each isolate was injected I/P into 3 healthy mice and chicks respectively. All were observed for 7 days for development of any clinical signs or fatality. The plasmid profiles were then compared with pathogenicity of each isolate to find out whether there is any relation between plasmid profiles and virulence of *S.* Typhimurium.

RESULTS AND DISCUSSION

During characterization all 22 isolates showed typical cultural, morphological and serological characteristics. During molecular confirmation, every one gave positive results with both genus and serotype primers (Figs 1, 2).

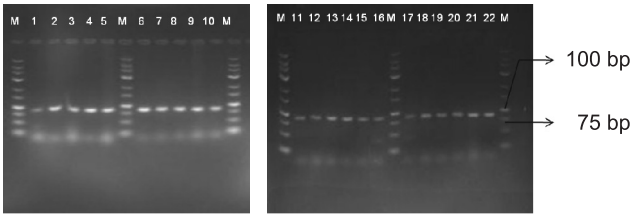

Fig. 1. PCR amplification of *fimA* primers (85 bp product).

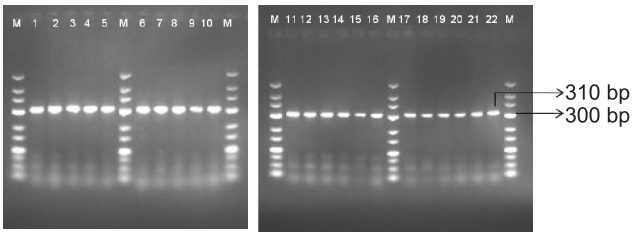

Fig. 2. PCR amplification of *STM4497 fr3* primers (310 bp product).

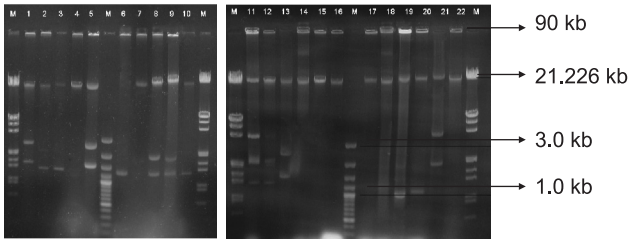

Fig. 3. Plasmid profiles of *S.* Typhimurium isolates.

Plasmid profiling

On plasmid profiling nine different sized bands (90.0, 50.0, 20.4, 3.5, 3.0, 2.5, 2.0, 1.5, and 1.2 kb) were detected with at least one plasmid in all 22 isolates. Similar molecular sized plasmids were reported earlier in *S.* Typhimurium isolated from outside India by many workers (Brunner 1983, Nakamura *et al.* 1986, Halawani and Shohayeb 2008). Ten different profiles were identified (Table 2) in which the most predominant profile (A) was characterized by the presence of 2 plasmids (90.0 and 20.4 kb). Similarly 18, 8, 7 and 4 different plasmid groups of *S*. Typhimurium among 198, 56, 25 and 23 isolates were reported by Baggesen *et al*. (1992), Singh *et al*. (2010), Halawani and Shohayeb (2008) and Abbassi- Ghozzi *et al*. (2012) respectively. We could not accurately determine the exact molecular weight of 2 large plasmids as they were above the limit of the molecular weight markers. Nevertheless, the molecular weight of large sized plasmids of *S.* Typhimurium isolates isolated by the same protocol were already reported as 90.0 and 50.0 kb (Rilay *et al*. 1983, Helmuth *et al*. 1985, Barrow and Lovell 1989, Baggesen *et al*. 1992, Bhattacharya *et al.* 2000, Liebana 2002, Majtan *et al.* 2005, Halawani and Shohayeb 2008, Singh *et al*. 2010, Abbassi Ghozzi *et al*. 2012). So the 2 high molecular weight plasmids obtained, were assigned as 90.0 and 50.0 kb. We could detect at least one plasmid in all isolates similar to observation of Nakamura *et al*. (1986) in Japanese isolates. The large molecular weight plasmid (90.0 kb) was shared by most isolates (82.2%). This 90 kb is often regarded as serovar specific plasmid of *S.* Typhimurium (Barrow and Lovell 1989, Liebana *et al*. 2002, Halawani and Shohayeb 2008). Compliant with our observation many authors (Rilay *et al*. 1983, Helmuth *et al*. 1985, Baggesen *et al*. 1992, Singh, *et al*. 2010, Abbassi Ghozzi *et al*. 2012) had reported the absence of this plasmid in some *S*. Typhimurium strains. Thus designation of this plasmid as "serotype specific" has to be verified by future studies. In the phylogram constructed based on the plasmid profiles (Fig. 4) the maximum dissimilarity distance between 2 types was 0.96496 units. The discrimination power of plasmid profiling was calculated as 0.89 showing the potentiality of this technique in the discrimination of Indian strains of *S*. Typhimurium. We couldn't correlate the presence of other plasmids with any host origin pointing out the potential hazard of interspecies sharing of these organisms. At the same time plasmids are important for the storage and

Table 2. Plasmid profiles among *S.* Typhimurium isolates of varied origin

Profile group		Banding pattern No. of isolates having the particular banding pattern					
		Poultry	Bovine	Human	Feed	Water	Coriander leaves
A	90 kb, 20.4 kb	1	3	-	1	-	1
B	90 kb,20.4 kb,50 kb	-	1	-	-	1	-
C	90 kb,20.4 kb,2 kb,1.2 kb	2	1	1	-	-	-
D	90 kb,20.4 kb,1.2 kb	2	-	-	-	-	-
E	90 kb,20.4 kb,3 kb,2 kb,1.2 kb	1	-	-	-	-	-
F	90 kb,20.4 kb,3.5 kb,2 kb	2	-	-	-	-	-
G	90 kb,1.2 kb	1	-	-	-	-	-
H	20.4 kb,1.2 kb	-	-	-	1	-	-
I	20.4 kb,2 kb,1.2 kb	1	-	-	-	-	-
J	20.4 kb,2.5 kb,1.5 kb	1	-	-	-	1	-

Table 3. Pathogenicity testing of *S.* Typhimurium isolates

Source	Culture code	Pathogenicity in mice within 7 days	Pathogenicity in chicks within 7 days
Poultry	E2393, E4227, E4896, E4885, E4841, E4885, E4896, E4256	3/3	3/3
	E2394, E2375	3/3	2/3
	E2622	1/3	0/3
	E809, E4638	0/3	0/3
	E2416, E4242, E4935	3/3	-
Bovine	E4946, E5158	2/3	-
	E4938	3/3	-
Human	E4658	3/3	-
Feed	E4231	0/3	-
	E4659	1/3	-
Water	E4490	2/3	-
	E2677	3/3	-
Coriander Leaves			

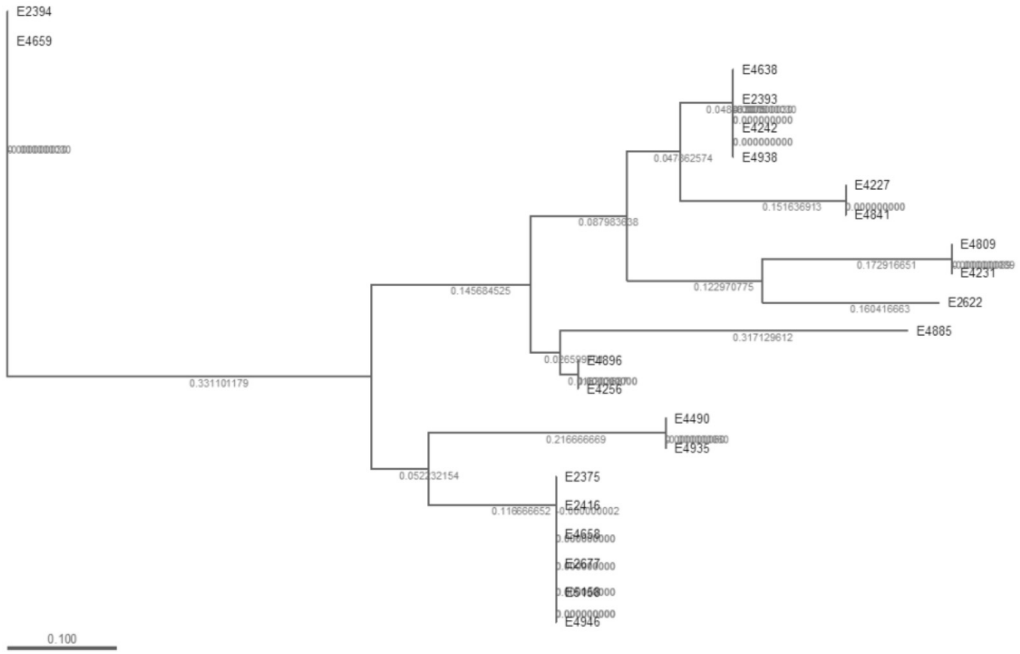

Fig. 4. Phylogram based on plasmid profiles of *S.* Typhimurium isolates.

dissemination of antibiotic resistance (Rychlik *et al*. 2006). Thus the extraordinarily high incidence of plasmids among Indian *S*. Typhimurium isolates raises the question of what biological function these plasmids must be performing in these bacteria which has to be explored further.

Pathogenicity testing of S. *Typhimurium isolates*

Out of 22 isolates tested for pathogenicity in mouse model, 14 induced death in all 3 mice within 24–48 h post inoculation after inducing nonspecific clinical signs like dullness and ruffled coat. Similarly, during pathogenicity testing in chicks out of 13 tested isolates, 8 induced death of all 3 chicks within 24–48 h (Table 3). It was worth to mention the isolates which were less or nonpathogenic in mice was nonpathogenic to chicks indicating the suitability of using mice for replacing chicks in virulence studies of *S*. Typhimurium.

Comparative analysis of plasmid profiling with pathogenicity

By the comparison of plasmid profiles with pathogenicity the 90.0 kb plasmid shared by most isolates was found to be absent in non virulent and less virulent isolates. Parallel to our observation Ahmer *et al*. (1999), Lesnick *et al*. (2001) and Tezcan-Merdol *et al*. (2001), have given the name virulence plasmid to this plasmid. Thus plasmid profiling proved to be a useful tool in pathotyping. But, it is possible that plasmid-free salmonella strains might have virulence genes in the chromosome (Fields *et al*. 1989). So inference on pathogenicity merely based on plasmid profiling has to be done vigilantly.

In conclusion, the present study confirmed the potential discriminatory power (0.89) of plasmid profiling along with extraordinarily high incidence of plasmids among Indian *S*. Typhimurium isolates. In addition the present study pointed out the potential hazard of interspecies sharing of these organisms revealing the urgent need for control measures. Moreover, this indicated the future use of 90 kb plasmid as a virulence marker of this serovar. At the same time it showed that the designation of this plasmid as serotype specific had to be reexamined.

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REFERENCES

Abbassi-Ghozzi I, Hammami S, Aissa R B, Urtaza J M, Boudabous A and Gtari M. 2012. Pulsed-field gel electrophoresis, plasmid profile and antimicrobial resistance pattern of *Salmonella* Typhimurium isolated from human and retail meats. *African Journal of Microbiology Research* **6**: 4680–6.

Ahmer B M, Tran M and Heffron F. 1999. The virulence plasmid of *Salmonella* Typhimurium is self-transmissible. *Journal of Bacteriology* **181**: 1364–8.

Baggesen D L, Olsen J E and Bisgaard M. 1992. Plasmid profiles and phage types of *Salmonella* Typhimurium isolated from successive flocks of chickens on three parent stock farms. *Avian Pathology* **21**: 569–79.

Barrow P A and Lovell M A. 1989. Functional homology of virulence plasmids in *Salmonella* Gallinarum, *S*. Pullorum, and *S*. Typhimurium. *Infection and Immunity* **57**: 3136–41.

Benincasa M, Pelillo C, Zorzet S, Garrovo C, Biffi S, Gennaro R and Scocchi M. 2010. The proline-rich peptide Bac7 (1–35) reduces mortality from *Salmonella* Typhimurium in mouse model of infection. *BMC Microbiology* **210**: 178– 86.

Bhattacharya A, Singh V P and Verma J C. 2000. Amplification of virulence gene, presence of 90 kb plasmid and virulence in mice and chicks in *Salmonella* Typhimurium (poultry isolates). *Indian Journal of Community Medicine* **21**: 107–10.

Bhowmick P P, Srikumar S, Devegowda D, Shekar M, Ruwandeepika H A D and Karunasagar I. 2012. Serotyping and molecular characterization for study of genetic diversity among seafood associated nontyphoidal salmonella serovars. *Indian Journal of Medical Research* **135**: 371–81.

Birnboim H C and Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**: 1513–23.

Brunner F, Margadant A, Peduzzi R and Piffaretti J C. 1983. The plasmid pattern as an epidemiologic tool for *Salmonella* Typhimurium epidemics: comparison with the lysotype. *Journal Infectious Diseases* **148**: 7–11.

Chapes S K and Beharka A A. 1998. Salmonella infections in the absence of the major histocompatibility complex II. *Journal of Leukocyte Biology* **63**: 297– 304.

Cohen H J, Mechanda S M and Lin W. 1996. PCR amplification of the *fimA* gene sequence of *Salmonella* Typhimurium, a specific method for detection of salmonella spp. *Applied and Environmental Microbiology* **62**: 4303–8.

Dieye Y, Ameiss K, Mellata M and Curtiss R. 2009. The salmonella pathogenicity island (SPI) 1 contributes more than SPI2 to the colonization of the chicken by *Salmonella* enterica serovar Typhimurium. *BMC Microbiology* **9**: 3.

El-Sebay N A, Gebreel H M, El-Zeedy S and Samy A A. 2012. Genotyping of some local isolates of genus salmonella using RAPD-PCR. *World Applied Sciences Journal* 17: 1377–85.

Fields P I, Groisman E A and Heffron F. 1989. A salmonella locus that control resistance to microbial protein from phagocytic cells. *Science* **243**: 1059–62.

Gallegos-Robles M A, Morales-Loredo A, Alvarez-Ojeda G, Vega-P A, Chew-M Y, Velarde S and Fratamico P. 2008. Identification of salmonella serotypes isolated from cantaloupe and Chile pepper production systems in Mexico by PCR-restriction fragment length polymorphism. *Journal of Food Protection* **71**: 2217–22.

Halawani E and Shohayeb M .2008. Epidemiological typing of *Salmonellla enterica* isolates causing acute food poisoning in Saudi Arabia based on plasmid profiles and antibiograms. *Advances in Biological Research* **2**: 68–77.

Helmuth R, Stephan R, Bunge C, Hoog B, Steinbeck A and Bulling E. 1985. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common salmonella serotypes. *Infection and Immunity* **48**: 175–82.

Hunter P R and Gaston M A. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology* **26**: 2465–6.

Kauffmann F. 1964. The world problem of salmonellosis. Das Kauffmann-White schema. pp 21–66. (Eds) E. van Oye. Dr. W. Junk Publishers, The Hague, The Netherlands.

Kim H J, Park S H and Kim H Y. 2006. Comparison of *Salmonella enterica* serovar Typhimurium LT2 and Non-LT2 salmonella genomic sequences and genotyping of salmonellae by using PCR. *Applied and Environmental Microbiology* **72**: 6142–51.

Lesnick M L, Reiner N E, Fierer J and Guiney D G. 2001. The salmonella *spvB* virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Molecular Microbiology* **39**: 1464–70.

Liebana E, Guns D, Garcia-Migura L, Woodward M J, Clifton-Hadley F A and Davies R H. 2001. Molecular typing of salmonella serotypes prevalent in animals in England: Assessment of methodology. *Journal of Clinical Microbiology* **39**: 3609–16.

Liebana E. 2002. Molecular tools for epidemiological investigations of *S. enterica* subspecies enterica infections. *Research in Veterinary Science* **72**: 169–75.

Mair S M, Nairz M, Bellmann-Weiler R, Muehlbacher, T, Schroll, A, Theurl, I, Moser P L, Talasz H, Fang F C and Weiss G. 2011. Nifedipine affects the course of *Salmonella enterica* serovar Typhimurium infection by modulating macrophage iron homeostasis. *Journal of Infectious Diseases* **204**: 685–694.

Majtan V, Majtanova L and Szaboova M. 2005. The virulence markers of *Salmonella enterica* serovar Typhimurium. Different phage-type strains isolated in Slovakia. *Folia Microbiology* (Praha) **50**: 519–23.

Nagappa K, Tamuly S, Brajmadhuri, Saxena M K and Singh S P. 2007. Isolation of *Salmonella* Typhimurium from poultry eggs and meat of Tarai region of Uttaranchal. *Indian Journal of Biotechnology* **6**: 407–9.

Nakamura M, Sato S, Ohya T, Suzuki S and Ikeda S. 1986. Plasmid profile analysis in epidemiological studies of animal *Salmonella* Typhimurium Infection in Japan. *Journal of Clinical Microbiology* **23**: 360–5.

Nhidza A F, Saidi B, Makaya P V, Kanyoka P, Niang I S and Savadye D. 2012. Distribution of SPV genes, plasmid profiles and pulsotypes of *Salmonella* Enteritidis isolates of animal and human origins in selected locations of Zimbabwe. *Journal of Clinical Medicine and Research* **4**: 63–74.

Popoff M Y, Bockemuhi J and Gheesling L L. 2004. Supplement 2002 (no.46) to the Kauffmann-White scheme. *Research in Microbiology* **155**: 568–70.

Riley L W, Diferdinando G T Jr, Demelfi T M and Cohen M L. 1983. Evaluation of isolated cases of salmonellosis by plasmid profile analysis: introduction and transmission of a bacterial clone by precooked roast beef. *Journal of Infectious Diseases* **148**: 12–7.

Rychlik I, Gregorova D and Hradecka H. 2006. Distribution and function of plasmids in *Salmonella enterica*. *Veterinary Microbiology* **112**: 1–10.

Singh B R. 2005. An overview of conventional and molecular tools for diagnosis of avian salmonellosis. National symposium on molecular techniques for diagnosis of major bacterial diseases. pp. 16–34. February 21–22. Izatnagar.

Singh B R, Agarwal M, Chandra M, Verma M, Sharma G, Verma J C and Singh V P. 2010. Plasmid profile and drug resistance pattern of zoonotic salmonella isolates from Indian buffaloes. *The Journal of Infection in Developing Countries* **4**: 477–483.

Taylor D E, Levine J G and Kouvelos K L. 1982. Incidence of plasmid DNA in salmonella strains isolated from clinical sources in Ontario, Canada, during 1979 and 1980. *Canadian Journal of Microbiology* **28**: 1150–7.

Tezcan-Merdol D, Nyman T, Lindberg U, Haag F, Koch-Nolte F and Rhen M. 2001. Actin is ADP-ribosylated by the *Salmonella enterica* virulence-associated protein SpvB. *Molecular Microbiology* **39**: 606–19.