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Research Article

Molecular Epidemiology and Characterization of *Salmonella Serovars* from Broilers in Haryana, India

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ABSTRACT

A total of 51 isolates of *Salmonella*, including 23 isolates of *Salmonella gallinarum*, 13 isolates of *Salmonella pullorum*, 9 isolates of *Salmonella enteritidis* and 6 isolates of *Salmonella typhimurium* were isolated and characterized from outbreaks of *Salmonella* infection in broilers from commercial poultry farms of Haryana state, India. Isolates were characterized at their genotypic level by plasmid profiling, restriction endonuclease analysis and PCR. Plasmid profiles and antibiotic resistance pattern were correlated. Eight different plasmid profiles were obtained with presence of large plasmid of size 85 kb in maximum number of isolates (70%). Study suggested that 85 kb plasmid might carry the genes responsible for virulence. No positive correlation between plasmid profiles and antibiotic resistance pattern was found. Restriction endonuclease analysis of genomic DNA showed a high degree of genome homogeneity suggesting a common grandparent source of infection. PCR was found to be sensitive, specific and fast method to know the virulence of *Salmonella* isolates. Study concluded that plasmid profiling along with restriction enzyme analysis can be used as epidemiological markers in back tracing infections especially in case of outbreaks. Need of hour is to start mandatory *Salmonella* testing of parent flocks, positive reactors should be removed from the flock and vaccination of parent flocks should be carried out for control of this fast spreading, zoonotically important vertically transmitted pathogen. Moreover, at government level, there should be some legislation for control of such infections from hatchery.

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INTRODUCTION

Bacterial infections of genus *Salmonella* are responsible for large number of diseases in poultry. These diseases cause significant economical loss to poultry farmers. Moreover infected poultry stocks are the main reservoirs of *Salmonella* that can be transmitted to humans through food chain (Joseph *et al.*, 1997). Since the year 2005, there is sudden increase of *Salmonella* infection in poultry flocks mainly broilers in Haryana, India (Kumar *et al.*, 2010). To explore reasons for this increasing trend of *Salmonella* infection, molecular epidemiology at genotypic level was conducted. Molecular epidemiology is the application of techniques of molecular genetics to type strains of infectious agents for epidemiological studies. Detailed strain identification is essential for successful epidemiological investigation of *Salmonella enterica* serovar outbreaks. Investigations have relied traditionally on serological methods antibiograms, Phage typing etc. Modern typing methods are based on characterization of the genotype of the organism. In this regard Plasmid profiles have become valuable markers in the epidemiological investigation (Ferris *et al.*, 1992; Joseph *et al.*, 1997).

Plasmid profile analysis has been used as a rapid method and has shown success in the discrimination of *Salmonella* strains (Taylor *et al.*, 1982). Characterization of *Salmonella enterica* serovar *gallinarum* and *pullorum* was performed by plasmid proofing and biochemical analysis (Christensen *et al.*, 1992). Search for plasmid associated virulence gene is of great epidemiological interest and Josh and his co-workers in 1999 detect this gene by PCR. Polymerase chain reaction (PCR) can particularly be a useful tool to provide rapid and definitive detection of avian *Salmonella* serotypes. Strains having 85 Kb plasmid proved to be virulent (Joseph *et al.*, 1999). An association between the presence of plasmid of an approximately 85-kilobase (kb) in *Salmonella enterica* serovar *gallinarum* and ability of strains to produce high mortality in chickens was reported (Barrow *et al.*, 1987). Epidemiological status of avian *Salmonella enterica* serovar infections in India was elucidated (Prakash *et al.*, 2005). The restriction endonuclease analysis of total DNA was found to be useful in differentiating field isolates of *Salmonella enterica* serovar *gallinarum* (Joseph and Singh 2000). Antibiogram study and plasmid profile analysis were conducted to find out the correlation of recently isolated *Salmonella* strains of Bangladesh (Khan *et al.*, 2005). In India, although the isolation of various *Salmonella* serovars from poultry has been regularly reported from different parts of the country but information about the molecular characteristics of these serovars is limited (Mariani *et al.*, 2001). Moreover there are very few reports to date on genotypic analysis of *Salmonella* spp. isolated from poultry in this region too.

In the present study, isolation was made from suspected outbreaks, attempts were made to find out the pattern of

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plasmids in isolates from different sources and find out the differences /relatedness to derive an epidemiological pattern at genotypic level of this infection in the region so as to recommend preventive methods for this economically important zoonotic disease of poultry.

MATERIALS AND METHODS

Sampling and isolating *Salmonella*

In the year of 2008 a total of 132 outbreaks were recorded from various poultry farms (mainly broilers) situated in Haryana, India. Samples were collected for isolation and 51 isolates of *Salmonella*, including 23 isolates of *Salmonella enterica* serovar *gallinarum*, 13 isolates of *Salmonella pullorum*, 9 isolates of *Salmonella enteritidis* and 6 isolates of *Salmonella typhimurium* were isolated from these outbreaks. Initially isolates were confirmed by culture characteristics and biochemical assay with the help of Hi Salmonella Identification kit (Himedia). Finally serotyping was performed at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli – 173204, Himachal Pradesh, India. Isolates were also characterized by scanning electron microscopy. Molecular characterization was performed by plasmid profiling, restriction enzyme analysis, PCR. The *in vitro* antimicrobial sensitivity patterns of *Salmonella* isolates to various antimicrobial agents, Chloremphenicol C (30 mcg), Amikacin Ak (30 mcg), Gentamicin G (10 mcg), Cephatoxime Ce (10 mcg) and Cephadroxil Cq (30 mcg) were determined by the disc diffusion technique (Bauer *et al*., 1996).

PLASMID PROFILING:

Plasmid DNA isolation:

Plasmid DNA was extracted using alkaline lysis method (mini prep) as described in Sambrook and Russel 2001. Briefly single colony of *Salmonella* spp. was inoculated in 3 ml BHI broth and was incubated at 37^{0}C for 16-18 hours with intermittent shaking. Out of this, 1.5 ml was centrifuged at 8200 X g for 5 minutes at 4^{0}C. Supernatant was removed. Bacterial pellet was resuspended in 100ul of ice cold alkaline lysis solution I by vigorous vortexing and incubated at 25^{0}C. Two hundred ul of freshly prepared alkaline lysis solution II was added to bacterial suspension and was mixed. Tube was kept on ice for 3-5 minutes. One hundred fifty ul of ice cold alkaline lysis solution III was added and dispersed through the viscous bacterial lysate by inverting the tube several times and tube was kept on ice for 3-5 minutes. Bacterial lysate was centrifuged at 8200 X g for 10 minutes at 4^{0}C. Equal volume of phenol: chloroform: isoamylcohol (25:24:1) was added in the Supernatant. Organic and aqueous phases were mixed by vortexing and then the emulsion was centrifuged at 8200 X g for 10 minutes at 4^{0}C. Aqueous upper layer was transferred to a fresh tube and equal volume of chloroform: isoamylcohol (24:1) was added to remove the traces of phenol. Organic and aqueous phases were mixed by vortexing and then the emulsion was centrifuged at 8200 X g for 10 minutes at 4^{0}C. Aqueous upper layer was transferred to a fresh tube. Nucleic acids were precipitated from the supernatant by adding equal volume of isopropanol and incubated at -20^{0}C for 15 minutes. Precipitated nucleic acids were collected by centrifugation at 8200 X g for 20 minutes at 4^{0}C. Supernatant was removed by gentle aspiration. Pellet was resuspended in 1 ml of 70% ethanol. DNA was recovered by centrifugation at 8200 X g for 20 minutes at 4^{0}C and supernatant was removed by gentle aspiration. Pellet was dried in the desiccators concentrated. Nucleic acids were dissolved in 20ul of tris EDTA (TE) (PH 8.0) containing 20 ug / ml DNase-free RNase A solution. DNA was stored at - 20^{0}C till further use.

Agarose gel electrophoresis

The isolated plasmids were separated in 0.7% agarose gel (Low LEO) in 0.5X Tris Borate EDTA (TBE) electrophoresis buffer. Ethidium bromide, 0.5 ug / ml (Sigma) was added in the gel and photograph was taken using digital camera (Canon Powershot A620) under UV transilluminator (Spectroline Biovision, USA) after electrophoresis. Molecular weight/size marker ranging from 125bp to 23.13kbp was run along with isolated plasmid DNA. Standard curve between log molecular size of DNA marker and its Rf. values was plotted and point to point method was used to calculate molecular size of plasmids.

Restriction enzyme analysis

Isolation of chromosomal DNA

Bacterial chromosomal DNA was isolated by the method outlined by Maloy *et al*., 1996 with certain modifications. Briefly, *Salmonella* isolate was grown in 300 ml of BHI broth, pelleted by centrifugation, washed twice in PBS and then in $T_{50}E_{50}$(50 mM Tris-HCL and 50 mM EDTA, pH 8.0), the bacteria were finally pelleted and resuspended in 10 ml of $T_{50}E_{50}$ in 50 ml sterile centrifuge tube. In the tube was added 1.250 ml of $T_{50}E_{50}$ containing 20 mg/ml of freshly prepared lysozyme. Tubes were incubated for 30 minutes at RT. Then 250ul of SDS was added in the tube followed by 1.250 ml $T_{10}E_1$ (10 mM Tris-HCI and 1 mM EDTA, pH 7.4) containing Pronase E (500 µg/ml stock in DGDW). The mixture was incubated at 37°C for 1.5 hours with intermittent shaking. Equal volumes of tris-HCI buffered phenol was then added and mixed by gentle rocking for 3 minutes and incubated at 37°C for 1 hour. The tubes were centrifuged at 3000 x g for 10 minutes at RT. Upper aqueous phase was collected into a clean centrifuge tube. The aqueous phase collected, was extracted twice with phenol: chloroform: isoamyl alcohol (25: 24: 1) by mixing 1: 1. The centrifuge tube was spun for 1 minute at RT and the upper phase was collected. To this 3M sodium acetate was added to a final concentration of 0.15 M. The DNA was precipitated by adding 2 volumes of ice-cold absolute alcohol. The precipitated DNA was collected by spooling on a sealed micropipette tip. Twirling of the sealed pipette tip released the spooled DNA in a tube containing 4 ml of $T_{10}E_1$. It was stored overnight at 4°C in a refrigerator. To the DNA preparation was added 200 µl of 10 mg/ml stock solution of RNase. After this, 3M sodium acetate to a final concentration of 0.15 M, was added and the DNA was precipitated with 2 volumes of cold ethanol. The precipitated DNA was spooled on a sealed pipette tip, pulled out on the tip, rinsed with 70% ethanol in another tube, air dried in the same tube and stored at -20^0 C. Further purification of the semipurified DNA was achieved by repeated phenol-chloroform treatment. Finally, the purified DNA was resuspended in endotoxin-free water and stored at -20^{0}C.

Restriction enzyme analysis

Purified DNA from three field isolates (*Salmonella gallinarum*) of different outbreaks were subjected to RE analysis with two enzymes namely *HindIII*, *EcoRI* (Fermentas) to find out the genomic linkage. Restriction enzymes namely *HindIII*, *EcoRI*, *PStI* , *Ssp1*and *Bstx1* (Fermentas) were also used to know which enzyme give interpretable banding patterns for typing *Salmonella* Gallinarum (T16). After digestion, all the samples were analyzed on 2.0 % agarose gel with 23 kb DNA ladder.

Polymerase chain reaction (PCR)

Ten field isolates of *Salmonella* including 5 isolates of *Salmonella gallinarum*, 2 isolates of each *Salmonella pullorum* and *Salmonella typhimurium* and one isolate of *Salmonella enteritidis* were subjected for PCR to amplify plasmid (85 kb) associated virulence gene as described earlier (Rexach *et al*., 1994). The details of primers (sigma) used are as follows:

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Gene	Primers	Primer sequence (5'-3')
Virulence-associated plasmid gene	Forward	TTG TAG CTG CTT ATG ATG GGG GGG
	Reverse	TGG AGA AAC GAC GCA CTG TAC TGC

Two microliters of the prepared DNA template was added to 25 ml of PCR reaction mixture in 0.2 ml thin walled microfuge tube. The reaction mixtures used in the PCR steps contained 10 X PCR buffer, 25 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate, 1.0 µl of forward primer, 1.0 µl of reverse primer (as indicated above), and 0.5 µl of Taq DNA polymerase. Plasmid DNA amplification was carried out in a Eppendorf thermocycler using an initial denaturation step of 94 ^{0}C for 4 min, followed by 32 cycles of amplification with denaturation at 94 ^{0}C for 50 s, annealing at 64 ^{0}C at for 60 s, and extension at 72 ^{0}C for 60 s, ending with a final extension at 72 ^{0}C for 5 min. Upon completion of PCR, the products were analyzed by ethidium bromide stained 1% agarose gel electrophoresis

RESULTS

Plasmid profiling

Among 23 isolates of *Salmonella Gallinarum*, 21 isolates (91.30%) possessed plasmids of different molecular weights and only two isolates (8.70%) were without plasmid. A common 85 kb large plasmid was present in 20 isolates and only one isolate was devoid of this plasmid. Small plasmids of size 1.5 kb and 2.5 kb were also present. Six isolates had only one plasmid of size 85 kb, while one isolate had one plasmid of size 1.5 kb alone. Rest of the isolates (fourteen) showed presence of multiple plasmids. Plasmid profile analysis was able to subtype the 23 isolates of *Salmonella gallinarum* into seven groups.

Eleven out of thirteen isolates of *Salmonella pullorum* contained plasmids of different molecular weights. Five isolates showed the presence of single large plasmid of size 85 kb, while three isolates possessed small plasmid of size 1.5 kb. A 2.5 kb plasmid was detected in one isolate (T35). Two isolates showed the presence of both large and small plasmids of size 85 kb and 1.5 kb. Six different plasmid profiles were obtained in serovar *gallinarum* while four plasmid profiles were seen in serovar *pullorum*.

In Nine isolates of *Salmonella enteritidis* two isolates showed presence of 2 large plasmids of size 90 kb and 85 kb. Three isolates possessed only one large plasmid of size 85 kb. In one isolate both 85 kb and 1.5 kb plasmids were present. Overall four different plasmid profiles were present.

In another zoonotically important serotype *Salmonella Typhimurium* out of six isolates, 4 isolates possessed only large plasmid of size 85 kb. Two isolates were devoid of any plasmid (Table 1, 2, 3, &4 and Fig.1a&b).

Table 1: Plasmid profiles of *Salmonella* Gallinarum (PPS) with antibiotic resistance pattern.

Sr. No.	Isolate No.	Plasmid profile	Resistance against antibiotics
1	T7	PPS II	C, AK, G, CE, CQ
2	T14	PPS II	C, CE, CQ
3	T16	PPS I	C, CE, CQ
4	T23	PPS I	CE, CQ
5	T28	PPS III	AK, CE, CQ
6	T37	PPS IV	CQ
7	T39	PPS V	AK, CE, CQ
8	T45	PPS IV	CQ
9	T47	PPS IV	C, AK, CE, CQ
10	T49	PPS V	C, AK, CE, CQ
11	T50	PPS V	AK, CE, CQ
12	T52	PPS I	C, AK, G, CE, CQ
13	T58	PPS I	CE, CQ
14	T61	PPS I	C, AK, G, CE, CQ
15	T62	PPS I	C, AK, G, CE, CQ
16	T63	PPS	AK, CE, CQ
17	T64	PPS	CE, CQ
18	T65	PPS VI	AK, G, CQ
19	T66	PPS II	AK,CQ
20	T67	PPS II	C, AK, G, CE, CQ
21	T68	PPS VI	AK, G
22	T69	PPS II	CE, CQ
23	T70	PPS II	AK, G, CE, CQ

PPS I= 85kb; PPSII= 85, 1.5kb; PPSIII= 1.5 kb; PPSIV= 90, 85, 1.5kb; PPSV= 90,85kb; PPSVI= 85, 2.5kb; PPSVII= 2.5kb; PPSVIII= 85, 2.5, 1.5kb.
C= Chloremphenicol, Ak= Amikacin, G= Gentamicin, CE= Cephatoxime, CQ=Cephadroxil

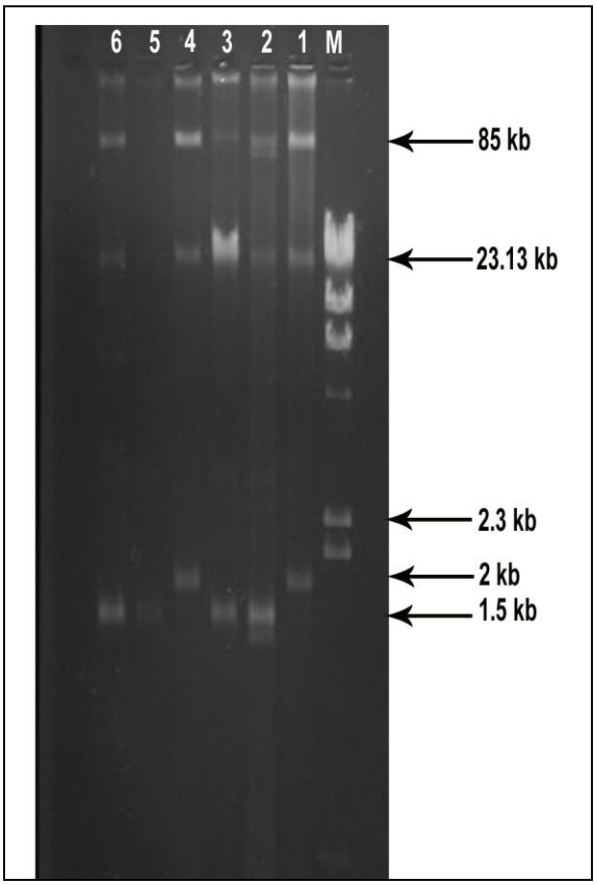

Figure 1. Plasmid profiles of *Slamonella* spp.
(Lane M = 23.13 kb DNA molecular size marker; Lane 1-6 field samples of *Salmonella* spp.)

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Table 2: Plasmid profiles of *Salmonella pullorum* (PPS) with antibiotic resistance pattern

Sr. No.	Isolate No.	Plasmid profile	Antimicrobial drug resistance
1	T8	PPS I	C, AK, G, CE, CQ
2	T9	PPS	CE, CQ
3	T10	PPS	G, CQ
4	T11	PPS I	C, AK, CE, CQ
5	T12	PPS I	C, CE, CQ
6	T13	PPS II	C, G, CE, CQ
7	T15	PPS I	C, AK, CE, CQ
8	T22	PPS I	C, AK, G, CE, CQ
9	T24	PPS II	CE, CQ
10	T25	PPS III	C, CE, CQ
11	T26	PPS III	C, CE, CQ
12	T30	PPS III	AK, CE, CQ
13	T35	PPS VII	CE, CQ

PPS I= 85kb; PPSII= 85, 1.5kb; PPSIII= 1.5 kb; PPSVII= 2.5kb.
C= Chloremphenicol, A= Amikacin, G= Gentamicin, CE= Cephatoxime, Q=Cephadroxil

Table 3: Relationship of Plasmid profiles of *Salmonella enteritidis* (PPS) with antibiotic resistance pattern

Sr. No.	Isolate No.	Plasmid profile	Antimicrobial drug resistance
1	T6	PPS I	C, AK, G, CE, CQ
2	T19	PPS I	C, AK, CE, CQ
3	T20	PPS II	AK, G, CE, CQ
4	T21	PPS I	C, CE, CQ
5	T27	PPS III	C, AK, CE, CQ
6	T29	PPS III	AK, CE, CQ
7	T31	PPSVIII	CE, CQ
8	T42	PPS V	C, AK, G, CE, CQ
9	T43	PPS V	AK, CE, CQ

PPS I= 85kb; PPSII= 85, 1.5kb; PPSIII= 1.5 kb; PPSV= 90,85kb; PPSVIII= 85, 2.5, 1.5kb.
C= Chloremphenicol, Ak= Amikacin, G= Gentamicin, CE= Cephatoxime, CQ=Cephadroxil

Table 4: Plasmid profiles of *Salmonella typhimurium* (PPS) with antibiotic resistance pattern

Sr. No.	Isolate No.	Plasmid profile	Antimicrobial drug resistance
1	T1	PPS I	C, AK, G, CE, CQ
2	T2	PPS	C, AK, G, CE, CQ
3	T3	PPS	C, AK, G, CE, CQ
4	T4	PPS I	C, AK, G, CE, CQ
5	T5	PPS I	AK, G, CE, CQ
6	T18	PPS I	C, G, CE, CQ

PPS I= 85kb;
C= Chloremphenicol, Ak= Amikacin, G= Gentamicin, CE= Cephatoxime, CQ=Cephadroxil

All the isolates of *Salmonella* spp. showed multiple drug resistance patterns. *Salmonella gallinarum* and *Salmonella enteritidis* isolates showed maximum sensitivity to Chloramphenicol. Gentamicin was found to be the second most effective antibiotic. *Salmonella pullorum* showed maximum sensitivity to Gentamicin (62.5%), where as *Salmonella typhimurium* isolates were 100% resistant to all antibiotics used in the study except Chloremphenicol which showed sensitivity for 33.4% isolates. Both *Salmonella enteritidis* and *Salmonella typhimurium* are zoonotically important serotypes and these isolates showed maximum resistance to commonly used antibiotics which is a cause of concern. However, there was no positive correlation between presence of plasmid and drug resistance pattern.

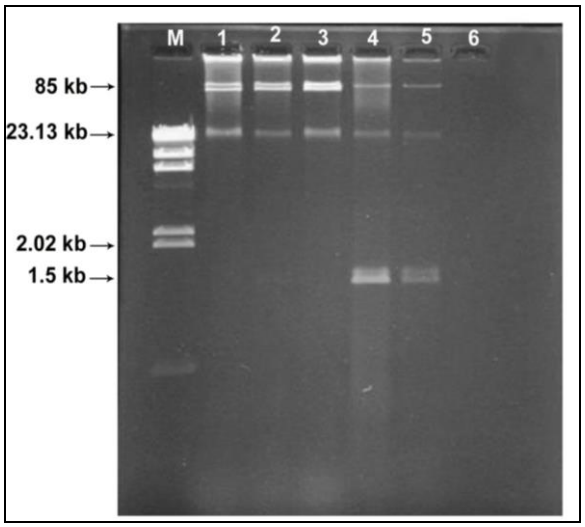

Figure.1 b. Plasmid profiles of *Slamonella* spp.
(Lane M = 23.13 kb DNA molecular size marker; Lane 1-6 field samples of *Salmonella* spp.)

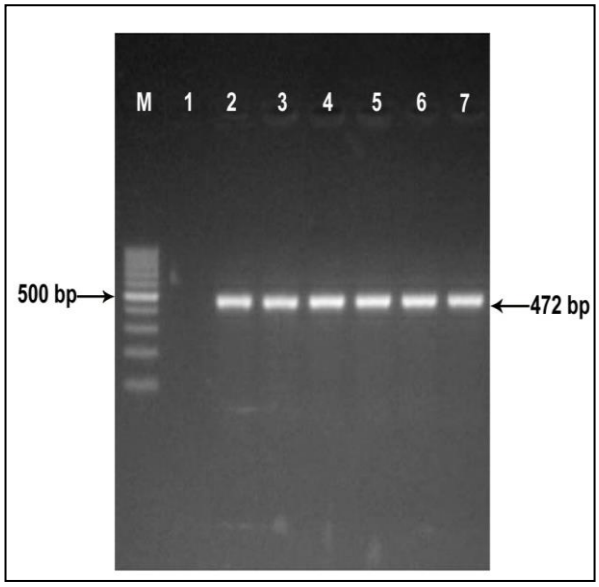

Figure 2. PCR results showing amplification of plasmid associated virulent gene in *Salmonella* spp.
(Lane M = 100 bp DNA molecular size marker; Lane 1 = *Salmonella* isolate without plasmid; Lane 2-7 = Isolates of *Salmonella* spp. having 85 kbp plsamid)

Restriction enzyme endonuclease analysis

Bacterial genomic DNA of 3 *Salmonella Gallinarum* isolates having purity 1.8 by Biophotometer showed complete digestion of genomic DNA with large number of bands. Similar banding pattern was seen in all the three isolates establishing the fact that these were similar at genomic level. When these isolates

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were analyzed for plasmids, it was observed that they had only single plasmid of the size 85 kb. These findings suggested that the isolates were similar at genomic level and might have originated from the single grandparent source. Findings were supported by retrospective study which revealed that most of the affected farms receive the poultry birds from a single hatchery. Digestion of *Salmonella* gallinarum (T16) with *EcoRI*, *HindIII* and *PstI* showed similar banding pattern with almost all the bands of high molecular weight. With *EcoRI* and *HindIII* last band was of the size approximately 1 kb. When banding pattern with *BstXI* and *SspI* was analysed, more discrete bands of lower molecular weight were observed. With *BstXI* last band was of the size approximately 100bp while with *SspI*, it was nearly of 150 bp.

Polymerase chain reaction (PCR)

All the ten isolates were having one common plasmid of size 85 kb and specifically amplified a product of approximately 472 base pair (bp), showing the presence of plasmid gene linked to virulence in *Salmonella* irrespective of the serotype (Table 5, Fig.2). PCR detection of plasmid-associated virulence gene, presence of 85 kb plasmid and high mortality in one to two week old chicks were positively correlated.

Table 5: PCR results along with presence of plasmids.

Sr. No.	Isolate No.	Serotype	PCR Result	No. of plasmids possessed	Molecular size of plasmids (kbp)
1	T5	*Salmonella typhimurium*	A	1	85
2	T6	*Salmonella enteritidis*	A	1	85
3	T13	*Salmonella pullorum*	A	2	85, 1.5
4	T16	*Salmonella gallinarum*	A	1	85
5	T18	*Salmonella typhimurium*	A	1	85
6	T22	*Salmonella pullorum*	A	1	85
7	T37	*Salmonella gallinarum*	A	3	90, 85, 1.5
8	T52	*Salmonella gallinarum*	A	1	85
9	T58	*Salmonella gallinarum*	A	1	85
10	T62	*Salmonella gallinarum*	A	1	85

A= Amplified 472bp virulent gene.

DISCUSSION

Detailed strain identification is essential for successful epidemiological investigation of *Salmonella enterica* serovar outbreaks. Plasmid profile analysis has been used as a rapid method and has shown success in sub-typing of *Salmonella* strains. Plasmids often encode properties such as antimicrobial resistance, invasiveness capability, toxins etc. Analyses on the bases of plasmid profiling revealed that in a given geographic area, an individual bacterial clone predominates (Van Embden *et al*., 1976; Helmuth *et al*., 1981). In Britain, plasmid profiling was first applied to know Salmonella epidemiology in 1979. Large plasmids of various sizes have been found in several serotypes of Salmonella, all of which produce a systemic type of disease in animals or humans. The role of 60-megadalton plasmid in the virulence of Salmonella typhimurium was demonstrated (Jones *et al*., 1982). An association between presence of 85-kilobase plasmid in Salmonella Gallinarum and ability of strains to produce high mortality in chickens was reported earlier (Barrow *et al*., 1987).

In the present study Salmonella isolates were characterized at their genotypic level by plasmid profiling, restriction enzyme analyses and PCR. Six different plasmid profiles were obtained in serovar *gallinarum* while four plasmid profiles were seen in serovar *pullorum*. A common large plasmid of size 85 kb was present in majority of the isolates along with the presence of small plasmids of size 1.5 kb and 2.5 kb. Presence of more than one plasmids detected in the present study is in agreement with the earlier observations (Rahman, 1999). Correlation between plasmid profiles and antibiotic resistance found to be useful in epidemiological characterization of *Salmonella* isolates (Bhattacharya *et al*., 2001; Radu *et al*., 1997). But in the present study there was no positive correlation between plasmid profiles and antibiotic resistance pattern. The results of the present study point towards the possibility of drug resistance-genes being present on the chromosomal DNA, in addition to the plasmids, since isolates which were devoid of plasmids also showed resistance to multiple antibiotics. However presence of plasmids correlated with virulence factors was previously reported (Oh *et al*., 2002; Singh *et al*., 1996) but various workers reported that drug resistance genes may also be present on chromosomes (Mariani *et al*., 2001). Multiple drug resistance was present in most of the isolates which might also be because of the presence of large number of plasmids in the isolates carrying drug resistance genes (Bakshi *et al*., 2003).

Plasmid profiling analyses along with restriction endonuclease analysis revealed that these isolates showed a high degree of genome homogeneity and might have originated from the single grandparent source. Digestion of genomic DNA of *Salmonella Gallinarum* with different restriction enzymes showed similar banding pattern with almost all the bands of high molecular weight. These observations conform to the observations of Tompkins *et al*. (1986). However *BstXI* and *SspI* yielded more discrete bands of lower molecular weight.

Plasmid associated virulence gene was detected by PCR irrespective of the serotype. Various other workers (Bhattacharya *et al*., 2001; Mariani *et al*., 2001) also detect *Salmonella* virulence-associated plasmid gene by amplifying 472 bp product. In the present study one common plasmid of 85 kb size was present in all the isolates which were positive by PCR. Retrospective study showed that the isolates with 85 kb plasmid were responsible for high mortality in the affected flock, suggesting the association of virulent gene with the presence of 85 kb plasmid. This is corroborated by the findings of other workers (Bhattacharya *et al*., 2001; Joseph *et al*., 2000; Rexach *et al*., 1994).

CONCLUSIONS

Study concluded that plasmid profiling along with restriction enzyme analysis can be used as epidemiological markers in back tracing infections especially in case of outbreaks. As 75% of isolates possess 85 kb plasmid, there is some common source of infection possibly at grandparent level. No positive correlation between plasmid profiles and antibiotic resistance pattern was

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found. The multi-drug resistance of the isolates suggests indiscriminate use of antimicrobials in the developing countries like India. PCR proved to be sensitive and effective method to detect virulent gene of *Salmonella* infection. So need of hour is to start mandatory *Salmonella* testing of parent flocks, positive reactors should be removed from the flock and vaccination of parent flocks may be carried out for control of this fast spreading, zoonotically important vertically transmitted pathogen. Moreover at government level there should be some legislation for control of such infections from hatchery.

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