International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 4 | OCT-DEC | 2018 | 154-159
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

PLASMID PROFILING OF *SALMONELLA TYPHIMURIUM* ISOLATES FROM COMMERCIAL POULTRY FARMS OF HARYANA

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ABSTRACT

Non-typhoidal salmonellosis is major cause of diarrhea in human and animals throughout the world leading to huge economic loss and mortality. Human Salmonellosis is sometimes traced back to animal source, majority of them from poultry and its products. Keeping this in mind, eleven strains of Salmonella typhimurium were isolated from different districts of Haryana; from broiler affected with Salmonellosis, which were revived and plasmid extraction was performed for profiling. The strains were classified into three plasmid types based on number and size of plasmids. Antibiotic sensitivity tests were also performed on all isolates and compared with plasmid. All plasmid types had 90kb plasmid whereas additional plasmid of size 110kb, 120kb and 23 kb were also observed in only two plasmid types. Plasmid type-1 and 2 had no major difference when checked for antibiotic resistance whereas plasmid type-3, having large size plasmids (110 and 120 Kbp) were found to exhibit resistance against the twelve antibiotics out of sixteen antibiotics used. Hence, high molecular weight plasmids may have role in antibiotic resistance and whereas plasmid between 50-100 kps may be serovar specific virulence genes.

KEY WORDS

Salmonella, plasmids, poultry, Haryana, Antibiotic, Virulence

INTRODUCTION

India is a developing country which has shown immense growth in poultry population over the past few years; from having only 307.07 million numbers (year 1992) to 729.2 million numbers (year 2012) as per 19th Livestock census-2012, where Haryana state contributes 5.37 (%) being number six producer of poultry in the country (1). One of the major challenges in growth of poultry is *Salmonella* spp. infection, whose control and containment is very important for reducing losses to poultry farmers and also to reduce the risk of infection in Humans.

Salmonella enterica (*S.enterica*) spp. is one of the most important bacterial pathogen causing fowl typhoid (*S.gallinarum*), pullorum disease and Salmonellosis. The most commonly reported serotypes are *S.enteritidis*, *S. typhimurium*, *S.gallinarum* and *S. pullorum*, out of which later two are host adaptive while former two serotypes are not limited to any host and affect not only animals but also human, making it one of the most important zoonotic pathogen worldwide as reported by Murugkar *et al*. (2005). According to World Health Organization (15), Salmonella is one of major four global cause of the diarrheal diseases and causes about 1.2 million illness, 23000 hospitalizations and 450 deaths in United States every year (6). The main control method for this bacterial infection is use of antibiotics, but as the pharmaceutical industry is making progress with development of new antibiotics, the *Salmonella spp.* bacteria is also making advancement by developing multiple antibiotic resistance, turning it into a superbug. One of the proposed mechanisms for development of resistance is by way of transfer of genes through

plasmids. These plasmids are extra chromosomal genetic material of bacteria which are capable of independent replication. These Salmonella plasmids may be classified into three groups based on molecular weights as serovar specific virulence plasmids, higher molecular weight plasmids and low molecular weight plasmids as per Rychlik *et al.* (2006). The role of plasmid profiling is that it helps in understanding molecular epidemiology of different outbreaks caused by same bacteria as reported by Humphrey *et al.* (1988) and Baggasen *et al.* (1992). The reports of plasmid profiling from India are available (Bakshi *et al.*, 2003, Singh *et al*., 2010., Kumar *et al*., 2013. and Sumithra *et al*., 2015), but are very few. The present study was undertaken to understand plasmid profile of *Salmonella typhimurium* isolates of commercial poultry farms of different parts of Haryana. Broilers, which are reared for meat purpose, have life of only 70-80 days but during this period to maintain production, they are given not only vaccination against viral disease but lots of antibiotics, to treat bacterial infections which spreads not only vertically from parent to chick but also horizontally through environment.

MATERIAL AND METHOD

Sample size and type

A total of eleven isolates of *Salmonella typhimurium* maintained in Nutrient agar at 4^{0}C at Department of Biotechnology, DCR university of Science and technology, Murthal, Sonipat (Haryana), India, were used for current study. These samples were collected from different regions (Hisar, Jind and Sonipat district) of Haryana state from broiler population at time of postmortem. All these *Salmonella typhimurium* isolates were got confirmed from Central research Institute, Kasauli, Himachal Pradesh (India). The details of the isolates used in this study are given in Table 1. These strains were revived on MacConkey Lactose (MLA) Agar and reconfirmed by gram negative staining.

Table 1: Plasmid detail of Salmonella Isolates

S. No.	Salmonella serotype	Source of isolate	Plasmid code	Plasmid types	Approx. size of Plasmid	Number of resistant antibiotics
1	*S.typhimurium*	Hisar	P-1	PT-1	90	4
2	*S.typhimurium*	Hisar	P-2	PT-1	90	4
3	*S.typhimurium*	Hisar	P-3	PT-1	90	5
4	*S.typhimurium*	Jind	P-4	PT-3	90,110,120	12
5	*S.typhimurium*	Jind	P-5	PT-1	90	5
6	*S.typhimurium*	Sonipat	P-6	PT-2	90,23	4
7	*S.typhimurium*	Hisar	P-7	PT-1	90	6
8	*S.typhimurium*	Hisar	P-8	PT-1	90	6
9	*S.typhimurium*	Hisar	P-9	PT-2	90,23	4
10	*S.typhimurium*	Hisar	P-10	PT-1	90	5
11	*S.typhimurium*	Jind	P-11	PT-1	90	6

Plasmid extraction

The plasmid DNA was extracted by using alkali lysis method as per Sambrooks and Russel (2001) with slight modifications. A single colony was picked and transferred to Luria Bertani (LB) broth (Hi-Media) and was incubated on shaker at 37^{0}C for 16-18 h. Two milliliters of grown culture was centrifuged at 7000 rpm for 5 min at 4^{o}C. To the pellet, 100µl alkali lysis solution (ALS)-I (25mMTris HCL pH 8.0, 50mM glucose, 10mM EDTA pH8.0) (Sigma Aldrich, India) containing proteinase-K and lysozyme was added and incubated at room temperature for 60 min. To the above solution, 200 µl ALS-II (0.2N Sodium hydroxide, 1% SDS) (SRL, India) was added and mixed thoroughly followed by addition of 150 µl ALS-III (3 M potassium acetate, 5M glacial acetic acid) (Merck) and centrifuged for 15 minutes at 10000 rpm at 4^{o}C. The obtained supernatant was transferred to a fresh tube and 500 µl chilled isopropanol (Merck) was added to it for precipitation of nucleic acid and centrifuged at high speed. Pellet was washed with ethanol (70%) (SRL) to remove all residual chemicals and dried pellet was dissolved in 50ul Tris EDTA (TE) buffer (pH 8.0) (Sigma Aldrich India) and stored till further use.

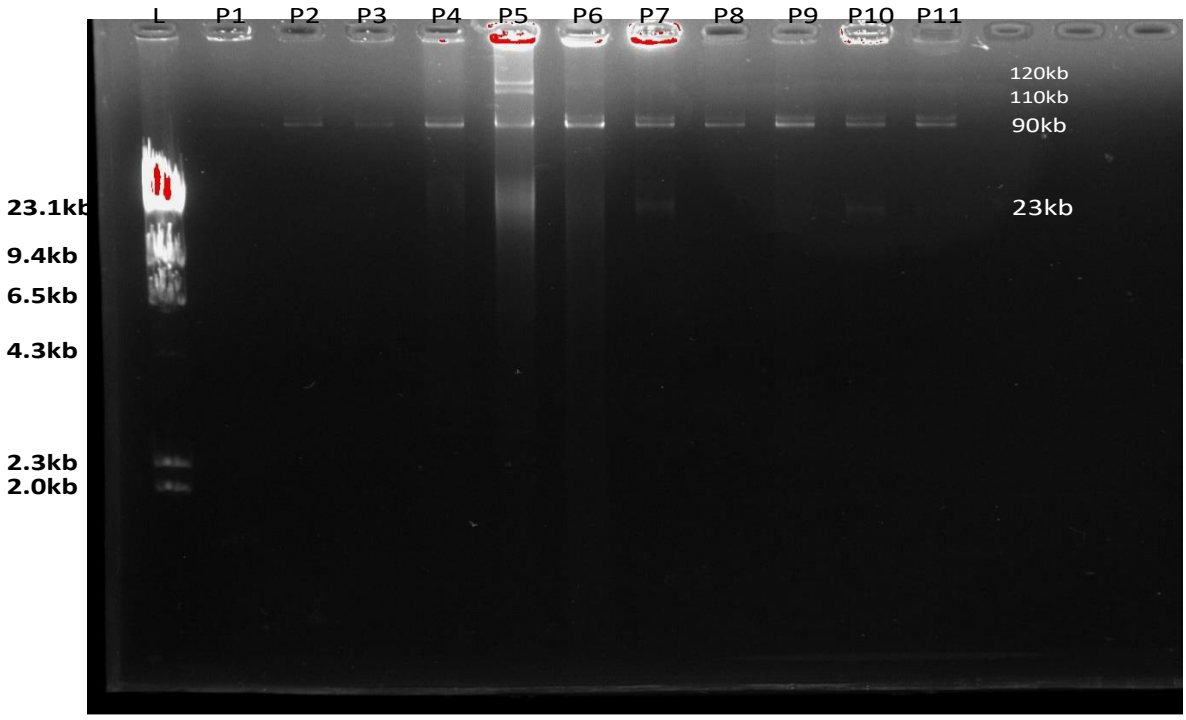

Fig1: Plasmid profile of *Salmonella tyhimurium* isolates.

L: lambda cut HindIII digest ladder, P-1 to P-11: plasmids of different isolates of S. *typhimurium*. P1 to P3, P6, P8, P9, P11; Size: 90Kbp, P5: 120kbp, 110kbp, 90 kbp, P7, P10: 90 kbp, 23 kbp.

Agarose Gel electrophoresis

Plasmids DNA were separated in 0.7% Tris Acetate EDTA (TAE) electrophoresis buffer (Hi-Media) by subjecting it to 50 V current for 150 minutes. The gel was stained with 0.5 µg/ml ethidium bromide (sigma Aldrich India) and the obtained bands were visualized under UV light using Gel Documentation system (BioRad, India). The molecular sizes of the bands were determined by comparing with molecular marker ranging from 125 kb to 23.13kb using lambda DNA-Hind III digest as DNA ladder (MBI Fermentas). Band size was determined by calculation of Rf value (distance travelled by DNA molecule/distance travelled by dye) by plotting log value of migration distance against known gel size.

Antibiotic sensitivity tests

A single colony was picked from culture and transferred to Nutrient broth (HiMedia) and incubated for 6-7 h at 37^{0}C. Culture was spread on Mueller Hinton Agar plates by using sterilized swabs and sixteen antibiotics disc were applied to it as per disc diffusion method (Bauer *et al*., 1996) and antibiotic sensitivity was recorded after 24 h incubation at 37^{0}C by measurement of zone of inhibition (mm). The antibiotic disc used were namely Co-trimethoxazole (25 µg), Oxytetracyclin (30 µg) , kanamycin (30 µg) , Cefotaxime (30 µg), Nitrofurantoin (300 µg), Ampicillin (10 µg), Amoxycillin (10 µg), Nalidixic Acid (30 µg), Norfloxacin (30 µg), Enrofloxacin (05 µg), Chloramphenicol (30 µg), Ciprofloxacin(05 µg), Amikacin (30 µg), Streptomycin (10 µg), Cefalexin (30 µg) and Gentamycin (10 µg) were selected for antibiotic profiling of *Salmonella* spp. Zone of inhibition (mm) were recorded after incubation at 37^{o}C for 24 h and classified as resistant and sensitive as per chart given with HiMedia antibiotic disc for reference.

RESULTS AND DISCUSSION

Out of a total of eleven *S.typhimurium* isolates studied, all were found to carry a 90kb plasmid. This band has another neighbouring band of approximately 85 kb which may be fragment of part of 90 kbp band rather than plasmid itself. Since no report is available for simultaneous presence of two plasmids of 80-85 kb and 90 kb, it is classified as one plasmid only. Additionally, two bands of higher molecular weight of approximately 110kb and 120kb was present in one of the eleven isolates (P-4). Additionally, a plasmid of approximately 23 kb was observed in two isolates (P-6 and P-9). Based on presence of plasmids, these are grouped into three plasmid types (PT) where PT-1 carried only one plasmid, PT-2 had two plasmids and PT-3 had three plasmids. Rychlik *et al*. (2006) reported that 50-100kb plasmids

are basically serovar specific virulence plasmids whereas higher molecular weight plasmid (more than 100kb) have role in antibiotic resistance. Since all strains in present study had approximately 90kb plasmid, they may be serovar specific plasmid to *S.typhimurium*, which may be required for virulence mechanism. This detection of 90 kb plasmid of *Salmonella spp.* from poultry in present study, agrees with the work of Salem *et al.* (2017). Mohamed *et al*. (2008) reported that *S. typhimurium* plasmids have molecular sizes of 3.8 and 90 kb whereas in *S. enteritidis* the plasmid size ranged between 4 and 65 kb. The plasmid of 90 kb was observed in all tested *S. typhimurium* strains and the plasmid size of 65 kb was detected in all tested *S. enteritidis* isolates.

Presence of plasmid of 90kb in all eleven isolates of S.typhimurium was found in agreement with observation of Sumithra *et al.* (2014) where almost all twenty-two strains of *S.typhimurium* were found to carry 90 kb plasmid. Similar study of Baggasen *et al*. (1992) reported that 94 kb plasmid was found in all but three strains of *S.typhimurium* circulating among parent stock chicken farm, where this plasmid was suggested as serovar specific virulence associated plasmid (pSLT). The additional plasmid of 120 kb reported was also found in one of the eleven strains in present study (PT-3). Kumar *et al.* (2013) reported that from Haryana state, out of fifty-one Salmonella strains isolated from broilers, six isolates belonged to *S.typhimurium* in addition to *S.gallinarum* (23), *S.pullorum* (13) and *S.enteritidis* (9). Among these six strains of *S.typhimurium* four were found to have 85 kb plasmid while two were devoid of any plasmid. It was also found that majority of strains carried 85kbp plasmid, which may be serovar specific virulence plasmid. Presence of common plasmid of approximately 90 kb was found in agreement with above study while difference was that none of the *S. typhimurium* strain in the present study was found without plasmid. This shows that almost similar type of strains may be circulating over the year with slight variation, as addition and loss of Salmonella plasmid, is a very well-known phenomenon. No direct co-relation between plasmids and antibiotic resistance was observed by Kumar *et al.* (2013) while in present study PT-1 had antibiotic resistance to 4-6 antibiotics, PT-2 had antibiotic resistance to four antibiotics were may not be a clear demarcation of presence of additional plasmid and antibiotic resistance. On the other hand, one strain (P-5) belong to PT-3 having addition plasmids of 110 and 120 kb, showed resistance to twelve antibiotics out of sixteen antibiotics used, which is sufficient to assume that PT-3 carried antibiotic resistance genes. In present study some very faints bands were excluded from analysis due to lack of clarity. In a different study, Singh *et al* (1996) reported that eight out of nine *S.gallinarum* isolates showed presence of 85 kb plasmid in addition to 2.5 kb plasmid in all nine isolates. Presence of a common 85 kb plasmid in almost all strains was suggested to have role in virulence and presence of a common 90 kb plasmid in present study is in partial agreement suggesting its role as serovar specific virulence plasmid. The same type of observation and role of common plasmid was also suggested by Barrow *et al.* (1989).

Plasmid pattern determination has proved to be a useful epidemiological tool in outbreak of Salmonellosis (Bezanson *et al.*, 1983 and Branner *et al.*, 1983). Based on presence of same size plasmid (90kb) from all isolates in present study, it is assumed that these outbreaks may be from same or related serotypes, which is *S.typhimurium*. One plasmid P-4 carried additional higher molecular weight plasmids, which may further help in development of antimicrobial resistance. In case, it spreads to other places, a new multiple antimicrobial resistant strain of *S.typhimurium* may proliferate in environment. More plasmid profiling in future may help in tracing spread of infection not only in poultry population but also in human population.

Singh *et al*. (2010) reported that fourty out of fourty-six strains of *Salmonella enterica* (belonging to eight serovars), had one or more plasmids. Virulence plasmids of around 35 kDa (approximately 53 Kb) were found in thirty-nine isolates from buffaloes on India. This was found in agreement with present study as 90 kb plasmid was found commonly in all the eleven strains. Although in the above study it was concluded that plasmid number had no direct correlation with antibiotic resistance pattern, the present study found that PT-3 needs to be seen as suggestive evidence for possible direct correlation between plasmid and antibiotic resistance as suggested by Rychlik *et al.* (2006).

Similar study from Malaysia by Douadi *et al*. (2010), reported that 74.4% strains of *S.typhimurium* of human and animal origin harbored one to six plasmids, out of which predominant plasmid was 90kb, which was in agreement with present study. A total of twenty-two

different plasmid profiles were observed in contrast to three plasmid types detected in present study. Plasmid was absent in eleven drug resistant strain in above study on contrary to present study where no strain was found having lost all the plasmid. Although both studies had *S.typhimurium* strains, difference in plasmid profiles may be mainly because of distinct geographical location, sample size and host range, which suggests that plasmid profile may be used as epidemiological tool for outbreak investigation and plasmid profiling of local circulating strains is imperative. The loss of large plasmid in above study of Douadi *et al.* (2010) was suggested to be due to shearing during extraction process in addition to co-precipitation with chromosomal DNA. This explanation was also in agreement with report of Hansen *et al.* (1978) while Olsen *et al.* (1994) suggested plasmid instability, a common phenomenon among *Salmonella* spp., as a reason for absence of plasmids.

CONCLUSION

Salmonella enterica serovar *typhimurium* plays a major role in non-typhiodal Salmonellosis in human and animals globally and causes a lot of economic loss. Plasmid profiling serves a very useful epidemiological tool for investigation of Salmonella outbreak. The detection of 90 kb plasmid present in all eleven strains of *S. typhimurium* isolated from *Salmonella* spp. affected broiler population of Haryana state at postmortem, may be a serovar specific virulence plasmid. Two additional plasmid profile were found where plasmid type-3 had additional large size plasmids of 110kb and 120 kb and was found resistant to twelve antibiotics out of 16 antibiotics applied, suggesting a correlation between large plasmid and antibiotic resistance. More detailed studies are needed to better understand infection progression in different areas and antibiotic resistance profiles.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We wish to express our sincere gratitude to Chairperson, Biotechnology Department, DCRUST, Murthal, Sonipat, Haryana for financial assistance.

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Received:06.08.18, Accepted: 09.09.18, Published:01.10.2018

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