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Prevalence and virulence gene profiles of *Salmonella* serovars isolated from animal origin

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Abstract
Salmonella is a foodborne pathogen having a worldwide public health concern. The present study was undertaken to characterize *Salmonella* species of animal origin based on cultural isolation, molecular confirmation of serovars and their virulence profiles. A total of 516 samples comprising poultry cloacal swabs (249), raw foods of animal origin (118 chicken samples, 65 mutton and 30 pork), poultry liver swabs (17) and poultry farm water samples (37) were examined for presence of *Salmonella* serovars. Overall prevalence of *Salmonella* isolates was found to be 4.06% (21/516) with highest prevalence in chicken samples (6/118, 5.08%) followed by cloacal swabs of poultry (12/249, 4.81%), mutton (2/65, 3.07%) and pork (1/30, 3.33%). All the isolates carried all the 7 virulence genes *i.e. invA, invH, sopB, sopE & stn* (100%), while *pefA* gene was found only in *S. typhimurium* isolates and *sefC* gene was found only in *S. enteritidis* isolates.

Keywords: *Salmonella* typhimurium, enteritidis, virulence genes profile

Introduction
Salmonella species is responsible for a wide range of acute and chronic diseases in both poultry and humans. Contaminated poultry products are among the most important sources for foodborne outbreaks in humans. *Salmonella* is reported more frequently from poultry and poultry products than from any other animal species. The genus *Salmonella* belongs to the family Enterobacteriaceae and consists of more than 2500 serovars. According to the CDC (Centre for Disease Control and Prevention, Atlanta), *Salmonella* alone affects about 1.4 million people each year in the United States with about 16,000 hospitalizations and more than 500 deaths annually. In 1996, the USDA, Economic Research Service estimated that the total costs for medical care and lost productivity, resulting from foodborne *Salmonella* infections of humans was between 0.6 – 3.5 billion dollars annually (USDA, 1996) [58]. Salmonellosis is a major health problem, worldwide and is responsible for high rates of morbidity. Infection with *Salmonella enterica* occurs mainly through the consumption of contaminated food and the estimated number of humans infections per year is greater than 93.8 million cases, with 155,000 deaths per year worldwide (Boyle *et al*., 2007; Majowicz *et al*., 2010 and Hendriksen *et al*., 2011) [14, 35, 26]. Genetically, *Salmonella enterica* is subdivided into 7subspecies based on biochemical criteria and DNA homology although, as many as 2500 serovars of *S. enterica* have been identified, the most common serotypes attributed to foodborne outbreaks are *Salmonella* Typhimurium (*S*. Typhimurium) and *Salmonella* Enteritidis (*S*. Enteritidis), which account for more than 75% of reported cases (Popoff, 2001) [42].
Salmonella pathogenicity depends on a variety of virulence factors that help the pathogen in adhesion, invasion, intracellular survival, fimbrial expansion, systemic infection, antibiotic resistance, toxin production and magnesium and iron uptake (Fardsanei *et al.*, 2017) [23]. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Some genes are involving in the adhesion and invasion *viz.*, *sef*, *pef*, *spv* or *inv* (Galen *et al.*, 1992; Krause *et al.*, 1992; Clouthier *et al.*, 1993 and Baumler *et al.*, 1996) [24, 34, 19, 11] and others are connected with the survival in the host system like *mgt*C (Blanc-Potard and Groisman, 1997) [12] or in the actual manifestation of pathogenic processes *viz.*, *sop*, *stn*, *pip* A, B, D (Wallis and Galyov, 2000; Chopra *et al.*, 1994 and Wood *et al.*, 1998) [61, 17, 63]. Some of the virulence genes *viz.*, *Salmonella* enterotoxin (*stn*) (Prager *et al.*, 1995 and Rahman, 1999) [44, 45] Salmonella Enteritidis fimbriae (*sef*) and plasmid-encoded fimbriae (*pef*) genes (Rahman *et al.*, 2000) [46] are being diagnosed by employing nucleic acid based diagnostic techniques.

Therefore the present study has been carried with the aim to study the prevalence and virulence gene profile of different *Salmonella* serovars isolated from different samples of animal origin.

Materials and Methods

Standard control and primers

ATCC (American Type Culture Collection) cultures of *Salmonella* Typhimurium (ATCC 14028) and *Salmonella* Eteritidis (ATCC 13076) were used as standard controls. Oligonucleotide primers were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad).

Sample collection

A total of 516 samples were collected from different sources including chicken meat samples (118), mutton (65) and pork (30) from retail shops, poultrty cloacal swabs (249) from retail shops and farms, liver swabs of poultry (17) from poultry diagnostic laboratories, water samples (37) from different poultry farms in and around Krishna district of Andhra Pradesh and they were collected in sterile polythene bags/ sterile swabs/ sterile bottle. Each bag/swab/bottle was labeled with sample number and particulars about samples. The samples were transferred in an ice box to the laboratory for further processing.

Ten grams each of raw meat sample collected from various sources were mixed with 90 ml of BPW (Merck, Germany) and homogenized within 24 h of collection. Cloacal swabs, liver swabs and 10 ml of water samples were transferred into 10 ml of BPW and later the samples were incubated at 37 ^{0}C for 24 h for enrichment. Enriched samples were transferred to selective enrichment broth like Rappoport Vasseliadis medium (RV medium) /selenite F broth and incubated at 42 ^{0}C for 18-24 h and later loop full of inoculum was streaked on XLD agar plates and incubated at 37 ^{0}C for 24 h. *Salmonella* specific colonies *i.e.* black centered red colonies were selected and they were taken into the TSB (Tryptic soy broth) and incubated at 37 ^{0}C for 24 h. Further they were confirmed by the biochemical, Latex agglutination (LA test) and multiplex PCR. The confirmed isolates of *Salmonella* were sent to Division of Bacteriology IVRI, Izzathnagar for serotyping.

Multiplex PCR (mPCR)

DNA was extracted from all the *Salmonella* isolates by using boiling and snap chilling (Suresh *et al*., 2018) method. 1.5 ml of enriched broths were taken into micro centrifuge tubes and centrifuged at 8000 rpm for 10 min. Supernatant was discarded, 50 µl of nuclease free water was added and placed in boiling water bath at 100 ^{0}C for 10 min. Immediately snap chilled for 10min and centrifuged at 10,000 rpm for 5min. The supernatant was taken as template and subjected to different mPCRs and the PCR products were subjected to gel electrophoresis using 1.5% agarose with ethidium bromide as fluorescent dye and visualized using Gel Documentation unit (BIORAD, USA).

Molecular confirmation of *Salmonella* Genus and serovars by MPCR

Salmonellae isolated from different samples by cultural methods were confirmed using *Salmonella* specific mPCR targeting random sequence gene, *fliC* & *sefA* genes for confirmation of *Salmonella* genus and *S.* Typhimurium *and S.* Enteritidis, respectively (Table-1). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 2.5 µl of 10x master mix (Go Taq Green Master Mix, Promega), 1µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation at 95^0 for 5 min; 35 cycles of 94^{0}C for 30 s, 56 ^{0}C for 90 s and 72 ^{0}C for 30 sec and a final elongation step at 72 ^{0}C for 10 min (Soumet *et al.*, 1999) [52].

Table 1: *Salmonella genus and* serovar specific m-PCR primers and sequences (Soumet *et al.* 1999) [52]

S. No	Primer	Primer sets	Primer sequence	Amplicon size (bp)
1.	*Salmonella*	ST 11	GCCAACCATTGCTAAATTGGCGCA	429
	Genus specific	ST15	GGTAGAAATTCCCAGCGGGTACTGG	
2.	*S.* Typhimurium *fliC*	Fli 15	CGGTGTTGCCCAGGTTGGTAAT	559
		Tym	ACTCTTGCTGGCGGTGCGACTT	
3.	*S.* Enteritidis *sefA*	Sef167	AGGTTCAGGCAGCGGTTACT	312
		Sef478	GGGACATTTAGCGTTTCTTG	

Detection of virulence genes in *Salmonella* isolates

All the confirmed *Salmonella* isolates from different sources were screened for the presence of virulence genes such as invA, *inv*H, *stn*, *sop*B, *sop*E, *sef*C and *pef*A genes (Table-2). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 2.5 µl of 10x master mix (Go Taq Green Master Mix, Promega), 0.5µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation at 94^0 for 5 min; 35 cycles of 94 ^{0}C for 30 s, 56 ^{0}C for 90 s and 72 ^{0}C for 120 sec and a final elongation step at 72 ^{0}C for 10 min (Choudhury *et. al.*, 2016) [18].

Table 2: Primers used for detection of virulence genes in *Salmonella* (Choudhury *et al.*, 2016) [18]

Target gene	primer	Primer sequence	amplicon size
*inv*A	*Inv*A-F	ACCACGCTCTTTCGTCTGG	942
	*Inv*A-R	GAACTGACTACGTAGACGCTC	
*inv*H	*Inv*H-F	TATAGCTGTCTTCCTGTCTT	305
	*Inv*H-R	ATGTATTGTGGATGTTCCTG	
stn	*Stn*-F	ATTGAGCGCTTTAATCTCCT	543
	Stn-R	GCTGTTGAATCTGTACCTGA	
*sop*B	*Sop*B-F	AGCATCTCTAAACGCTACTG	470
	*Sop*B-R	GCTTCTATCACTCAGCTTCA	

*sop*e	*Sop*E-F	GGTAGGGCAGTATTAACCAG	254
	*Sop*E-R	TTTATCTCCCTAGGTAGCCC	
*pef*A	*Pef*A-F	GCCAAAGTACTGGTTGAAAG	185
	*Pef*A-R	TATTTGTAAGCCACTGCGAA	
*sef*C	*Sef*C-F	GGCAGGTCCAAAACTATACA	609
	*Sef*C-R	GCGATAACGAAACACCATTT	

Results and Discussion

Out of 516 samples comprising foods of animal origin and other sources (249 Poultry cloacal swabs, 118 Chicken, 65 Mutton, 30 Pork, 17 Liver swabs of poultry and 37 poultry farm water) analyzed, 21 samples were found positive for *Salmonella* spp. with overall prevalence of 4.06%, the prevalence of the *Salmonella* in various samples was ranging from 5.08% in chicken followed by 4.81% in poultry cloacal swabs, 3.33% in pork and 3.07% in mutton. All the water samples and poultry liver swab samples were found negative for *Salmonella*.

All 21 *Salmonella* isolates were subjected to LA test (Hi *Salmonella* Latex kit, (Hi media). A clearly visible agglutination is seen for all 21 *Salmonella* isolates. *S.* Typhimurium ATCC 14028 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively.

The overall prevalence of *Salmonella* in the present result was in accordance with earlier research works done in different states of India, on animals and foods of animal origin where 4.58% (Mekwana *et al.*, 2015) [36], 6.1% (Samanta *et. al.*, 2014) [50] and 2.5% (Nair *et. al.*, 2015) [39] prevalence of *Salmonella* spp. were reported. However Murugkar *et al.* (2005) [37] and Kaushik *et al.* (2014) [31] reported a higher prevalence of *Salmonella* spp. in foods of animal origin and animal samples i.e. 14.5% and 23.7%, respectively.

Among the 21 positive *Salmonella* isolates, *S.* Typhimurium (7) was the most dominant serovar, followed by *Salmonella* group II (7), *Salmonella* group G (3), *Salmonella* Enteritidis (2), *Salmonella* Daytona and *Salmonella* Linderburg (one each).

Salmonella group II, considered as *Salmonella enterica* subspp. *salamae*, comprising of *Salmonella* Sofia, *Salmonella* Tranoroa, *Salmonella* Hagenbeck, *Salmonella* Nairobi serovars will be commonly associated with cold blooded animals but can be an occasional pathogen in man and other animals (Wuthe, 1969) [64]. It may also be responsible for severe infection in the young, very old or immuno-compromised patients who then require hospitalization. Most of the infections with this subspecies were accompanied by gastroenteritis, with systemic infection apparent only in a very few cases. Although it is generally believed that cold-blooded animals are the major reservoirs of *S. enterica* subsp. *salamae*, it is also important to consider for food-borne infections (Angulo and Swerdlow, 1995 and CDC, 1999) [5, 16].

Salmonella groupG includes serovars like *S.* Poona, *S.* Worthington, *S.* Mississippi, *S.* Grumpensis, *S.* Atlanta, *S.* Cubana, *S.* Wichita etc. (Bridges and Scott, 1935) [15]. *S.* Poona infections associated with pet turtle exposure have been reported. Nine outbreaks of *S.* Poona linked to the consumption of contaminated (water melon & cantaloupe) food between 1998 and 2008 in different places of USA (Patil Waller, 2015) [41] have been reported. *S.* Cubana epizootic was reported in animals due to feeding of synthetic starter food and was also isolated from diarrhoeic infants in Cuba (Seligmann *et al*., 1945) [51].

Serovars like *S.* Typhimurium, *S.* Enteridis and *S.* Daytona belong to family *Salmonella enterica* sub. spp. *enterica*. Among *Salmonella enterica* subsp *enterica*, serotypes *S.* Typhimurium and *S.* Enteritidis are the most important agents of foodborne salmonellosis in humans (Baay and Huisin'tveld, 1993 and Tan and Shelef, 1999) [8, 55]. It was estimated that approximately 75% of human salmonellosis cases were due to consumption of contaminated food products such as beef, pork, poultry, eggs (Kent *et al.*, 1981) [32] and meat products like sausages (Wall *et al.*, 1994) [60].

Present work revealed 4.81% (12/249) prevalence of *Salmonella* in poultry cloacal swabs, which was in agreement with the 4% prevalence as reported by Gracia *et al*. (2011) [25], 6.9% by Amini *et al*. (2010) and 5.8% by Jafari *et al*. (2007) and findings of present study were higher than 0.6% as reported by Rodriguez *et al*. (2017). In contrast, higher prevalence of 12.5%, 15%, and 84% were reported by the Eyigor *et al*. (2002) [22] Samanta *et al*. (2014) [50] and Ramya *et al*. (2012) [47].

Out of 12 *Salmonella* isolates recovered from the poultry cloacal swabs, 6 were belonging to *Salmonella* group II, 3 were *Salmonella* group G, 2 were *S.* Enteritidis and one *S.* Daytona. The prevalence of *S.* Enteritidis in cloacal swabs of poultry (0.8%, 2/249) in present study was very low when compared to the prevalence of 2.8%, 51.4%, & 62% as reported by Gracia *et al*. (2011) [25], Amini *et al*. (2010) [4] and Eyigor *et al*. (2002) [22], respectively. Present study revealed (6/249) 2.40% of *Salmonella* group II in poultry cloacal swabs and almost similar prevalence rate of 4% (4/100) was reported by Ahmed *et al*. (2011) [1].

Six samples (5.08%) out of 118 chicken samples examined were positive for *Salmonella* spp. which correlates well with the results of Saha *et al*. (2016) [49] who reported a prevalence rate of 4%, whereas different studies conducted in India and China reported higher prevalence of 12.5%, 23.7%, 33.33%, 45.2% as per Dogru *et al*. (2009) [21], Kaushik *et al*. (2014) [31], Balakrishnan *et al*. (2018) [9] and Bai *et al* (2015) [7], respectively and lower prevalence of 1%, 3.2% and 3.7% were reported by many authors like Wang *et al*. (2009) [62], Issa *et al*. (2017) [28] and Yoon *et al*. (2014) [66], respectively.

Out of 6 *Salmonella* isolates recovered from the chicken samples, 5 (4.23%, 5/118) were *S.* Typhimurium and 1 (0.84%, 1/118) *S.* Linderburg. The results were in agreement with Kaushik *et al*. (2014) [31] and Anumolu *et al*. (2012) [6] who also reported 6% and 2% prevalence of *S.* Typhimurium in chicken meat samples.

Out of 65 mutton samples evaluated, 2 (3.07%, 2/65) samples revealed the presence of *Salmonella* spp. S. Typhimurium and *Salmonella* group II). This was almost in agreement with the findings of Dabassa (2013) [20] and Yadav *et al*. (2006) [65] who reported 2.7 % and 3.0% in mutton samples respectively while, higher prevalence rates of 11% and 43% were reported by Zweifel *et al*. (2004) [68] and Bonke *et al*. (2012) [13] and low prevalence of 1.1% was reported by Nouichi and Hamdi (2009) [40] in mutton.

Out of 17 poultry liver swabs processed, no *Salmonella* isolate was detected while 6.3%, 35.1% and 23.33% of prevalence were reported by Al- Nakhli *et al*. (1999) [3], Islam *et al*. (2006) [27] and Karim *et al*. (2017) [30], respectively. Good managemental practices of poultry flock at the farm level may be the reason for absence of *Salmonella* in liver

swabs collected during P.M examination.

Out of 30 pork samples processed, only one sample was positive for *S.* Typhimurium (3.33%, 1/30) which was in correlation with the findings of work done by TEAGASC (The Agriculture and Food Development Authority) in Ireland and Kiran *et al.* (2016) [33] where *Salmonella* spp. prevalence was reported to be 3.3% and 1.57%. In contrast to the present study, higher prevalence of 47.75% and 58.1% were reported by Villal Pando- Guzmani *et al.* (2016) [59] and Zaidi *et al.* (2006) [67].

Out of 37 poultry farm water samples processed, no single *Salmonella* was detected. The finding of the present study was in correlation with the findings of Alali *et al.* (2010) [2], who also reported similar results in poultry water samples of Georgia. Barros *et al.* (2001) [10] and Traore *et al.* (2015) [57] also reported the non-existence of *Salmonella* in water samples of different broiler chicken farms of Finland. While some authors like Poppe *et al.* (1991) [43] and Samanta *et al.* (2014) [50] reported a higher prevalence rate of 12.3% and 20% of *Salmonella* serovars in poultry farm water samples of Canada and West Bengal, respectively. Absence of *Salmonella* in poultry farm water samples may be due to the supply of *Salmonella* free water to the birds and proper disinfection and treatment of water or may be due to the presence of *Salmonella* at a very low level, below the detection limit (10 CFU/ml) of the isolation/cultural methods used in the present study (Alali *et al.*, 2010) [2].

The PCR based approach has been successfully used for the detection of specific virulence genes in *Salmonella* spp. (Swamy *et al.*, 1996) [54]. The different virulence genes detected by PCR were *invA*, *invH*, *sopB*, *sopE*, *stn*, *pefA* and *sefC*. All the isolates were found to be carrying *invA*, *invH*, *sopB*, *sopE* and *stn* (100%) genes while variability was observed among *pefA* (33.33%) and *sefC* (16.66%) genes. *pefA* gene was found only in *S.* Typhimurium and *sefC* only in *S.* Enteriditis. The results of present study were in correlation with the work done by Choudhury *et al.* (2016) [18], who reported that 100% *Salmonella* isolates collected from different parts of India carried *invA*, *invH*, *sopB* and stn genes. Muthu *et al.* (2014) [38] reported 79.5% prevalence of *stn* gene among different human clinical *Salmonella* serovars collected in and around Chennai, India and reported absence of *pefA* among screened *Salmonella* Enteritidis isolates.

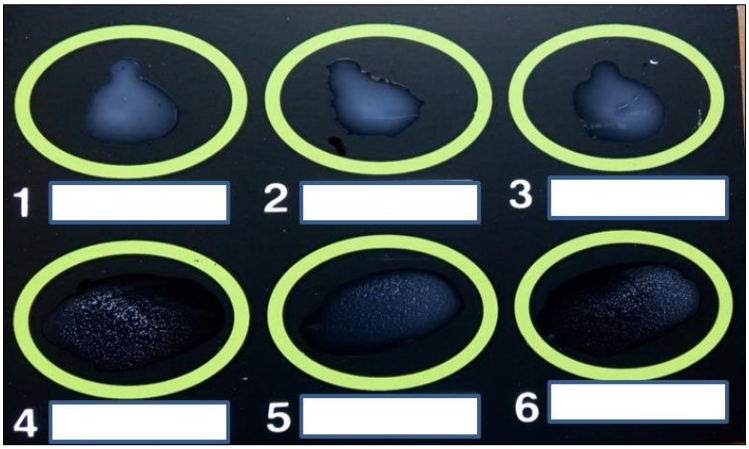

Fig 1: Latex agglutination test kit results for Salmonella spp.
Well 1-3 Negative reaction (1-E. coli ATCC 25922, 2 and3 –Salmonella negative samples),
Well 4 Positive control showing clear agglutination (S. Typhimurium ATCC14028),
Well 5 & 6 Salmonella isolates showing clear agglutination reaction

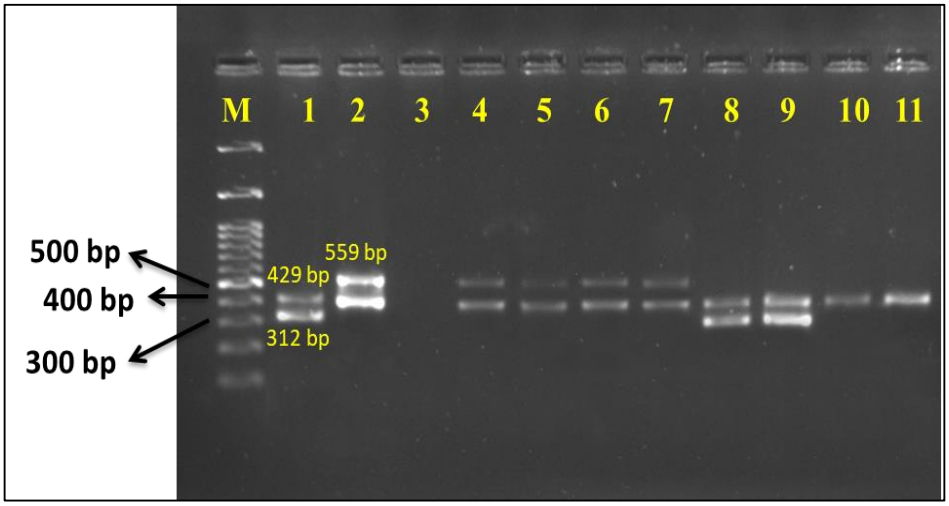

Fig 2: Gel photograph of m-PCR showing bands for Salmonella genus, S. Typhimurium & S. Enteritidis, Lane M Molecular weight marker (100bp), Lane 1 Positive control of S. Enteritidis ATCC 13076 (312 bp and 429 bp), Lane 2 Positive control of S. Typhimurium ATCC14028 (429 bp and 559 bp), Lane 3 Negative control, Lane 4 -7 S. Typhimurium positive isolates from different samples (chicken, mutton and pork), Lane 8 & 9 S. Enteritidis positive isolates from poultry cloacal swabs, Lane 10&11 Salmonella genus positive isolate obtained from poultry cloacal swabs (429bp)

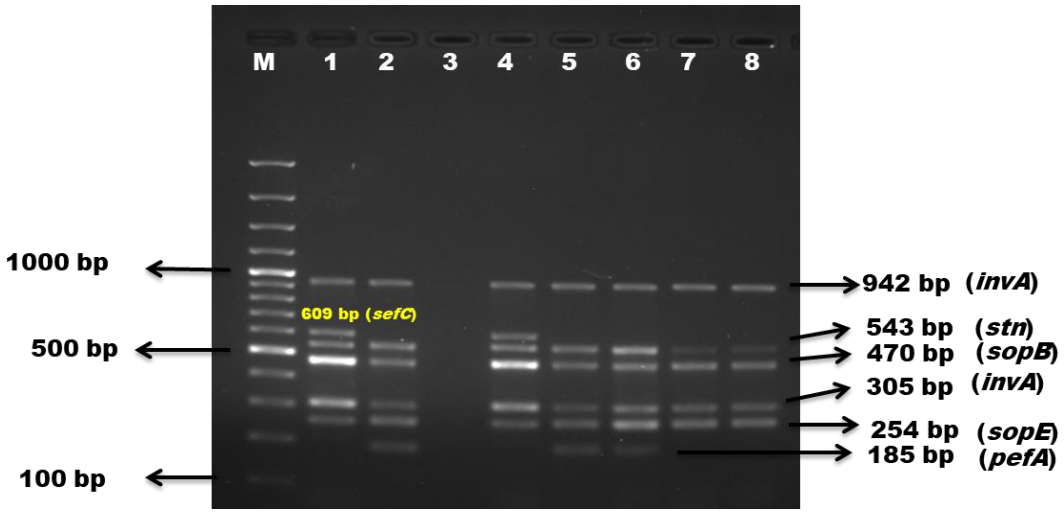

Fig 3: m-PCR showing different virulence genes in Salmonella spp,
Lane M Molecular weight marker (100bp),
Lane 1 Positive control of S. Enteritidis showing ATCC 13076 invA, invH, sopB, sopE, stn & sef C,
Lane 2 Positive control of S. Typhimurium ATCC 14028 invA, invH, sopB, sopE, pefA & stn,
Lane 3 Negative control,
Lane 4 S. Enteritidis isolate showing invA, invH, sopB, sopE, stn & sefC genes,
Lane 5 & 6 S. Typhimurium isolate showing invA, invH, sopB, sopE, pefA & stn genes,
Lane 7&8 Salmonella Daytona isolate samples showing invA, invH, sopB, sopE and stn genes

Conclusion

The high frequency of presence of virulence genes that were investigated highlights the pathogenic potential of the studied *Salmonella* serovars which have been causing disease in humans due to consumption of contaminated foods of animal origin.

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