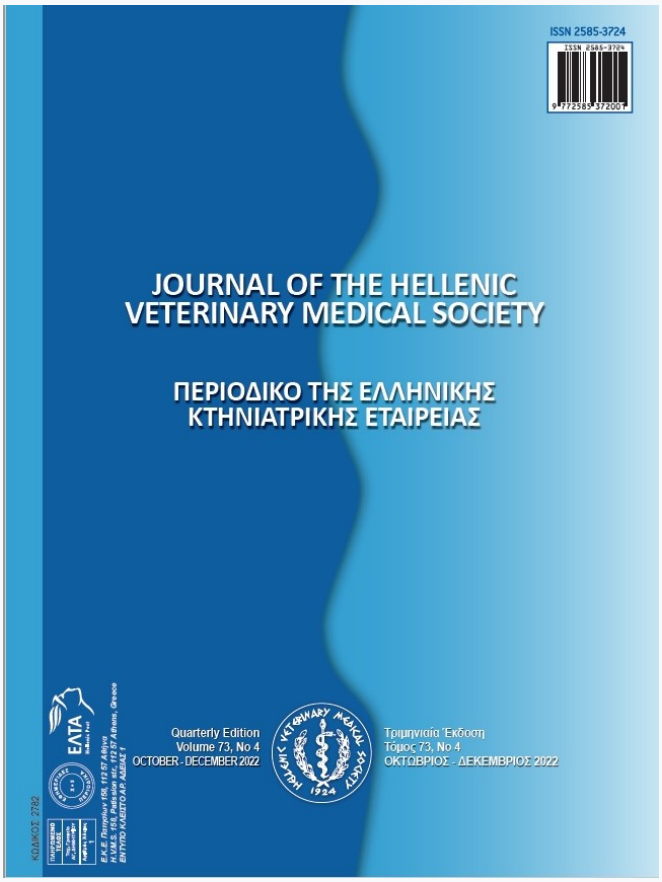
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 4 (2022)



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doi: 10.12681/jhvms.27474

To cite this article:

Rahat, S., Ahmad, K., & Ali, A. (2023). Molecular identification of virulence features of Salmonella enterica isolated from fresh chicken meat sold at retail shops and markets in Peshawar, Pakistan. *Journal of the Hellenic Veterinary Medical Society*, *73*(4), 4773–4778. https://doi.org/10.12681/jhvms.27474

J HELLENIC VET MED SOC 2022, 73 (4): 4773-4778
ΠΕΚΕ 2022, 73 (4): 4773-4778

Research article
Ερευνητικό άρθρο

Molecular identification of virulence features of *Salmonella enterica* isolated from fresh chicken meat sold at retail shops and markets in Peshawar, Pakistan

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ABSTRACT: *Salmonella enterica* is an important agent of food borne illnesses in humans. Poultry meat and products are easily contaminated with different strains of *Salmonella enterica*. Consumption of such food causes salmonellosis in human. The aim of present investigation was to estimate the prevalence of *Salmonella enterica* in fresh chicken meat available in retail shops and markets at District Peshawar, Pakistan and survey the prevalence of virulence associated genes in the isolated *Salmonella enterica*. Fresh chicken meat samples were collected from retail shops and markets at District Peshawar, Pakistan. The samples were processed for isolation of *Salmonella enterica* using selective media. Presumptive isolates were confirmed as *Salmonella enterica* via different biochemical tests and by amplification of *invA* gene. Ninety samples were contaminated with *Salmonella enterica* out of 150 chicken meat samples. Isolates were tested for the presence of eight different virulence genes *invA, spiA, cdtB, prgH, orgA, lpfC, sopB,* and *pefA*. The prevalence of virulence genes was: *invA* (100%), *lfpC*(48.8%),*sopB* (44.4%),*orgA* (30%), 2*spiA* (26.6%),*pefA* (21.1%), *prgH* (20%) and *cdtB* (6.6%). Genetic profiles P1-P26 were identified based on the occurrence of virulence genes among the isolates. The findings of this study highlight an alarming public health risk and demand for strict bio-safety hygiene measures to be taken.

Key words:*Salmonella enterica*; Salmonellosis; pathogenesis; virulence genes; meat

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Date of initial submission: 10-07-2021
Date of acceptance: 05-05-2022

INTRODUCTION

Salmonella enterica subspecies *enterica* is the leading cause of foodborne illnesses worldwide. Mostly salmonellosis is caused by non-typhoidal Salmonellae (NTS) that leads to life threatening conditions (Agron et al,. 2001; Piccini and Montomoli, 2020). Poultry meat and meat products easily get contaminated by *S. enteric* that are major sources of salmonellosis in human worldwide (Amavisit et al., 2001). Pathogenicity of *S. enterica* is due to the presence of different virulence genes. With the help of such genes, *Salmonella* can invade and destroy cells ((Latasa et al., 2012, Skyberg et al., 2006; Smith and Bayles, 2006; Zou et al., 2011).

Salmonella species possess many virulence genes that are located in *Salmonella* pathogenicity islands (SPIs). The most common pathogenicity islands are SPI1, SPI2, SPI3, SPI4 and SPI5. Collectively, these pathogenicity islands carry a total 60 virulence genes. These genes are responsible for adhesion, invasion, and multiplication of the pathogen inside the host cell. The *invA* gene participates in adhesion and invasion while *mgtc5* mostly has role in survival inside the host cell (Oliveira et al., 2003).The *invA* genes is also associated with the structure of TTSS (Type Three Secretion System) and considered as a target gene for confirmation of *S. enterica* at genus level (Nikaido, 2003; Beceiro et al., 2013).Along with *invA* gene some other genes such as *Salmonella* outer proteins (*sop*) allow *Salmonella* to invade the host cell (Nayak et al., 2004; Huehn et al., 2010; Choudhury et al., 2016; Swamy et al., 1996). Pilus regulatory genes (*Prg)* operon is mainly composed of three genes i.e., *PrgH, PrgI,* and *PrgK* (Murugkar et al., 2003; Kubori et al., 1998). All these genes play crucial role in pilus assembly and its localization in membrane. The oxygen-regulated gene (*OrgA)* is involved in invasion of serovar *S. typhimurium* inside the host cells. The cytolethal distending toxin B (*cdtB*) is an important toxin found in many serovars of *S. enteric* that is involved in intestinal pathogenesis through cell cycle arrest, apoptosis, and cytoplasm distension in a range of mammalian host cells (Smith and Bayles., 2006).

Characterization of virulence genes is essential for better understanding the pathogenicity mechanisms of zoonotic *S. enterica*. Various studies from different parts of the world have focused on investigating the prevalence of virulence associated genes of *S. enterica* isolated from poultry and poultry products (Dione et al., 2011; Zou et al., 2011; Borges et al., 2013). Such data are not available in Pakistan. Hence, the current study was aimed at investigating the existence of virulence genes in *S. Enteric* recovered from chicken meat simples collected from different locations in Peshawar, Pakistan. To our knowledge, this is the first study from Pakistan on the occurrence of virulence genes in *S. enterica* isolates of chicken meat origin.

MATERIALS AND METHODS

This study was conducted at the Center of Biotechnology and Microbiology, University of Peshawar, Pakistan. A total of ninety isolates of *S. enterica* were isolated from 150 fresh chicken meat samples collected from different poultry markets and retail shops in Peshawar, Pakistan during 2014-2016.

Isolation and identification

For isolation of *S. enterica*, all samples were processed by enrichment and plating on selective media. Presumptive colonies of *S. enterica* were further confirmed by different biochemical tests according to standard procedures as previously described (Parija, 2006).

Molecular identification of *S. enterica*

DNA purification kit (GeneJET, K0721) was used for extraction of DNA. For genus level identification the *invA* gene was amplified via conventional PCR (Chiu and Ou 1996). A reaction mix(25µl) had 4µl master mix (BIORON, Cat. No. 1200126), 1µl of each primer (0.5µ M), 2 µl of template DNA and 18µl water (Sigma-Aldrich, US). The thermo cycler was set to initial denaturation for 1 min at 94^{0}Cfollowed by35 cycles of denaturation at 94^{0}C for 1 min, annealing at 53^{0}C for 2 min and extension at 72^{0}C for 3 min. Finally, reaction was kept for 7 min at 72^{0}C. Amplified product of *invA* gene was analyzed using 1.5% agarose gel and 100 bp DNA ladder (BIORON,304105). Previously isolated *S. enteric* isolates were used as positive controls.

Detection of virulence genes

The occurrence of eight virulence genes (*invA*, *spiA*, *cdtB*, *prgH*, *orgA*, *lpfC*, *sopB*, and *pefA*) was analyzed by PCR (Skyberg et al., 2006). A PCR reaction mix of 25 µl contained4 µl master mix (solis-BioDyne,04-12-00126), 1 µl DNA template, 1 µl (0.5 µM) of each primer and 18 µl molecular grade water. Initial denaturation was set to 95^{0}C for 5 min that was followed by 30 cycles of denaturation at 94C for 1 min, annealing at 66^{0}C for 60 sec and extension

at 72^{0}C for 90 sec. An additional final extension at 72^{0}C for 10 min was also used. The products were analyzed using 1.5% agarose gel and a100 bp DNA ladder (Bioron, Cat no.304105) as size marker. The genetic profile of virulence genes i.e., co-occurrence of different virulence genes among the isolates was designated as P1, P2, P3 etc.

RESULTS

Out of 150 fresh meat chicken samples collected randomly at different locations in Peshawar, 90 samples (60%) were positive for *S. enterica*. Isolates were confirmed as *S. enterica* by different biochemical tests and amplification of *invA* gene via PCR. The target virulence genes (*invA*, *spiA*, *cdtB*, *prgH*, *orgA*, *lpfC*, *sopB*, and *pefA)* were detected in the isolates with variable frequencies. Virulence genes profiles were named as P1-P26 based on the occurrence of the different virulence genes among the isolates (Table-1). Among all the isolates, 90(100%) had *invA* gene, 44(48.8%) possessed *lfpC* gene,40(44.4%) possessed *sopB* gene, 27(30%) possessed *orgA* gene, 24(26.6%) possessed *spiA* gene, 19(21.1%) possessed *pefA* gene, 18(20%) possessed *prgH* gene and 6(6.6%) possessed *cdtB* gene. 15.5% had five genes and 21.1% had four genes simultaneously. 72.2% isolates harbored two virulence genes.

DISCUSSION

This study confirmed a high prevalence of *S. enterica* in meat samples collected from local markets at Peshawar, Pakistan. Similar results have been reported previously from Greece that showed a very high (97 %) contamination of chicken samples with

Table 1. Virulence genes profiles of *S. enterica* isolates

No of resistance genes detected	Virulence gene	Genetic profile	No of isolates	Strain ID
One	*invA*	**P1**	25	B71, D63, L1, S2K, E2, E4, E6, E7, E9, E10,E12, E13, E14, E15, E17, E19, A1.2, A2, A2.3, 8, 10,11,12, 20, 14T
Two	*invA, sopB*	**P2**	11	FB, P2I, P4I, PIS, M61, E16, 9, 22, 8T, 9T, C5
	invA, lfpC	**P3**	12	F7, D61, C62, P3S, P2S, L2, E1, E3, E18, 13, 28T, 29T
	invA,orgA	**P4**	03	P5I, 25P, P3I
	invA, prgH	**P5**	02	E8, A3
	invA,pefA	**P6**	01	A1
Three	*invA, lfpC, spiA*	**P7**	01	2FP8
	invA,lfpC, sopB	**P8**	04	2M61, 3N62, L3, 14
	invA,orgA, sopB	**P9**	01	26P
	invA, lfpC, orgA	**P10**	01	15
	invA,orgA, pefA	**P11**	01	27
	invA,sopB, spiA	**P12**	01	28
Four	*invA,orgA, prgH, sopB,*	**P13**	01	S2G
	invA, lfpC, prgH, spiA	**P14**	02	INA, FD
	invA, lfpC, orgA, spiA	**P15**	01	PR7S
	invA, lfpC, pefA, spiA	**P16**	01	IS9
	invA,lfpC, sopB, spiA	**P17**	02	IS3, IS5
	invA,lfpC, orgA, sopB	**P18**	01	C63
	invA,lfpC, prgH, sopB	**P19**	01	P1I
	invA,lfpC, orgA, prgH	**P20**	01	S2M
Five	*invA,lfpC, prgH, sopB,spiA*	**P21**	03	F8S, S2A, S2I
	invA, lfpC, orgA, prgH, sopB	**P22**	01	IN3
	invA, lfpC, orgA, sopB, spiA	**P23**	04	211S, FIS, IS6, FA
	invA, orgA, prgH, sopB, spiA	**P24**	01	S2E
Six	*invA,lfpC, orgA, prgH, sopB, spiA*	**P25**	05	IN9, 201S, S2F, 1N14, IS4
Seven	*invA, lfpc, OrgA, PefA, PrgH, SopB, SpiA*	**P26**	03	S2C, INIS, S2B

Salmonella (Karabaxoglou et al. 1996). The high contamination of chicken meat with *Salmonella* in the current study could be attributed to the unhygienic processing and storage of chicken meat in the local markets. These findings are alarming from health point of view as these bacteria are well equipped to cause *Salmonella* infections in human upon consumption of contaminated chicken meat or meat products as has been reported previously (Marin and Lainez, 2009; Papadopoulos et al., 2016,Zdragas et al., 2012). The isolated bacteria were also found to possess eight different virulence genes and many isolates had multiple types of virulence genes. Similar results have been reported previously involving clinical or other environmental and food samples (Uchiya and Nikai, 2008). This data confirmed that chicken meat isolates may cause human infections because of presence of similar virulence genes as reported in clinical isolates (Borges et al., 2013). All isolates were found positive for *invA* gene that play significant role in the host recognition and internalization of *Salmonella* inside the host epithelial cell during invasion process.

Fimbrial virulence genes such as *lpfA, lpfC,* and *pefA* have crucial importance in binding, attachment, and adhesion of the bacterium to enterocytes (Schechter and Lee, 2000; Chuanchuen et al., 2007; Miranda et al., 2008; Lekshmi et al., 2017). In current study *lpfC* and *pefA* showed high prevalence of 48.8% and 21.1% respectively as compared to past studies (Borges et al., 2013). The *cdtB* gene encodes an important toxin which plays a significant role in inducing apoptosis of the infected host cells. Only 6.6% isolates were found positive for *cdtB* gene as reported in previous studies (Tatavarthy and Cannons, 2010; El-Tawab, 2017). Some effectors proteins such as *orgA*, *prgH* and *sipB* genes have a strong association with TTSS (Tatavarthy and Cannons, 2010; Borges et al., 2013; El-Tawab, 2017; Kim and Lee, 2017). In our findings *orgA* and *prgH* were found with prevalence of 26.6% and 20% respectively. Borges et al also reported similar genes in different *Salmonella* strains isolated from various food samples (El-Tawab, 2017). For better survival inside the host cells especially in presence of macrophages, genes such as *msgA, spiA, pagC,* and *tolC* are crucial. In current study *spiA* gene was detected in 26.6% isolates like previous findings (Tatavarthy and Cannons, 2010). The *sopB* gene that is mainly associated with prophages and helpful in construction of TTSS was found in 30% isolates. Current data is in close agreement with previous reports (Borges et al., 2019).

CONCLUSIONS

The presence of *Salmonella enterica* and its virulence genes in poultry meat is a great alarm for public health because these pathogens could be easily disseminated from poultry to human communities via food web. Hence, proper management and preventive measures need to be taken to prevent the spread of these pathogenic stains to human population. The study also had some limitations which were: This study covered a specific geographical location and the results could not be generalized. The study was conducted in 2014-16 and we do not have data for serovars. The result could, hence, be interpreted accordingly.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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