

ORIGINAL ARTICLE

Identification and Characterization of *Salmonella* Species Isolated from Broiler Chickens

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Received: 04 August 2021 | Accepted: 24 August 2021 | Published online: 28 August 2021

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Abstract

Salmonellosis is one of the most important problems facing poultry industry and a critical food safety hazard. In the present study the prevalence of avian Salmonellosis was studied in different farms of broiler chickens in Beni Suef Governorate, Egypt during the period from January to April 2020. A total of 140 samples were collected from slaughtered diseased or freshly dead broiler chickens aged from one to 35 days. Bacteriological examination revealed that 7.14% of the samples were *Salmonella* positive. Serotyping of *Salmonella* isolates showed that *S. kentucky*, *S. blegdam* and *S. virchow* were recognized at rates of 40%, 30% and 30%, respectively. Antimicrobial susceptibility test revealed that all salmonella isolates were multidrug resistant (MDR). All isolates were resistant to oxytetracycline (100%) while 90% were resistant to amoxicillin-clavulanic acid, cefotaxime, sulfamethoxazole-trimethoprim and norfloxacin. On the other hand, 80% of isolates were sensitive to fosfomycin and nitrofurantoin. Results of screening of some MDR isolates by multiplex PCR for detection of some virulence genes showed that all the tested isolates (100%) had *invA*, *stn*, *spvC* genes meanwhile *pefA* was not detected in any isolate.

Keywords

Multidrug resistance, *Salmonella*, *invA*, *stn*, *spvC*

1. Introduction

Salmonellosis is an important bacterial disease usually infects poultry flocks. It is caused by a wide variety of *Salmonella* species (Haider et al. 2004). It represents a critical food safety hazard (Vinueza-Burgos et al. 2019). Also *Salmonella* is one of the most important zoonotic foodborne pathogens (WHO 2016). *Salmonellae* infections in poultry can be grouped into two categories; the first includes two non-motile serotypes, *S. pullorum* and *S. gallinarum*,

that causes pullorum disease and fowl typhoid, respectively (Barrow and Neto 2011). The second includes infections with the numerous motile *Salmonella* serotypes referred collectively as paratyphoid salmonellae. Although infections of poultry with *Salmonella enterica* serovars are very common, they seldom cause acute systemic disease except in highly susceptible young birds subjected to stress conditions (Gast et al. 2003). Outbreaks of human salmonellosis are associated with the

consumption of poultry products contaminated with *S. enteritidis* and *S. typhimurium* (Vose et al. 2011).

Although antimicrobial agents are important tools to treat clinically diseased birds and to maintain the healthy and productivity of birds, the misuse of antimicrobials as well as their prophylactic application in poultry industry is considered the cause of drug resistance (Phillips et al. 2004). Resistance of salmonellae to antimicrobials is considered a serious problem worldwide having a very important public health concern. The emergence and spread of these antimicrobial resistances is a complicated problem caused by many interconnected factors (Radwan et al. 2020). In-vitro antimicrobial susceptibility testing of pathogens provides valuable guidance to the veterinarian in the selection of appropriate antimicrobial agent (Radwan et al. 2016). Moreover, it can be used in detection of MDR isolates. Surveillance data showed an obvious increase in salmonellae antimicrobial resistance in the recent days than the past (Su et al. 2004).

There is a strong correlation between the existence of several virulence genes and the pathogenicity of salmonellae (Radwan et al. 2016). The majority of the salmonella isolates from human and food origin harbored the *invA*, *stn*, *sopB* and *sopE1* virulence genes (Zou et al. 2012).

This study aimed to investigate the antimicrobial resistance and molecular characterization of virulence-associated genes of pathogenic salmonellae isolated from broiler chickens.

2. Material and Methods

2.1. Sample collection

A total of 140 samples were collected aseptically from broiler chickens aged from one to 35 days collected from Beni Suef Governorate, Egypt during the period from January up to April 2020. These chickens were suffering from respiratory manifestations and/or gastrointestinal problems. Swabs from heart, lung, liver, and yolk sac showed gross lesions were used for bacteriological examination.

2.2. Isolation and identification of salmonella species

The collected samples were inoculated under aseptic condition into buffered peptone water (BPW) and

incubated aerobically at 37°C for 24 hours then inoculated into Rappaport-Vassiliadis (RV) broth and incubated aerobically at 41°C for 24 hours. A loopful from the culture media was streaked onto MacConkey's agar and xylose lysine deoxycholate (XLD) agar media, and incubated aerobically at 37°C for 24 h. Suspected pale colonies on MacConkey's agar and red with black centers on XLD agar were subjected to further tests for identification. These isolates were identified as *Salmonella* species based on their colony morphology and biochemical tests according to the schemes described by Collee et al. (1996) and Quinn et al. (2002).

2.3. Serological identification of *Salmonella* spp.

Salmonella serotyping was carried out using slide agglutination test using salmonella antisera to detect the somatic (O) and flagellar (H) antigens according to white Kauffmann-Leminor scheme as described by Grimont and Weill (2007).

2.4. Antibacterial sensitivity testing

Disk diffusion technique was carried out in concordance to CLSI (2018). All salmonella isolates were tested for their susceptibility to 9 different antimicrobial discs (Himedia, Turkey) represented different antimicrobial classes of veterinary concern including amoxicillin/clavulanic acid (30 µg), cefotaxime (30 µg), doxycycline (30 µg), fosfomycin (200 µg), gentamicin (10 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), oxytetracycline (30 µg) and sulfamethoxazole-trimethoprim (25 µg). Resistance to three/or more antimicrobials of different groups was considered as multidrug resistance (MDR) according to Magiorakos et al. (2012).

2.5. Polymerase chain reaction (PCR) for *Salmonella* isolates

PCR was applied on 5 MDR salmonella isolates for detection of 4 virulence genes (*pefA*, *stn*, *invA*, *spvC*). DNA extraction was performed using QIAamp DNA mini kit according to the manufacturer's instruction. Primers sequences and amplified products for the targeted genes for salmonella isolates were illustrated in Table (1). Temperature and time conditions of the primers during PCR were shown in Table (2) according to Emerald Amp GT PCR master-mix Takara) kit.

Table 1. Primers of virulence genes used in PCR.

Target gene	Primers direction	Primers sequences	Amplified segment	Reference
<i>pefA</i>	F	TGT TTC CGG GCT TGT GCT	700 pb	Murugkar et al. (2003)
	R	CAG GGC ATT TGC TGA TTC TTC C		
<i>stn</i>	F	TTG TGT CGC TAT CAC TGG CAA CC	617 pb	
	R	ATT CGT AAC CCG CTC TCG TCC		
<i>invA</i>	F	GTG AAA TTA TCG CCA CGT TCG GGC AA	284 pb	Oliveira et al. (2003)
	R	TCA TCG CAC CGT CAA AGG AAC C		
<i>spvC</i>	F	ACC AGA GAC ATT GCC TTC C	467 pb	Huehn et al. (2010)
	R	TTC TGA TCG CCG CTA TTC G		

Table 2. Cycling conditions of the different primers during PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	cycles No.	Final extension
<i>pefA</i>	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	40 sec	45 sec.		10 min.
<i>stn</i>	94°C	94°C	59°C	72°C	35	72°C
	5 min.	30 sec.	40 sec	45 sec		10 min.
<i>invA</i>	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	30 sec	30 sec		7 min.
<i>spvC</i>	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	40 sec	45 sec.		10 min.

3. Results

3.1. Prevalence of salmonella in the examined broiler chickens

Out of 140 broiler chicken samples, 10 salmonella isolates were recovered with a prevalence rate of 7.14%.

3.2. Serotyping of Salmonella isolates.

Results of serotyping of 10 *Salmonella* isolates were represented in table (3). Out of 10 *Salmonella enterica* isolates, 3 serotypes were identified. *Salmonella kentucky* was the most prevalent serotype; represented as 4 isolates (40%) followed by *Salmonella blegdam* and *Salmonella virchow*; 3 isolates (30% for each).

Table 3. Serotypes and antigenic structure of Salmonellae recovered from broiler chickens.

Serotypes	No.	%	Serogroup	Antigenic structure	
				O	H (H1:H2)
<i>Salmonella kentucky</i>	4	40%	C1	8, 20	i : z6
<i>Salmonella blegdam</i>	3	30%	H	6, 8	g, m, q : -
<i>Salmonella virchow</i>	3	30%	C1	6, 7, 14	r : l, 2
Total No. of isolates	10	100			

%. was calculated according to the total number (No.) of isolates (n=10).

3.3. Antimicrobial susceptibility testing

The results of *in-vitro* susceptibility testing (Table 4) showed that salmonella isolates were completely resistant to oxytetracycline (100%). A high percentage of isolates were resistant to amoxicillin-calvulanic acid, cefotaxime, sulfamethoxazole-trimethoprim and norofloxacin (90% for each) as

well as doxycycline (80%) and gentamicin (50%). On the other hand, a high percentage of isolates were sensitive to fosfomycin and nitrofurantoin (80% for each). All isolates were MDR (100%). The average MDR index (MDRI) for all *Salmonella* isolates was 0.677.

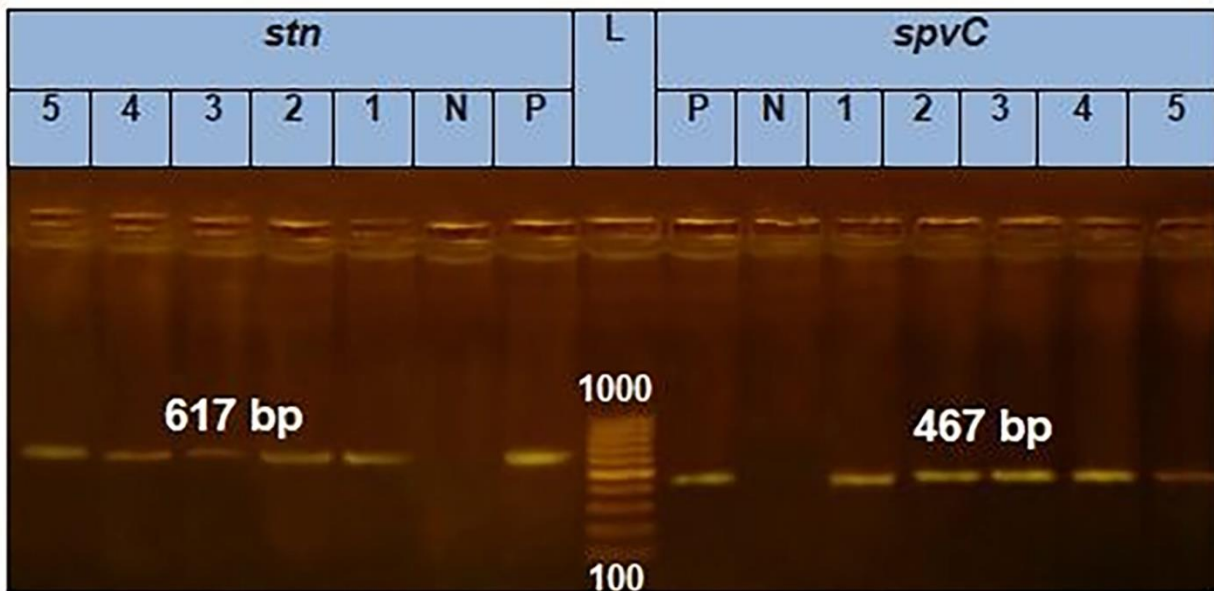


Fig. 2. PCR amplification of the *stn* gene at 617bp and the *spvC* gene at 467bp fragments. Lanes 1–5 showed positive amplifications of *stn* and *spvC* genes. **P** = Positive control; **N** = negative control; **L** = 100-bp DNA molecular size ladder.

4. Discussion

Salmonella infection causes extensive mortality in poultry flocks, leading to serious financial and economic losses in poultry production, as well as posing a public health concern (Pedersen et al. 2002). Identification and genotyping of salmonella isolates are important for the epidemiological surveillance and outbreaks investigation (Hassan et al. 2018).

In the present study, 10 *Salmonella* spp. were isolated from the 140 samples of internal organs of broiler chickens manifested respiratory signs and/or gastrointestinal problems with a prevalence rate 7.14%. This result was similar to Sedeik et al. (2019) who found that the prevalence of salmonella was 7.5% in diseased and freshly dead broiler chickens collected from different farms in El-Gharbia, El-Behera, Kafr-Elshikh, Alexandria and Marsa-Matrouh Provinces. Also, nearly similar findings; 7.8% and 7.7%, were recorded in previous studies (Shang et al. 2018; Lassnig et al. 2012). Meanwhile, this result was lower than that detected by Temelli et al. (2012); 30% and Islam et al. (2014); 49.91%.

The predominant serovars differ from one geographic area to another. Serotyping of all salmonella isolates revealed that all isolates belonged to *S. enterica* subsp. *enterica* and 3 serotypes were identified. *S. kentucky* (40%) followed by both *S. blegdam* and *S. virchow* (30% for each). These results run parallel to

that obtained by Djefal et al. (2018) who detected *S. kentucky* at rate of 49% and lower than Hegazy (2002) who detected *S. kentucky* at rates of 62.2%. Also Fagbamila et al. (2017) found that *S. kentucky* was the dominant serovar isolated from chicken.

Antimicrobial therapy is one of the primary control measures for reducing the morbidity and mortality caused by infectious bacterial diseases. They also used as growth promoters at sub-therapeutic doses for maintaining health and productivity of birds thus reduce the great economic losses in the poultry industry (Bogomazova et al. 2020; Radwan et al. 2020). Due to the similarity of animal and human antimicrobial agents, there is a high risk for developing MDR strains, thus, they must be properly used in veterinary medicine (Seifi et al. 2015; Khaltabadi Farahani et al. 2018).

In the current study, all salmonella isolates (n=10) were tested for their antimicrobial susceptibility for different antimicrobial drugs for detection of the appropriate drug for treatment as well as for detection of the MDR isolates for further analyses. Results illustrated in Table (4) revealed that salmonella isolates were mostly resistant to most of the antimicrobials used while they showed high sensitivity to fosfomycin and nitrofurantoin only.

The current results run hand to hand with those recorded by **Radwan et al. (2016)** who reported high sensitivity against fosfomycin (75%). Meanwhile, 58.3% of isolates were resistant to amoxicillin-clavulanic. Moreover, **Radwan et al. (2018)** recorded complete resistance to trimethoprim-sulphamethoxazol. Also, **Hassan et al. (2018)** reported high resistance to sulphamethoxazole-trimethoprim (76.9%) meanwhile high sensitivities were observed to gentamycin (73.1%).

The result of MDR *Salmonella* isolates as 100% was similar to that detected by **Radwan et al. (2018)**; 100% and nearly similar to those detected by **Donado-Godoy (2010)**; 98%, **Radwan et al. (2016)**; 75%, and **Hassan et al. (2018)**; 92.3% . A lower percentage was detected by **Fallah et al. (2013)**; 34.1%. MDR mechanisms in *Salmonella* isolates were associated with mobile genetic elements (plasmids, integrons or transposons) that can be transferred among bacteria and enhance the spreading of these resistance genes in the bacteria (**Radwan et al. 2020**). Also **Dhanani et al. (2015)** reported that all salmonella isolates of broiler chicken carried multidrug efflux pump systems regardless of their antibiotic susceptibility profile. So there is an increasing risk of spread of antimicrobial-resistant *Salmonella* to public health (**Youn et al. 2017**).

Salmonellae are mostly invasive bacteria having various systems for penetration and interaction with the epithelial mucosa for systemic invasion (**Galan 2001**). Indeed, a multiple virulence-associated genetic regions termed salmonella pathogenicity islands (SPI) which encoding multiple virulence factors and essential for salmonella pathogenicity have been identified. The most two important SPIs are SPI-1 and SPI-2 which encode structural proteins that form needle-like complexes allowing the insertion of the bacterial proteins inside the host cells modulating both the cellular functions and immune pathways (**Galan 2001**). There are at least 60 genes related to SPIs; many of such genes are encoded on SPI-1 while the majority is located on the chromosome or on large virulence-associated plasmids (**Hassan et al. 2018**). SPI-1 is responsible for the invasion of epithelial cells and macrophage apoptosis induction (**Rhen and Dorman 2005**). The *invA* is located in SPI-1 (**Amavisit et al. 2003**). *Salmonellae* lacking SPI-1 have significantly lower invasiveness ability than *invA*-positive strain (**Li et al. 2011**).

Salmonella invA gene was considered one of the most popular PCR target sequences and its amplification was recognized as an international standard for detection of salmonella and considered as target genes for the detection of salmonellae at the genus level and it is important in its pathogenesis (**Radwan et al. 2016**). The *invA* gene encodes a specific protein present in the bacterial inner membrane which is essential for invasion of the host epithelial cells (**Darwin and Miller 1999**). In addition, fimbriae are important for salmonella pathogenicity as they promote the salmonella attachment to epithelial cells. Pef fimbria is encoded by the *pef* operon (**Murugkar et al. 2003; Ammar et al. 2016**). Other chromosomal gene like *stn*, encoding enterotoxin production has been shown to be a causative agent of diarrhea also *stn* is involved in the regulation of bacterial cell membrane integrity (**Huehn et al. 2010; Osman et al. 2010; Thung et al. 2018**). In addition, *spvC* is located on a self-transmissible virulence plasmid, which facilitates the systemic spread of *Salmonella*, highlighting the potential high virulence of the salmonella isolates and contributes to the colonization of deeper tissues among other functions (**Abd El-Aziz 2018; Rozwandowicz et al. 2018**).

In the current work, PCR was also applied on 5 MDR *Salmonella* to detect 4 genes. The results illustrated in **Table (5)** and **Figs. (1, 2)** revealed that all the tested *Salmonella* isolates harbored *invA*, *stn* and *spvC* genes meanwhile no isolates harbored *pefA* gene.

Concerning *invA* gene finding, similar results were obtained in other studies in Egypt (**Radwan et al. 2016; Hassan et al. 2018; Awad et al. 2020**) as well as other studies worldwide (**Amini et al. 2010** in Iran, **Campioni et al. 2012** in Brazil; **Mphuthi et al. 2020** in South Africa).

Also *stn* and *spvC* virulence genes results were similar to those reported by **Ahmed et al. (2016); ElSheikh et al. (2019); Awad et al. (2020)**. Meanwhile, **Diarrassouba et al. (2007)** recorded lower rates especially *spvC* gene which was detected in 42% of the isolates while **Ammar et al. (2016)** reported that only 5.88% of tested isolates were *spvC* positive. Also **Khaltabadi Farahani et al. (2018)** reported *spvC* as 37.6%.

In the current study *pefA* gene was completely absent in the tested isolates. This was similar to the result obtained by **Elkenany et al. (2019)** and **Elhariri et al. (2020)** in which *pefA* gene was not detected. But

there were different results recorded in previous studies (Retamal et al. 2015; 93%; Ahmed et al. 2016; 6.7%).

5. Conclusion

The presence of multidrug resistance pathogens occur due to the misuse of the antibiotics. In this study, all salmonella isolates were MDR. Furthermore, different virulence associated genes were analyzed using multiplex-PCR.

6. Authors Contributions

All authors contributed equally to study design methodology, interpretation of result and preparing of the manuscript.

7. Conflict of interest

The authors declare no conflict of interest.

8. References

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How to cite this article: Hassan WH, Hassan HS, Hassan WMM, Shany SAS, Osman GSI. Identification and characterization of *Salmonella* species isolated from broiler chickens. *J Vet Med Res.*, 2021; 28(1): 21–29. <https://doi.org/10.21608/jvmr.2021.89244.1041>