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RESEARCH ARTICLE

Characterization of Paratyphoid *Salmonellae* Isolated from Broiler Chickens at Sharkia Governorate, Egypt

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Abstract

Paratyphoid *Salmonella* has emerged as a global problem for humans and poultry. Therefore, in this study we investigated the occurrence, serological, antimicrobial and molecular characteristics of paratyphoid *Salmonella* isolated from chicken flocks at Sharkia Governorate during 2015-2016. The prevalence of paratyphoid *Salmonella* among the 150 suspected flocks was 32.6 % (49/150), the highest rate (41/49; 80%) was among young ages (1-10 days old) and the lowest rate (8/49; 16 %) among older ages (11- 21 days old). The highest recovery was from liver (30.66 %), followed by spleen (25.33 %), caecum (20 %) and yolk sac (15.7 %). Serotyping of 49 *Salmonella* isolates revealed 11 different serogroups, with *Salmonella* Typhimurium was the most prevalent one (24.49 %), followed by *Salmonella* Kentucky (18.36 %) and *Salmonella* Enteritidis (14.28 %). The most sensitive antibiotics were apramycin (82 %) and ciprofloxacin (65 %). Multidrug resistance (MDR) was significant to ampicillin, gentamycin and cefotriaxone in all *Salmonella* isolates. All phenotypically identified MDR *Salmonella* were found to possess *inv*A, *hil*A, *pef*A (100%) and *avr*A (95 %) genes by polymerase chain reaction (PCR), confirming that these virulence genes are important virulence markers for rapid diagnosis of *Salmonella* infection.

Keywords: Multidrug resistant *Salmonella*, Virulence genes, Serotyping, Chicks.

Introduction

Salmonellosis is a serious problem and a public health risk [1], causing high economic losses in poultry industry due to high mortality in young chicks and debilitating effect predisposing to other diseases [2]. Avian *Salmonella* infection occurs in the form of acute or chronic disease caused by genus *Salmonella*, of the family *Enterobacteriaceae* [3]. Paratyphoid *Salmonellae* include more than 2400 serovars [4], causing pasty diarrhea, inappetence, dehydration, growth retardation, blindness and lameness in one week old broiler chicks. The main gross lesions are hepatomegaly with necrotic foci, splenomegaly, pericarditis, panophthalmitis, persistent yolk sac and arthritis [5]. Paratyphoid infection affects chickens at any age and of any type. In young birds, high mortality rates may reach 80% or higher while older ages, over 3 weeks old, paratyphoid infection rarely causes mortality but the survivors become carriers and excrete the organisms in the environment [6].

The pathogenicity of *Salmonella* depends on a set of virulence associated factors harbored by the bacterium, the bird and its environment. Adhesion and penetration of the bacterium into intestinal mucosa is a prerequisite for systemic infection [7]. The infection usually starts by ingestion, followed by intestinal colonization and penetration of the mucosal epithelium which results in a systemic infection with colonization in the spleen and liver [8]. *Salmonella* pathogenicity islands (SPIs) are large clusters of chromosomal conserved virulence genes in pathogenic *Salmonella* spp. that are absent in nonpathogenic spp. [9]. Several pathogenicity

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islands have been identified in *Salmonella* Typhimurium, *S.* Dublin and *S.* Enteritidis [10, 11]. SPI-1 encodes a type III secretion system (TTSS), which is required for the uptake of *Salmonella* by intestinal epithelial cells [12, 13]. The deletion of this island resulted in avirulent mutant by the oral route of infection but are virulent by the intravenous route, indicating that SPI-1 is required for intestinal invasion but not for systemic infection [14]. SPI-2 is essential for intramacrophage survival [15] and is required for the systemic phase of infection [16]. SPI-3 encodes the mgtCB operon that is required for both intramacrophage survival and growth in Mg+2-limiting conditions [17]. *Salmonella* of serotypes Dublin, Pullorum, Gallinarum, Choleraesuis, Abortusovis, and some strains *of* Typhimurium and Enteritidis harbor virulence plasmids that encode genes required for the ability to cause systemic disease [18, 19].

Salmonella infection can be detected via bacterial isolation, identification, serological tests [20] and polymerase chain reaction (PCR) which is more accurate especially in case of rough strain lacking O-antigen [21]. Efforts to reduce *Salmonella* include guidelines adoption for antibiotic misuse, continuous antimicrobial susceptibility surveillance to identify the changing pattern of *Salmonella* resistance [22] and alternative additives other than antibiotics as probiotics, prebiotics, synbiotics and organic acids [23]. Therefore, the aim of this study was to investigate the occurrence, serological, antimicrobial and molecular characteristics of paratyphoid *Salmonella* isolated from chicken flocks at Sharkia Governorate, Egypt.

Materials and Methods

Bird examination and sample collection

A total of 150 broiler farms (Cobb, Ross, Arbor Acres, Sasso and Balady breeds) during 2015-2016 outbreaks were divided as 100 (1-10 days old) and 50 (11-21 days old) with a history of whitish diarrhea and mortality were examined by clinical and postmortem (PM) examination for *Salmonella* infection. Pooled samples including three of each organ; liver, spleen, caecum (150 for each) and unabsorbed yolk sac (70) were aseptically collected and transferred in ice box to the avian bacterial pathogen laboratory of Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Bacterial isolation and identification

Samples were cultivated on buffered peptone water (Oxoid, UK) at 37 ºC/18 h, from which 0.1 mL was transferred to a 10 mL Rappaport Vassiliadis broth (Oxoid, UK) at 41.5 ºC/24 h. Ten microliter of broth culture were inoculated onto xylose lysine deoxycholate agar (XLD) and MacConkey's agar (HiMedia™ Laboratories, India) at 37 ºC/24 h. Pure colonies were microscopically examined, stabbed into semisolid agar for motility detection and biochemically identified by urea hydrolysis, triple sugar iron agar (TSI), lysine decarboxylase and indole production, methyl red test, Voges-Proskauer test, and citrate utilization test (IMVC) (Oxoid, UK). Isolates were preserved on tryptic soy agar (TSA) slants (Oxoid, UK) [24].

Serogrouping

Forty nine isolates were subjected to serotyping using polyvalent and monovalent somatic (O) and flagellar (H) *Salmonella* antisera via slide and tube agglutination test, respectively at the serology unit of the Faculty of Veterinary Medicine, Benha University, Egypt [25].

Antibiotic sensitivity test

The recovered isolates were tested for antibiotic susceptibility on Muller Hinton agar by disc diffusion method according to clinical and laboratory standard institute (CLSI) guide lines. Swabs from standardized suspension of colonies (match 0.5 McFarland standard) were streaked evenly on Mueller Hinton agar plate (Oxoid, UK). The antibiotic discs (thiamphenicol, 30 µg; florphenicol, 30 µg; ampicillin, 10 µg; erythromycin, 15 µg; apramycin, 15 µg; ceftriaxone, 30 µg; doxycycline, 30 µg; gentamicin, 10 µg; ; spectinomycin, 100 µg and ciprofloxacin, 5 µg, Oxoid, UK) were distributed evenly and firmly pressed on media. The plates were inverted and incubated for 18 h at 37° c. The diameter of inhibition zone was measured to the nearest mm with a ruler and interpreted according to CLSI breakpoints [26].

DNA Extraction and PCR assay for virulence genes

Twenty MDR isolates including (5 *S.* Typhimurium, 3 *S.* Enteritidis, 3 *S.* Kentucky, 2 *S.* Molade and one each of *S.* Infantis, *S.* Takoradi, *S.* Papuana, *S.* Labadi, *S.* Tsevie, *S.* Larochelle and *S.* Angers) were tested using two biplex PCR one for *inv*A and *pef*A and other for *hil*A and *avr*A. DNA was extracted using QIAamp kit (Qiagen, Germany). Test was done according to EmeraldAmp GT PCR mastermix (Takara, Europe) instructions**.** Thirty µl of each amplified samples, negative and positive control were gel electrophorized for 30 min and examined by UV-transilluminator [27]. The primer sequences, their thermal cycling condition for both PCRs and their amplicon sizes are shown in Table 1.

Table 1: Oligonucleotide primer sequences, amplicon sizes and cycling conditions

Gene	Function	Primer sequence (5' -3')	Amplicon size(bp)	Reference	Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension
					35 cycles				
***Pef*A**	plasmid encoded fimbriae	TGTTTCCGGGCTTGTGCT CAGGGCATTTGCTGATTCTTCCR	700	[5]	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.
***inv*A**	responsible for invasion	GTGAAATTATCGCCACGTTCGGGC TCATCGCACCGTCAAAGGAACC	284	[17]					
***hil*A**	hyper invasive locus	CATGGCTGGTCAGTTGGAG CGTAATTCATCGCCTAAACG	150	[18]	4˚C 5 min.	94˚C 30 sec.	58˚C 40 sec	72˚C 45 sec	72˚C 10 min.
***avr*A**	effector protein of TTSS	CCTGTATTGTTGAGCGTCTGG AGAAGAGCT TCGTTGAATGTCC	422	[19]					

Results

Clinical and PM findings of examined chickens

Diseased broiler chicks showed whitish diarrhea, progressive somnolence and mortality. Acute cases were septicemic, while chronic cases showed necrotic foci in the liver, enlarged gall bladder, spleen and liver, unabsorbed yolk sac, cheesy cecal cores and urates-filled ureters (Figure 1).

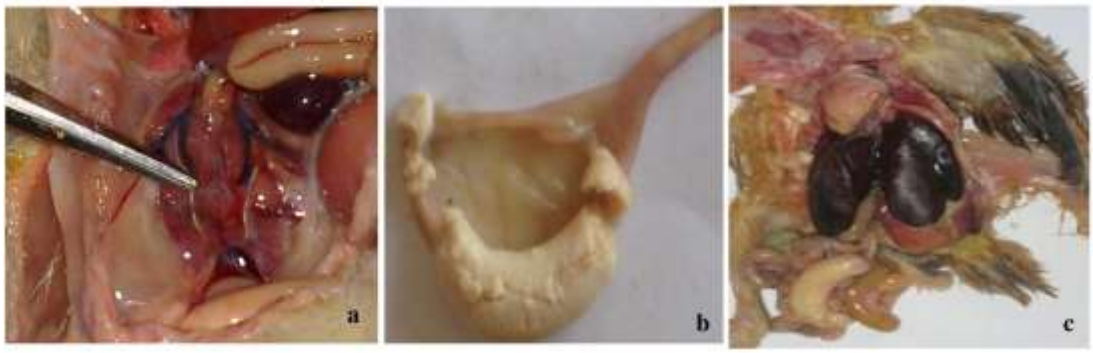

Figure 1: Congested spleen and urates filled ureters of 10 days old Hubbard chick (a); cheesy caecal core (b); swollen caecum and congested liver of 2 week old balady chick (c).

Salmonella isolation, identification and serotyping

The prevalence of paratyphoid Salmonella among the 150 suspected flocks was 32.6 % (49/150), the highest rate was (41/49; 83.6 %) among young ages (1-10 days) and the lowest rate was (8/49; 16.4 %) among older ages (11-21 days). The highest recovery was from liver (30.66 %), followed by spleen (25.33 %), caecum (20 %) and yolk sac (15.7 %). *Salmonella* spp. were Gram negative bacilli showed pale colonies on MacConkey's and reddish colonies with black center on XLD agar**;** urease negative; IMVC - + - + ; red/ yellow (acid butt/alkaline slant) with black color due to H_2S production on TSI agar and violet slant/ violet butt with black color on lysine iron agar. Serogrouping of 49 *Salmonella* isolates by slide agglutination test using specific monovalent and polyvalent O and H *Salmonella* sera revealed 11 different *Salmonella* serotypes (Table 2).

Table 2: Serotypes of *Salmonella* isolates, their prevalence and multidrug resistance

Flock No.	Isolates	%	Group	Antigenic structure		Resistance to antibiotics =				No. of	
				O	H	4	5	6	7	8	9
123	*S.* Angers	2.04	C3	8, 20	Z35: Z6	-	1	-			
20, 35, 67, 90, 101, 107, 144	*S.* Enteritidis	14.2	D1	1, 9, 12	g, m: -	-	-	-		7	
23, 57, 83, 105	*S.* Infantis	8.1	C1	6, 7, 14	r: 1,5	-	-	1	2	1	
3, 51, 69,73,85,100,118, 127, 146, 140	*S.* Kentucky	18.3	C3	8, 20	i: Z6	-	1	2	5	1	-
26, 93	*S.* Labadi	4.08	C3	8, 20	d: Z6	-	-	-			2
78	*S.* Larochelle	2.04	C1	6, 7	d: 1,6	1	-	-			
42, 64, 88, 110, 128	*S.* Molade	12.2	C2	8, 20	Z10: Z6	-	-	-		2	4
49, 72, 150	*S.* Papuana	6.1	C1	6, 7	r: e, n, Z15	-	-	1	2		
17, 114, 136	*S.* Takoradi	6.1	C2	8, 20	i: 1,5	-	-	-	-	2	1
53	*S.* Tsevie	2.04	B	4, 5	i: e, n, z15	-	-	1	-	-	
8, 12, 32, 59, 81, 97, 104, 119, 125, 131, 133, 149	*S.* Typhimurium	24.4	B	1, 4, 5, 12	i: 1,2	-	3	3	2	4	
					Total no (%)	1 (2)	5 (10)	8 (16)	11 (22)	17 (35)	7 (14)

Antimicrobial resistance

Salmonella isolates were resistant to ampicillin, gentamycin and cefotriaxone (100 %), followed by doxycycline (96 %), florfenicol (84 %), thiamphenicol (76 %), spectinomycin (73 %) and erythromycin (63 %). Sensitivity was highest to apramycin (82 %) and ciprofloxacin (65 %). Multidrug resistance was observed in all isolates at least to 4 antibiotics and approximately half (49 %) of the isolates were resistant to 8-9 drugs (Table 2).

Molecular detection of Salmonella virulence genes by PCR

The 4 virulence markers; *inv*A, *hil*A, *avr*A and *pef*A revealed bands of 284, 150, 422 and 700 bp., respectively (Figures 2and 3) and detected in all 20 MDR *Salmonella* serotypes (100%), except *avr*A (95 %).

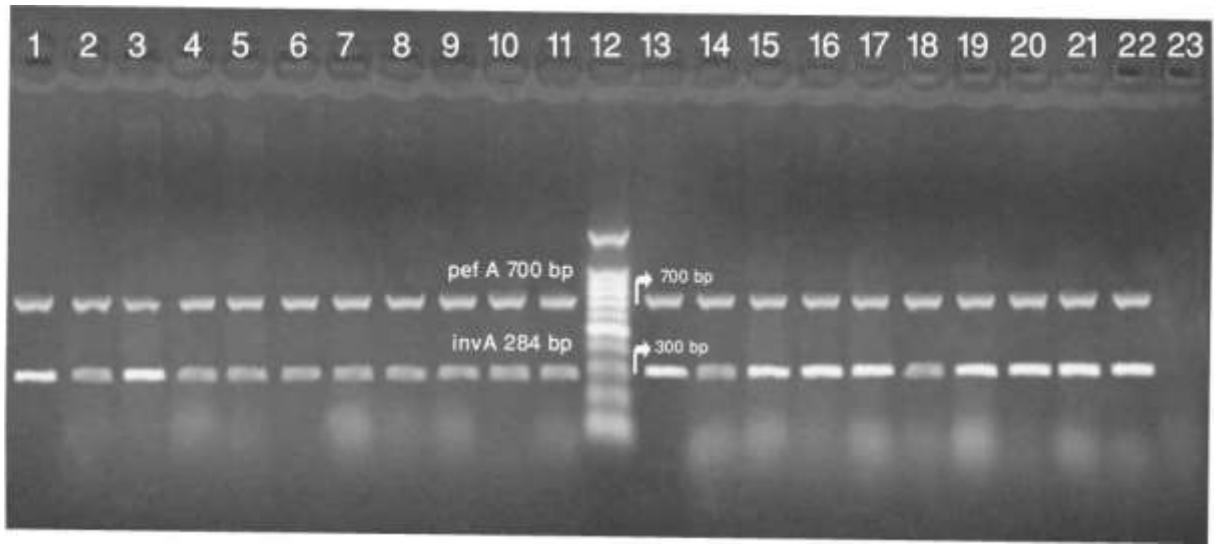

Figure 2: Agrose gel for amplicons generated in duplex PCR for detection of *pef*A *and inv*A genes of *Salmonella* spp. lanes 1-11, 13-21, samples positive for *pef*A (700 bp) and *inv*A gene (284 bp), lane 12, 1.5 kb DNA ladder, lane 22, positive control (*S.* Enteritidis a local lab strain), lane 23, negative control (*E. coli* DH5α).

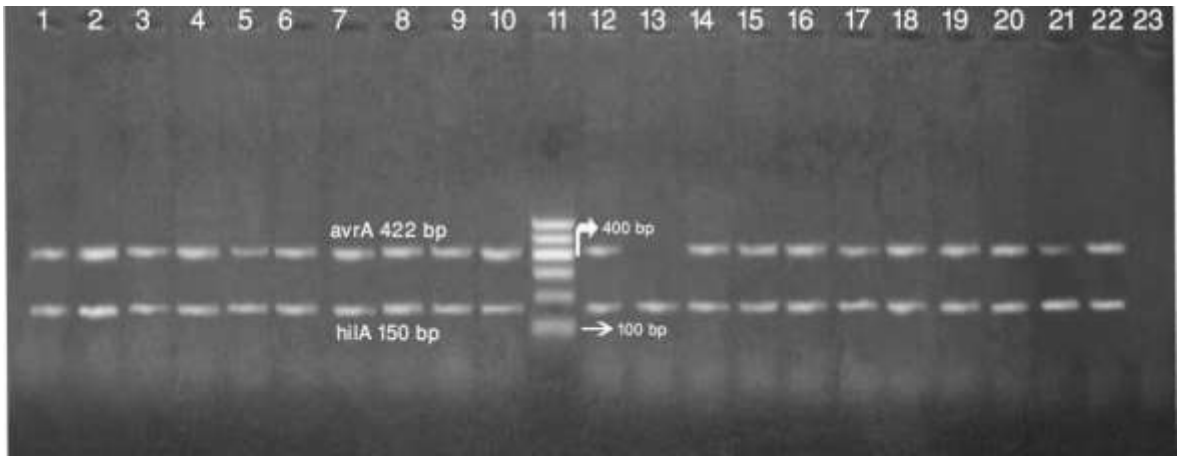

Figure 3: Agrose gel for amplicons generated in duplex PCR for detection of *avr*A and *hil*A of *Salmonella* spp. lanes 1-10, 12-21, samples positive for *avr*A gene (422 bp) and *hil*A (150 bp) except sample in lane 13 that was negative for *avr*A gene, lane 11, 0.6 kb DNA ladder, lane 22, positive control, (*S.* Enteritidis a local lab strain), lane 23, negative control (*E. coli* DH5α).

Discussion

In the present study, 150 broiler chicken flocks revealed high isolation rate (32.6 %) of *Salmonella*, that was higher than those recorded by Ammar *et al.* [28] (17 %) and El-Zeedy *et al.* [29] (4.1 %) respectively. This may be explained by low biosecurity measures inside farms and possibility of disease transmission via different reservoirs and workers in farms [5].

A higher percentage (41 %) of *Salmonella* isolation was recorded at 1-10 days old, while lower percentage (16 %) was recorded at 11-21 days old. This could be attributed to acquisition of protective microflora in young birds that either competes with *Salmonella* for intestinal receptors or produces antagonistic factors [٣٠].

Forty nine *Salmonella* isolates were serologically identified into 11 serotypes by slide agglutination test using specific

monovalent and polyvalent O and H antisera. *S.* Typhimurium was the most prevalent one (24.49 %), followed by *S.* Kentucky (18.36 %), *S.* Enteritidis (14.28 %), *S.* Molade (12.24 %), *S.* Infantis (8.16 %), *S.* Takoradi, *S.* Papuana (6.12 %, for each), *S.* Labadi (4 %), while *S.* Tsevie, *S.* Larochelle and *S.* Angers were the least ones (2 % , for each).

Our results agreed with Abd El- Tawab *et al.* [31], who reported low percentage of *S.* Papuana, *S.* Takoradi, *S.* Labadi, and *S.* Angers, each as 2.3 %, while disagreed with Moussa *et al.* [32] who reported that *S.* Enteritidis was the most predominated serotype in Saudi Arabia (55.6 %), followed by *S.* Typhimurium (22.2 %). Our results were lower than those obtained by Abd- El-Ghany *et al.* [33] who reported that *S.* Enteritidis was the most prevalent one (37.25 %), followed by *S.* Typhimurium (29.41 %), *S.* Infantis (19.6 %), while *S.* Tsevie (3.92 %) and *S.* Kentucky (7.84 %) were the least ones. The high prevalence of multiple paratyphoid *Salmonella* serotypes emphasizes the need to develop appropriate preventive and control measures to minimize their presence in chicken flocks and its potential transmission to humans in Egypt.

MDR *Salmonella* isolates were detected in our study to at least 4 antibiotics while 49 % of isolates where resistant to 8-9 antibiotics and also by Shah and Korgo, [34] to 11 antibiotics and Zahraei *et al.* [35] to more than 8 antibiotics. A lower resistance was recorded by Kusumaningrum *et al.* [36] and Taddele *et al.* [37] to at least 2 antibiotics. All *Salmonella* isolates were resistant to ampicillin, gentamycin and cefotrixone (100 %). This is not surprising because these antibiotics are commonly used in humans and poultry. Another factor is the antibiotic misuse by poultry producers including subtherapeutic doses, unauthorized use without prescription and usage as preventive tool in poultry, leading to development of enteric flora resistance, from which pathogenic *Salmonella* may acquire and transfer resistance to human's strains through food chain leading to emergence of multidrug resistance *Salmonella* [38].

Cefotrixone and ampicillin resistances were higher than those recorded by Learn *et al.* [39] in Malaysia (78%) and Parvej *et al.* [40] in Bangladesh (87%). On contrary, Habrun *et al.* [41] found that 99.3 % of *Salmonella* isolates were sensitive to gentamycin.

A higher sensitivity rate of *Salmonella* to ciprofloxacin (65 %) was recorded in this study that was lower than Munawwar *et al.* [42] (87.88 %). Molbak *et al.* [43] has reported an increased quinolone resistance in *Salmonella*. Controversy, no *Salmonella* resistance was observed from broiler carcasses in Barazil [44]. Florfenicol and doxycycline resistance was recorded in this study as 84 % and 96 %, respectively that partially matched with Ghoddusi *et al.* [45] as 72 % and 100 %, respectively. Molecular characterization of pathogenic organism is of most importance to diagnose, characterize and understand the pathogenesis of the disease in order to facilitate its control. Therefore, the present study aimed to investigate the PCR protocol as a rapid method for the identification of paratyphoid *Salmonella*. Paratyphoid *Salmonella* has several virulence genes include plasmid encoded fimbriae (*pef*A), hyper invasive locus (*hil*A), involved in adhesion and invasion [46]. In addition to, *avr*A an effector protein of type III secretion system (TTSS) complex, limiting the host's inflammatory responses via induction of macrophage apoptosis [47] and *inv*A, a conserved gene in all *Salmonella* spp. responsible for invasion [48] and located on SPI-I with *hil*A gene in all MDR *Salmonella*, indicating that *Salmonella* may exhibit several determinants to induce pathogenicity [49].

Our PCR result detected *inv*A and *hil*A genes in all *Salmonella* serovars (100%) that matched with Lampel *et al.* [50], Shabnam and Kwai, [51], respectively, and Malorny *et al.* [52] for both genes, indicating the specificity of *inv*A gene as *Salmonella* specific and virulence marker. The obtained results were matched with the traditional microbiological identification methods, confirming that *inv*A and *hil*A PCR is rapid, sensitive and specific method for diagnosis of *Salmonella* infections in poultry.

The *avrA* gene was detected in 95 % of *Salmonella* serotypes by PCR, which was similar to Hopkins and Threlfall [53]. On the

other hand, Ben-Barak *et al.* [47] considered the high frequency of *avr*A gene was only in the most important *Salmonella* serovars. The obtained results found *Pef*A gene in all *Salmonella* isolates (100 %), which was higher than Murugkar *et al.* [46](89 %).

Conclusion

The prevalence of paratyphoid *Salmonella* among the examined flocks at Sharkia Governorate was (32.6%) with the highest rate among 1-10 days old chicks. Serotyping revealed eleven different *Salmonella* serotypes with *S.* Typhimurium as the most prevalent serotypes. MDR was recorded in 49 % of the examined *Salmonella* isolates. The prevalence of *inv*A, *hil*A and *pef*A genes were 100% and *avr*A (95%), indicating their importance as genus specific primers and thus can be used for rapid diagnosis of *Salmonella* infection in poultry.

Conflict of interest

The authors declared that they have no conflict of interest.

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الملخص العربى

توصيف سالمونيلا باراتيفويد المعزولة من دجاج التسمين بمحافظة الشرقية بمصر

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برزت سالمونيلا الباراتيفود كمشكلة عالمية للإنسان والدواجن. ولذلك قمنا في هذه الدراسة بالتحري عن تواجد والخصائص المصلية والجزيئية و مقاومة المضادات الحيوية للسالمونيلا باراتيفويد المعزولة من قطعان الدجاج في محافظة الشرقية خلال الفاشيات ٢٠١٥-٢٠١٦م. كان معدل الإصابة بالسالمونيلا باراتيفويد بين ١٥٠ قطيعًا مشتبهًا به ٣٢.٦ ٪ و في الأعمار الصغيرة كانت نسبة العزل أعلى بنسبة ٨٤ % (١-١٠ أيام) وأدنى معدل (١٦ ٪) في الأعمار الأكبر سنا (١١-٢١ يوما). كان أعلى نسبة عزل من الكبد (٣٠.٦٦ ٪)، تليها الطحال (٢٥.٣٣ ٪)، الأعورين (٢٠ ٪) وكيس المح (١٥.٧ ٪). كشفت الأنماط المصلية لـ ٤٩ عزلة من السالمونيلا عن ١١ مجموعة مصلية مختلفة، وكان سالمونيلا تيفيميوريم هو الأكثر انتشارًا (٢٤.٤٩٪)، يليه سالمونيلا كنتاكى (١٨.٣٦ ٪) وسالمونيلا انترتيديز (١٤.٢٨ ٪) وكانت المضادات الحيوية الأكثر حساسية هي الأبراميسين (٨٢ ٪) والسيبروفلوكساسين (٦٥ ٪). كانت المقاومة متعددة العقاقير للأمبيسلين والجنتاميسين والسيفوتزياكسون في جميع عزلات السالمونيلا. جميع معزولات السلمونيلا متعددة مقاومة العقاقير تحتوى على *inv*A و *hil*A و *pef*A بنسبة ١٠٠ ٪ وكذلك *avr*A بنسبة ٩٥ ٪ بواسطة تفاعل البلمرة المتسلسل، مما يؤكد أن هذه الجينات من جينات الضراوة المحدده والمهمة للتشخيص السريع لعدوى السالمونيلا.