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Application of PCR-based serogrouping of *Salmonella* enterica serotypes isolated from the poultry in Egypt

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Abstract

Salmonella spp. remains a significant concern in poultry flocks due to its potential impact on both animal health and public health. In this study, a comprehensive approach to isolate, serotype, and identify Salmonella strains from poultry flocks was employed. A total of 350 samples were collected, including internal organs liver, lung and cecum, from poultry flocks. Salmonella isolates were isolated and characterized according to established protocols. Conventional serotyping was performed based on the Kauffman–White scheme using Salmonella antisera, and the results were classified into various serogroups. To enhance the efficiency of serotyping, a multiplex PCR assay targeting both O and H antigens was developed. DNA extraction from collected samples followed the boiling method the multiplex PCR assay used specific primers for various serogroups. Out of the 350 samples tested, approximately 14.2% were positive for Salmonella. A total of 50 Salmonella isolates were serotyped, and the strains were categorized into several serogroups including B, D1, E1, C1, and C3. The multiplex PCR assay successfully identified O and H antigens, revealing a prevalence of serogroup B (46%) and D1 (32%) strains. Interestingly, none of the Salmonella strains exhibited the Vi antigen. This investigation showcased the precision and effectiveness of the multiplex PCR technique in identifying *Salmonella* strains. Notably, the multiplex PCR yielded outcomes surpassing those of traditional serotyping using the Kaufmann White scheme, all within a notably reduced time frame. In conclusion, the study highlights and presents a rapid and reliable method for serotyping and identifying *Salmonella* strains using multiplex PCR. This approach has the potential to expedite the control and prevention of Salmonella transmission, contributing to improved poultry health and reduced public health risks associated with Salmonella infections. The rapidity and accuracy of the multiplex PCR method make it particularly suitable for international efforts to monitor and manage Salmonella outbreaks effectively.

Keywords: Serotyping; *Salmonella,* PCR, flagellar antigens

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Introduction

There are many bacterial infections affect poultry flocks butsalmonellosis considered the most common bacterial disease, furthermore, it can affect public health through poultry byproducts, consequently it has multiple negative effects on the national economy in all sectors for poultry producers, and for human illness which impair productivity so all these for-mentioned reasons act as a financial burden on individuals income and the national budget (Gast and Porter,2020)

Salmonella represents one of the most common zoonotic causative agents, it takes the second place after *Campylobacter* which are responsible for gastro intestinal disturbance in human, the greatest level of *Salmonella* in food has been recorded among poultry meat especially in under cooked food, so it is mandatory continual tracing of *Salmonella* infection in poultry farms, during slaughtering, processing, preparation and catering(EFSA, 2019), also Center for diseases control and prevention (CDC) reported a human salmonellosis outbreak as a result of contact with infected backyard poultry (CDC, 2017)

Salmonella belongs to family *Enterobacteriaceae* and divided into 2 species, *S. enterica* and *S. bongori*, , *S. enterica* subdivided into 6 subspecies, *S. enterica* subspecies enterica contains more than 2500 serovars which are mostly responsible for illness in animals (Grimont and Weill, 2007; Gast and Porter, 2020).*Salmonella Enteritidis* & *Salmonella Typhimurium* are the most commonly distributed serovarsin poultry all over the world (Gand*et al.*, 2020;Gast and Porter, 2020), moreover a lot of countries reported these serovars from human cases as in USA.(Bugarel*et al.*, 2017; Gast and Porter, 2020), however Pullorum disease is caused by *Salmonella pullorum* and *Salmonella gallinarum* which are strictly host adapted to poultry so can produce severe systemic infection, severe septicemic pictures and extreme deaths.(Gand*et al.*, 2020;Gast and Porter, 2020).

Demographic rate in Egypt is high, furthermore, high prices of red meat in the Egyptian market lead to increase consumption of poultry and poultry byproducts so we resort to import to meet consumer’s needs. Imported one day old poultry participate in the building unit in grandparents and breeders flocks in the poultry sector, it is well known that international trade in poultry act as transporter for salmonellosis to human (FAO, 2002),also infection with Salmonella lead to drastic economic losses, consequently they may infect human, the imported one day old chicks are kept in the quarantine for about 7-10 days until the results of isolation and serotyping of received samples to RLQP to be released, and upon the identified serotype the decision is taken. Therefore rapid identification of serotyping is important and the traditional method for*Salmonella* serotyping is based on that *Salmonella* has three antigens: somatic (O), flagellar (H), and virulence (Vi) (Grimont and Weill,2007), but *Salmonella* serovars may show cross-reactivity between them which may interfere with conventional serotyping (OIE 2022),also molecular typing by PCR has many advantages than serological typing as it is faster, more accurate and help in serotyping of un-typed isolates such as rough strains or lack a flagellar antigen also, in such cases of mono and biphasic strains, Vi antigen may mask O somatic antigen, on the other hand, conventional serotyping needs an expensive antisera, well trained experts in addition to probability of cross

reactions between serotypes (Lu¨deritz*et al.*,1996; Lim and Thong, 2009; Munnoz*et al.*, 2010;Prendergast *et al.*, 2012) .

The aim of this study is to apply multiplex PCR which allow rapid identification of the most common serogroups of *Salmonella* that are frequently reported from poultry farms and help in the epidemiological mapping and surveillance projects for the salmonellosis , additionally determine the salmonellae which are frequently found along with the whole chain of food starting from farm to fork, besides also to confirm the most significant *Salmonella* serovars by aid of multiplex PCR detect groups A, B, C1, D, E and Vi positive strains and PCR for flagellar antigens “a�">, “b” and “d”. Beside that the primary aim of this study is to introduce and validate an innovative approach for the rapid and accurate identification of Salmonella strains prevalent in poultry flocks in Egypt

Materials and Methods

Sampling

Three hundred and fifty (350) samples from poultry flocks, by collecting the internal organs (liver, lung) and cecum. Study was done on different ages including apparently healthy and diseased chickens, different criteria such as congestion, necrosis in the liver, bronzy liver, congestion in the heart, omphalitis, typhlitis, and enlarged ceca.

Isolation and characterization of salmonellae

Salmonella spp. were isolated and identified according to (ISO 6579-1:2020).

Conventional Serotyping of *Salmonella* isolates

Serotyping was done according to ISO 6579-3: 2014 and classified according to the Kauffman–White scheme (Grimontand Weill, 2007) by Sifin *Salmonella* antisera (Sifin).

Multiplex PCR for O and H antigens

The collected samples have been extracted following the instructions of the boiling method (Lim and Thong, 2009). The identification of the O and H antigens has been done using multiplex conventional PCR using the primers in table (1), that have been mixed with Thermo Reddy Mix™ PCR Master Mix kit (thermo-cat no. AB-0575/LD), as mentioned in its work instruction. The prepared PCR reactions have been incubated in a thermocycler of applied biosystem as follow: Initial Denaturation 95°C 2 min, 40 cycles of Denaturation at 95°C for 40 sec , annealing 55 C for 40 sec , and extension at 72°C for 1 min., then final Extension at72°C for 10 min. the amplified products have been electrophorized in agarose gel electrophoresis system with agarose gel 1.5% , then got the results by gel documentation system as bands of different molecular sizes for typing antigens, as illustrated in (Lim and Thong, 2009).

Results

Prevalence of Salmonella

About 50 Salmonella isolates were recovered from 350 poultry flocks 14.2%

Conventional serotyping

Fifty (50) *Salmonella* isolates were serotyped. The strains belong to serogroup B (*Salmonella* Typhimurium =16, *Salmonella* Stanley=1, *Salmonella* Indiana=1, *Salmonella* Ayinde=2, *Salmonella* Yaounde=1, *Salmonella* Ljublijana =2), also other strains belong to serogroup D1 (*Salmonella* Enteritidis=16), strains belong to serogroup E1(*Salmonella* London=1, *Salmonella* Lamber hurst =3) , strains belong to serogroup C1 (*Salmonella* Eingedi=1), strains belong to serogroup C2 (*Salmonella* Newport=1, *Salmonella*

Cremieu=1), strains belong to serogroup C3 (*Salmonella* Kentucky=4) .

Identification of O grouping in *Salmonella* by multiplex PCR& and detection of Vi antigen

All the50 target *Salmonella* strains gave 100% matching to the original identification, as they showed positive amplifications for all the used primers according top articular serogroups; groups A, B, C1, D & E, as follow: B (n = 23, 46%), D1 (n = 16, 32%), E (n = 4, 13.2 %),C1 (n = 1, 8 %) and none of the *Salmonella* strains were positive for Vi antigen.

Identification of H typing in *Salmonella* by multiplex PCRs for flagellar antigens.

When this PCR was applied on the 50 target *Salmonella* strains, only (n = 3, 6%) produce the expected bands size of flagellar antigens (Hd) while (H: a, b) were not amplified.

Table (1): The primers of the O and H antigen typing for multiplex PCR assay (Lim and Thong, 2009).

Target	ID	Primer Sequence	Amplicon size (bp)
B group	F-rfbJ	CCAGCACCAGTTCCAACTTGATAC	662
	R-rfbJ	GGCTTCCGGCTTTATTGGTAAGCA	
D group	F-tyv	GAGGAAGGGAAATGAAGCTTTT	614
	R-tyv	TAGCAAACTGTCTCCCACCATAC	
Vi strains	F-vi	GTTATTCAGCATAAGGAG	439
	R-vi	CTTCCATACCACTTTCCG	
A & D group	F-prt	CTTGCTATGGAAGACATAACGAACC	256
	R-prt	CGTCTCCATCAAAAGCTCCATAGA	
C1 group	F-wzxC1	CAGTAGTCCGTAAAATACAGGGTGG	483
	R-wzxC1	GGGGCTATAAATACTGTGTTAAATTCC	
E group	F-wzxE1	TAAAGTATATGGTGCTGATTTAACC	345
	R-wzxE1	GTTAAAATGACAGATTGAGCAGAG	
H:a, H:b and H:d	H-for	ACTCAGGCTTCCCGTAACGC	423
	Ha-rev	GAGGCCAGCACCATCAAGTGC	
	Hb-rev	GCTTCATACAGACCATCTTTAGTTG	551
	Hd-rev	GGCTAGTATTGTCCTTATCGG	
oriC	P1	TTATTAGGATCGCGCCAGGC	163
	P2	AAAGAATAACCGTTGTTCAC	

Table (2) . *Salmonella* Strains used for comparing PCRs results and conventional serotyping.

Serotype	Total no. of strains	Antigenic formula	Ogroup	H 1 antigen	No. of strains tested positive									
					rfbJ	tyv	vi	prt	wzxC1	wzxE1	H:a	H:b	H:d	P1-P2
ST	**16**	**1 , 4 ,[5],12 i 1,2**	**B (O4)**	**I**	**16**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	16
SE Enteritidis	**16**	**1,9,12 g,m**	**O:9 (D1)**	**g,m**	**-**	**16**	**-**	**16**	**-**	**-**	**-**	**-**	**-**	16

SK Kentucky *	**4**	**8 ,20 i z6**	**C3 (08)**	**I**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	4
S Newport *	**1**	**6,8,20 e,h 1,2 [z67], [z78]**	**C2 (6.8)**	**e,h**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	1
S Stanley	**1**	**1,4,[5],12,[27] d 1,2**	**B (O4)**	**D**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**1**	1
S London	**1**	**3 , {10}{15} l,v 1,6**	**O:3,10 (E1)**	**l,v**	**-**	**-**	**-**	**-**	**-**	**1**	**-**	**-**	**-**	1
S Lamberhurst	**3**	**3,10 e,h e,n,z15**	**O:3,10 (E1)**	**e,h**	**-**	**-**	**-**	**-**	**-**	**3**	**-**	**-**	**-**	3
S Indiana	**1**	**1,4,12 z 1,7**	**B (O4)**	**Z**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	1
S Ljublijana	**2**	**4,12,27 k e,n,x**	**B (O4)**	**K**	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	2
S Cremieu *	**1**	**6,8 e,h 1,6**	**C2 (6.8)**	**e,h**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	1
S Ayinde	**2**	**1,4,12,27 d z6**	**B (O4)**	**D**	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**2**	2
S Yaounde	**1**	**1 ,4,12,27 z35 e,n,z15**	**B (O4)**	**z35**	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	1
S Eingedi	**1**	**6,7 f,g,t 1,2,7**	**O:7 (C1)**	**f,g,t**	**-**	**-**	**-**	**-**	**1**	**-**	**-**	**-**	**-**	1

* Non target *Salmonella* strains

Discussion

Salmonella is one of the major bacterial infections in the poultry sector, it causes dramatic losses in farms, as they are transmissible either horizontally or vertically, furthermore, it may infect human via food chain causing food poising (EFSA 2012;Gast and Porter, 2020).It is widely found that importation of chicks is universally found, Egypt represents one of the countries that deals in birds importation, which may allow transferee of several types of microorganism as Salmonellae from various regions all-over the world, (Osman *et al.*,2010).

Salmonellae were prevalent by 14.2% which is nearly similar to what is reported by (Ammar*etal.*,2019) from broilers in Egypt by 15·6%, also *Salmonella Enteritidis* and *Salmonella Typhimurium* are the predominant serovarsin our study which in accordance with (Ammar*et al.*,2019).

There are a lot of salmonella serotypes recovered in the recent years in Egypt and may pose problems and take a long time to be serotyped especially in huge poultry

sectors. *Salmonella Enteritidis* and *Salmonella Typhimurium* are the most predominant serovars from imported birds which is in accordance with (Osman *et al.,*2010; Ezzeldeen *et al.,*2013; Osman *et al.*, 2014, EFSA, 2019)

It has been previously mentioned that *Salmonella* has more than 2500 serovars, so serological typing is important as they are different in their pathogenicity, virulence and host adaptation, the serological typing is based on detection of O somatic antigen (surface polysaccharide) and H flagellar antigen to recognize the *Salmonella* serotype (Grimont and Weill, 2007; Liu *et al.*, 2014).*Salmonella* serotyping is a main tool in epidemiological studying, surveillance programmes and outbreaks (Shu-Kee*et al.*,2015), conventional serotyping has different weak points as cross reactivity and judging agglutination results by human naked eye; all these obstacles push the development of new research projects to find an alternative tool for conventional serotyping as *Salmonella* identification from pre enriched culture by PCR (Osman *et al.*, 2010)

So, in our study we use the previously modified protocol for *Salmonella* serogrouping by PCR which is designed by (Lim and Thong, 2009) to identify *Salmonella* isolates into serogroups and enable rapid identification of *Salmonella* serogroups from poultry samples to judge on them. Multiplex PCR sero-grouping approach was used mainly targeting the identification of strains which are of public health significance such as SE and ST sothe rapid accurate serotyping is mandatory to save especially in the critical field problems.

Multiplex PCR used for *Salmonella* sero-grouping as examined previously by (Herrera-Leon *et al.*, 2007; Levy *et al.*, 2008; and Thong,2010andSantos *et al.*,2020), consequently, the current study involved primers as designated by (Lim and Thong, 2009), we achieved 100% agreement between the serotyping and PCR sero-grouping results(Lim and Thong, 2009andNori and Thong,2010), also we showed 100% specificity of the primers as reported by (Nori and Thong,2010).

In the present study, all conventionally serotyped Salmonellae which has a specific O serogroup primer in the existing primer set were positive which are in the same context with that reported by (Nori and Thong,2010 and Dalyan, *et al.*,2013). *Salmonella* Typhiand Paratyphi strains aren't detected in poultry field which carry Ha, Hb and Viflagellar antigens were not existing in this work; so, those genes couldn't be detected. P1-P2 primers are considered as internal control, they are distinct and exclusive for *Salmonella* detection and to assure validity of test, also they can differentiate *Salmonella* from other species, it has been detected in 100% of all tested *Salmonella* isolates, as reported by (Marlony*et al.*2003and Lim and Thong, 2009),

Conclusion

*Salmonella*s erogrouping by multiplex PCR is a valuable tool in *Salmonella* identification. This method is anappropriate way to be applied, as it takes only few hours in comparison to conventional serotyping method to promote control of Salmonella spread between different countries all over the world

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Conflict of interest

In accordance with the guidelines of transparency and integrity in research, all authors involved in this study declare that there are no actual or potential conflicts of interest that could influence, or be perceived to influence, the findings and outcomes presented in this research. The authors affirm that this study has been conducted with impartiality and objectivity, solely driven by the pursuit of scientific knowledge and advancement in the field.

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