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Original Research

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An Overview of the Current Situation of Salmonellosis in Pigeons, Household Chickens, and Commercial Broilers with a Special Reference to a Customized Vaccine Developing Trial

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Abstract

Salmonellosis caused by virulent multidrug-resistant strains is a great concern of the poultry industry; thus, the prevalence, resistance genes profile, and development of customized vaccines were evaluated in poultry species. Thus, 100 cloacal swabs from pigeons, household chickens, and commercial broilers were collected from Sharkia governorate, Egypt; 31% were positive for *Salmonella* serovars confirmed by the *inv*A gene. All isolates were examined for antimicrobial susceptibility for detecting antibiotic resistance genes on plasmid by PCR, which demonstrated *qep*A, *mcr*1, *tet*A(A), and *flo*R genes (100%) for each, while *sul*I and *aad*A1 genes were 93.8% and 87.5%, respectively. A phylogenetic tree was constructed to illustrate the evolutionary relationships between different strains in Egypt. Additionally, a bivalent killed *Salmonella* vaccine comprising two isolated serovars of *Salmonella enterica* (*S.* Typhimurium and *S.* Enteritidis) was developed and evaluated for its efficacy in specific pathogen-free (SPF) chickens. Vaccinated chickens developed a high humeral response measured by ELISA. Moreover, the protection rate of the developed vaccine in the challenge test was 85%, with less fecal shedding. The findings suggest that customized vaccines prepared from local circulating strains can be used as an alternative effective control tool against salmonellosis.

KEYWORDS
Poultry, *Salmonella* spp., MDR, Resistance genes, Sequence, Customized vaccine.

INTRODUCTION

Poultry industry is one of the fastest growing agribusinesses in Egypt, which is the most important and patronized in terms of number and quality development. Poultry are exposed to various diseases, such as salmonellosis, the most common foodborne zoonosis, constituting a worldwide primary public health concern. Salmonellosis in humans is related to contaminated poultry, highlighting the possibility of the organism transmission through the food chain (Djeffal *et al*., 2018). For several years, some serovars may be predominant in country, which may disappear and be substituted by other serovars.

The horizontal transfer of *Salmonella*-contaminated rodents, fomites, infected equipment, litter, excrement, feed, and diseased chicks affected poultry (Tabo *et al*., 2013). During the rearing period, *Salmonella* spp. can transmit to poultry through wild birds, domestic animals, and personnel. Furthermore, after laying, contaminated eggshells act as vertical transmission of parent flocks through transovarian transmission. Feed is the most common factors for the lateral spread of the organisms, which the poultry environment is contaminated by localized organisms of the carriers gut that shed *Salmonella* intermittently (Pande *et al*., 2016).

Salmonella spp. infections are becoming more common, and the emergence of antimicrobial resistance (MDR) from both human and poultry sources globally raises concerns about an international public health issue (Lapierre *et al*., 2020). The majority of virulence-associated plasmids in *Salmonella* spp. carry transfer functions, making plasmids one of the mobile genetic elements that contain resistance genes of single or multiple antimicrobial agents and act as a horizontal transfer of genetic material among bacteria and between related or different species (Ammar *et al*., 2015).

Monitoring, biosecurity, management, feed additives, and vaccines were the most important for controlling salmonellosis in chickens. These methods have been effective in lowering the occurrence of *Salmonella* in farms, human food poisoning, and the use of antibiotics during production (Hofacre *et al*., 2021). Vaccines were an effective prevention control for *Salmonella* (Desin *et al*., 2013) and the induction of antigen-specific antibodies was mainly responsible for their early protective efficacy. The beneficial effects of inactivated *Salmonella* vaccine were confirmed by the homology of the O antigens which observed in the infecting strain (Deguchi *et al*., 2009). Chickens that get inactivated *Salmonella* vaccinations require protective immunity that prevents *Salmonella* from colonization in organs and lowers the amount of pathogen in feces (Crouch *et al*., 2020 a).

Sequencing technology revealed a wealth of information regarding the species, pathogenicity, serovar, virulence, antibiotic resistance, and bacteria subtype. Partial-genome sequencing (PGS) was the high-quality sequence data in laboratories public health because it detected the clinical strains of antimicrobial resistance and virulence genes. PGS could also alter the field of

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genomics (Oakeson *et al*., 2017). PGS can determine the route of disease transmission within a population and give information on the expected source. It is crucial to analyze the strains to detect genetic alteration in microorganisms during an outbreak (Gilchrist *et al*., 2015). Identification of outbreak clusters and effective inference of phylogenies from the sequencing data are two critical applications for PGS (Ahrenfeldt *et al*., 2017).

Consequently, this study aimed to isolation of some *Salmonella* spp. and evaluate customized vaccines against locally isolated *Salmonella* strains as antibiotic alternatives with regard to PGS and antimicrobial resistance genes on plasmid to control salmonellosis.

MATERIALS AND METHODS

Collection of samples

Cloacal swabs (100 swabs/facility) were collected from diseased flocks that suffered from salmonellosis symptoms as (inappetance, depression, ruffled feathers, closed eyes, loud chirping, white diarrhea, vent pasting, gasping, lameness) from [commercial broiler farm (n = 40), household chickens (n = 40), and pigeons (n = 20)]. Within 24 hours, the collected samples were tested at the Reference Laboratory of Veterinary Quality Control of Poultry Production (RLQP) Sharkia branch and stored at 4ºC to 8ºC.

Isolation and identification

Salmonella species were isolated in accordance with ISO/6579.1. 2017/Amd.1. 2020.

Serological identification

Salmonella serotype isolates were determined according to Patrick and Francois (2007).

Testing for antibiotic susceptibility

The antimicrobials were comprised of aminoglycosides (kanamycin (K), 10 µg; streptomycin (S) 10 µg), fourth-generation cephalosporins (cefepime, FEP 30 µg), second-generation fluoroquinolones (norfloxacin (NX) 10 µg), aminopenicillin (ampicillin (AMP), 10 µg), polymyxins (colistin (CT) 10 µg), chloramphenicol (florfenicol (FLO), 30 µg), tetracycline (tetracycline (T) 30 µg; doxycycline (DO) 30 µg), and sulfamethoxazole-trimethoprim (SXT, 25 µg), which were provided by Bioanalyse®, Turkey. The inhibitory zones were measured according to Clinical and Laboratory Standards Institute (CLSI 2020) guidelines.

Conventional PCR technique

Plasmid extraction

Luria-Bertani (LB) broth (4 ml) was added to a loopful of harvested bacteria at 37ºC overnight in a shaker incubator, which was centrifuged for 13000 rpm/10 min. According to the manufacturer's instructions for the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), the pelleted bacteria were suspended in 250 µl of buffer and 50 µl of elution buffer was added to the plasmid DNA.

Chromosomal extraction

200 µl sample suspension was treated at 56ºC / 10 min with 200 µl lysis solution and 10 µl proteinase K. The lysate was added to 200 µl ethanol (100%), which was rinsed and centrifuged, then according to manufacturer's instructions, 100 µl elution buffer was added to the nucleic acid.

Amplification and analysis

Oligonucleotide primers (Table 1) were provided by Metabion (Germany) which utilized in 25 µl reaction that contained 12.5 µl EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl primer of each, 5.5 µl water, and 5 µl DNA template in Applied Biosystems 2720 thermal cycler for reaction. For gel analysis, 1.5% agarose gel electrophoresis was prepared to separate PCR products (Applichem, Germany, GmbH), each product (20 µl) was placed into a gel slot. Fragment sizes were evaluated by a GeneRuler 100 bp ladder (Fermentas, Germany), the picture and data were examined using software of gel documentation system (Alpha Innotech, Biometra).

invA gene sequencing

Further, purified products were used for nucleotide sequencing through BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) and purified by Centrisep spin column, (Thermo Fisher,Waltham, MA, USA). Sequencing was performed by ABI PRISM® 3100 Genetic Analyzer (Life Tech-

Table 1. Primers sequences, target genes, and amplicon sizes.

Target gene	Function of target gene	Primers sequences	Amplified segment (bp)	Reference
*inv*A	*Salmonella* spp. Conserved virulence gene	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	Oliveira *et al*. (2003)
*flo*R	Florfenicol resistance gene	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494	Doublet *et al*. (2003)
*mcr*1	Mobile colistin resistance gene	CGGT CAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	308	Newton-Foot *et al*. (2017)
*qep*A	Quinolone resistance gene	CGTGTTGCTGGAGTTCTTC CTGCAGGTACTGCGTCATG	403	Cattoir *et al*. (2008)
*sul*I	Trimethoprim *sul*famethoxazole resistance gene	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	Ibekwe *et al*. (2011)
tetA(A)	Tetracycline resistance gene	CCTTATCATGCCAGTCTTGC ACTGCCGTTTTTTCGCC	576	Sabarinath *et al*. (2011)
*aad*A1	Aminoglycosides resistance gene	TGATTTGCTGGTTACGGTGAC CGCTATGTTCTCTTGCTTTTG	284	Clark *et al*. (1999)

nologies, USA). Further, the obtained nucleotide sequences were assembled and edited using Bio-edit programme version 7.2.5 (Hall *et al*., 2011). The NCBI website (http://www.ncbi.nlm.nih.gov/blast/) was used for a Blast search, the pairwise nucleotide sequences and percent identity were aligned by BioEdit version 7.0 (Hall, 2004) with other strains representing different clades as well as vaccine strains against obtained from the National Center for Biotechnology Information (NCBI). The Phylogenetic analyses were conducted out by MEGA 6 (Tamura *et al*., 2013), General Time Reversible (GTR) substitution were the best models with estimate of proportion of invariable sites (I) and Gamma distribution (G), a moderate strength neighbor-joining approach, and 1000 bootstrap repeats (Kumar *et al*., 2016).

Vaccine preparation according to Charles et al. (1994).

For pure colonies, *S.* Typhimurium and *S.* Enteritidis were cultured at 37°C/24 hours on SS agar, then on tryptone soya broth at 37°C/24 hours. Using total colony count technique, the bacterial solution of each strain was adjusted to 10^{10}CFU/ml, followed by centrifugation at 5000 rpm at 4°C/ 30 min. Each strain count was adjusted to be 10^{10}CFU/0.5 ml, making a separate final suspension from *S.* Typhimurium and *S.* Enteritidis. All bacterial suspensions were inactivated with agitation and formalin (0.3%). The bacterial suspensions were combined with Montanide ISA78 VG (SEPPIC®, France) in a ratio of 70 adjuvant: 30 antigens.

Experimental design

From Koom Osheem, Fayum, Egypt, 100 SPF one-day-old chicks were housed in batteries with a network floor. During the 10-week observation period, chicks were reared under optimal temperature, humidity, ventilation, and a 24-hour constant light schedule (Burnham *et al*., 2002; Evans *et al*., 2012), following the recommendations of NRC (1994). Ten chicks were used as a safety of the prepared vaccine, and 90 were divided equally into three groups as in the experimental design, as shown in Table 2.

Group A was vaccinated with the prepared bivalent vaccine, Group B was vaccinated with trivalent *Salmonella* vaccine in commercial product (positive control group), and Group C included control non vaccinated chicks.

Ethical approval

The experimental study was done according to an approved protocol by the Ethical Committee of the Animal Health Research Institute (AHRI), Ministry of Agriculture (ARC), Giza, Egypt.

Quality control of the prepared vaccine

Sterility test for oil adjuvant and bivalent vaccine (Bekele and Assefa, 2018)

Montanide ISA 78 VG was sterilized at 160°C / one hour. These experiments were conducted at 37°C/7 days using VF, Thioglycolate, Tryptic Soy Broth, Tryptose Agar, and Sabouraud Agar. After verifying the growth inactivation procedure, the bacterial biomass was properly emulsified using the adjuvant Montanide oil. As a final step, the inactivated culture was sterilized and rendered safe.

Purity test (OIE, 2012)

The prepared vaccine was tested for bacterial and fungal contamination.

Safety test (OIE, 2012)

Ten-one-day-old SPF chicks were given a double field dosage (1 ml) of the prepared vaccine for two consecutive weeks. The chicks were monitored daily for any appearance of local responses, clinical signs, or mortality.

Potency test

As shown in Table 2, three groups and subgroups were created from 90 one-day-old SPF chicks. Each group of 30 birds was subdivided into three subgroups (10 birds for each strain). At 14 days old, the first group was injected with 0.5 ml subcutaneous (S/C) by the locally prepared bivalent inactivated vaccine, and then a booster dose was given three weeks later. The second group was injected with the trivalent *Salmonella* vaccine in the market (positive control group). The third group was kept as negative unvaccinated blank control.

Then, two subgroups in each group were challenged individually with 1 ml containing 108 CFU/each strain of *S.* Typhimurium and *S.* Enteritidis by crop gavage three weeks after the booster dosage, while one subgroup kept unchallenged. Following the challenge, the inoculated chicks were observed for three successive weeks. Protection was determined by the severity of the clinical symptoms, mortality, and shedding of the challenge organisms from cloacal samples. ELISA assays were used to evaluate and measure humeral immune responses, so the blood samples (2 ml/bird) from the wing vein were collected before and after each immunization (once per week).

Table 2. Experimental design

Chicken groups	No. of Chicks	Subgroup	1st vaccination (age/weeks)	2nd vaccination (age/weeks)	Challenge (3 weeks after 2nd vaccination)
Group A	10	*S.* Typhimurium	2	5	*S.* Typhimurium
	10	*S.* Enteritidis	2	5	*S.* Enteritidis
	10	unchallenged group	2	5	Unchallenged group
Group B	10	*S.* Typhimurium	2	5	*S.* Typhimurium
	10	*S.* Enteritidis	2	5	*S.* Enteritidis
	10	unchallenged group	2	5	Unchallenged group
Group C	10	*S.* Typhimurium	-	-	*S.* Typhimurium
	10	*S.* Enteritidis	-	-	*S.* Enteritidis
	10	Unchallenged group	-	-	Unchallenged group

RESULTS

Prevalence of Salmonella serovars

Thirty-one/over a hundred flocks (31%) were positive for *Salmonella* spp. [household chickens, 35% (14/40); commercial broilers, 25% (10/40); pigeons, 35% (7/20)]. Serotyping of the isolates indicated that *S.* Typhimurium was the prevalent serotype (25.8%; 8/31), followed by *S.* Enteritidis (22.6%; 7/31), *S.* Kentucky, *S.* Pullorum, and *S.* Gallinarum at 9.7% (3/31) prevalence rate, and *S.* Virchow, *S.* Infantis, and *S.* Inganda strain at 6.5% (2/31) prevalence rate and *S.* Agama at 3.2% (1/31) prevalence rate, as shown in Table 3. PCR was performed to emphasize the isolated strains by targeting the relevant species conserved (*inv*A) gene (100%).

Phenotypic of antimicrobial resistance profiles

High resistance rates were detected for norfloxacin, colistin, and florfenicol (100%, 93.5%, and 87.1%), respectively, followed by ampicillin, streptomycin, and doxycycline (83.9%), then kanamycin (80.6%), trimethoprim/sulphamethoxazole (71%), and tetracycline (67.7%), while the lower rate of resistance as detected against fourth-generation cephalosporins (cefepime) (25.8%), as shown in Figure 1.

All 31 *Salmonella* isolates (51%) were categorized as pan-drug-resistant (PDR), which is defined as nonsusceptibility to all categories of antimicrobial agents: seven from household chickens (50%), five from commercial broilers (50%), and four from pigeons (57%). All extensively drug-resistant (XDR) isolates were resistant to at least two antimicrobial categories, but not more than one agent (n = 9; 29%) was recovered from four household chickens (28%), two from commercial broilers (20%) and three pigeons (42.8%), as shown in Table 4. Thirty-one isolates (100%) were multidrug-resistant to ≥3 antimicrobials.

Resistance genotypic profile of Salmonella isolates

According to the phenotypic resistance profile, 16 multi-drug-resistant isolates were selected for studying the genotypic resistance profile by PCR on plasmids. *mcr*1 gene of colistin resistance was detected (100%) of tested *Salmonella* isolates. Florfenicol, tetracycline, and quinolone resistance were confirmed through the identification of *flo*R, *tet*A(A), and *qep*A resistance genes (100%) for each in tested isolates, followed by *sul*I (93.8%) resistant gene of trimethoprim-sulfamethoxazole and *aad*A1 resistant gene (87.5%) of aminoglycosides (Table 5).

Phylogenetic analysis

The *inv*A gene sequence from three selected household

Table 3. Prevalence of *Salmonella* serovars.

Serovars	Source of samples			Total (%)
	Household chicken (%)	Commercial broiler (%)	Pigeon (%)	
S. Typhimurium	4/40 (10%)	2/40 (5%)	2/20 (10%)	8/31(25.8%)
S. Enteritidis	3/40 (7.5%)	2/40 (5%)	2/20 (10%)	7/31(22.6%)
S. Kentucky	1/40 (2.5%)	2/40 (5%)	0/20 (0%)	3/31(9.7%)
S. Pullorum	1/40 (2.5%)	1/40(2.5%)	1/20 (5%)	3/31 (9.7%)
S. Gallinarum	2/40 (5%)	0/40 (0%)	1/20 (5%)	3/31 (9.7%)
S. Virchow	2/40 (5%)	0/40 (0%)	0/20 (0%)	2/31 (6.5%)
S. Infantis	1/40 (2.5%)	1/40 (2.5%)	0/20 (0%)	2/31 (6.5%)
S. Inganda	0/40 (0%)	1/40 (2.5%)	1/20 (5%)	2/31 (6.5%)
S. Agama	0/40 (0%)	1/40 (2.5%)	0/20 (0%)	1/31 (3.2%)
Total	14/40 (35%)	10/40 (25%)	7/20 (35%)	31/31(100%)

Table 4. Resistance patterns of *Salmonella* isolates against antimicrobial agents.

Resistant patterns	Isolates source			Total 31 (%)
	Household chicken (n.=14)	Commercial broiler (n.=10)	Pigeon (n.=7)	
Ten drugs	7	5	4	16 (51.6)
Nine drugs	2	1	2	5 (16.1)
Eight drugs	2	1	1	4 (12.9)
Seven drugs	2	2	0	4 (12.9)
Less than seven drugs	1	1	0	2 (6.5)

Table 5. Genotypic resistance profiles of *Salmonella* spp. isolates.

Source of isolates	Resistant genes (%)					
	*mcr*1	*qep*A	*flo*R	*sul*I	*tet*A(A)	*aad*A1
Household chicken (7)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	6 (85.7)
Commercial broiler (5)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	4 (80)
Pigeon (4)	4 (100)	4 (100)	4 (100)	3 (75)	4 (100)	4 (100)
Total (%)	16 (100)	16 (100)	16 (100)	15 (93.8)	16 (100)	14 (87.5)

chicken, commercial broiler and pigeon isolates were examined and submitted to the Gene Bank database which assigned the accession no. (OQ134094, OQ134095 and OQ134096), respectively (Table 6). The phylogenetic analysis of *Salmonella enterica* serovar Typhimurium strain was clustered into two major branches (group A, B). Group A divided into three subgroups, the strains under study clustered with (subgroup 3) which contained Egyptian, USA, Iran, Pakistan, china, and Indonesia strains, while the other two subgroups contained Egyptian strains only. Group B containing the Egyptian strains isolated recently at 2020

Table 6. Source modifier tabulates for *inv*A gene isolates and strains sequences retrieved from GenBank for alignment, phylogenetic analysis, and tree construction for different isolates.

Strain name	Accession no.	Identification of *S.* Typhimurium isolates and strain
SS8	LC318976	*Salmonella enterica* subsp. *enterica* serovar Ouakam *inv*A gene, partial sequence, broiler, strain: SS8, Iran, 2017
SS23	LC321985	*Salmonella enterica* subsp. *enterica* serovar Typhimurium *inv*A gene, partial sequence, broiler, strain: SS23, Iran,2017
Azh1	KC197068	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain Azh1 *inv*A gene, partial cds, wild bird, Egypt, 2013
KCID11	OL581591	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain KCID11 *Inv*A (*inv*A) gene, partial cds, chicken, Indonesia, 2022
MeganVac1	CP112994	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain MeganVac1 chromosome, chicken, USA, 2022
ZONb	MF953388	*Salmonella enterica* strain ZONb, (*inv*A) gene, partial cds, grilled chicken, Egypt, 2018
Vet CU11	MF566062	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain Vet CU11, (*inv*A) gene, partial cds, poultry, Egypt,2018
2AlxB	KJ718882	*Salmonella enterica* subsp. *enterica* strain 2AlxB, (*inv*A) gene, partial cds, broiler, Egypt,2014
MAW1	MH688053	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain MAW1, (*inv*A) gene, partial cds, poultry, pakistan, 2019
SS4	LC318972	*Salmonella enterica* subsp. *enterica* serovar Typhimurium *inv*A gene, partial sequence, strain: SS4, broiler, Iran, 2017
SS11	LC318979	*Salmonella enterica* subsp. *enterica* serovar Typhimurium *inv*A gene, partial sequence, strain: SS11, broiler, Iran, 2017
SS9	LC318977	*Salmonella enterica* subsp. *enterica* serovar Ouakam *inv*A gene, *inv*asion protein A, partial sequence, strain: SS9, broiler, Iran, 2017
1104-65	CP110201	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 1104-65 chromosome, complete genome, China,2022
CVM N17S380	CP082706	*Salmonella enterica* subsp. *enterica* serovar 4,[5],12: i:- strain CVM N17S380 chromosome, complete genome, turkey, USA, 2021
XSK	CP113538	*Salmonella enterica* strain XSK chromosome, complete genome, China, 2022
ZLQ	CP113535	*Salmonella enterica* strain ZLQ chromosome, complete genome, China, 2022
50BehB	KJ718887	*Salmonella enterica* subsp. *enterica* strain 50BehB (*inv*A) gene, partial cds, broiler, Egypt, 2014
Egy Vet CU31	KX524152	*Salmonella enterica* subsp. *enterica* serovar Heidelberg strain Egy Vet CU31 (*inv*A) gene, partial cds,broiler, Egypt, 2017
DK3	MF678538	*Salmonella enterica* subsp. *enterica* serovar Heidelberg strain DK3 (*inv*A) gene, partial cds, broiler, Egypt, 2018
Faw-SE-EG014	KP843557	*Salmonella enterica* subsp. *enterica* strain Faw-SE-EG014 (*inv*A) gene, partial cds, beef meat product, Egypt, 2015
70BehB	KJ718879	*Salmonella enterica* subsp. *enterica* strain 70BehB (*inv*A) gene, partial cds, broiler, Egypt, 2014
Vet CU9	MF566060	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain Vet CU9 (*inv*A) gene, partial cds, poultry, Egypt, 2018
Vet CU6	MF566058	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain Vet CU6 (*inv*A) gene, partial cds, poyltry, Egypt, 2018
Vet CU4	MF566056	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain Vet CU4 (*inv*A) gene, partial cds, poultry, Egypt, 2018
MAW2	MH688054	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain MAW2 (*inv*A) gene, partial cds, poultry, Pakistan, 2019
Sal2/GA2020	MT662114	*Salmonella enterica* subsp. *enterica* serovar Typhimurium isolate Sal2/GA2020 (*inv*A) gene, partial cds, meat product, Egypt, 2021
S2122	CP110657	*Salmonella enterica* subsp. *enterica* strain S2122 chromosome, complete genome, China,2022
Sal1/GA2020	MT662113	*Salmonella enterica* subsp. *enterica* serovar Typhimurium isolate Sal1/GA2020 (*inv*A) gene, partial cds, meat product, Egypt,2021
Sal2/GA2020	MT662114	*Salmonella enterica* subsp. *enterica* serovar Typhimurium isolate Sal2/GA2020 (*inv*A) gene, partial cds, meat product, Egypt, 2021

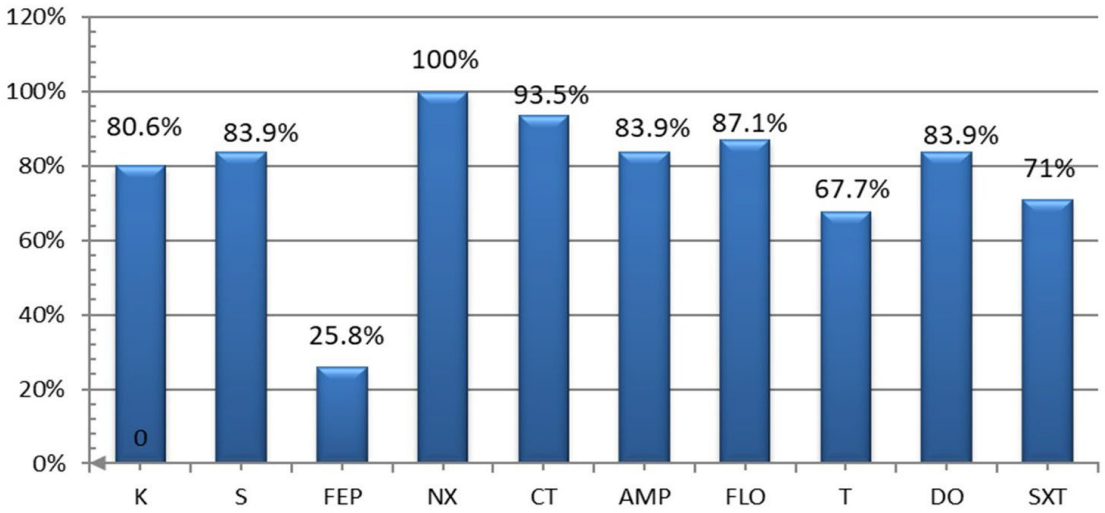

Fig.1. Percentages (%) of antimicrobial-resistant *Salmonella* isolates. K: kanamycin; S: streptomycin; FEP: cefepime; NX: norfloxacin; CT: colistin; AMP: ampicillin; FLO: florfenicol; T: tetracycline; DO: doxycycline; SXT: trimethoprim/sulphamethoxazole.

clustering with China strain S2122 (accession no. CP110657) and Pakistan strain MAW2(accession no. MH688054.1) (Figure 2). The homology percentage of deduced nucleoid identity between our examined Egyptian strains and other strains showed high homology ranged from (100% to 97.3%) with accession no.(OQ134094, OQ134095, OQ134096, SS8, OL581591, CP112994, KJ718882, KC197068),while other Egyptian strains isolated at 2018 with accession no. (MF953388 and MF566062) gave (80.9% to 79.4%) identity (Figure 3). These results confirmed the high conservation level of the *inv*A gene for them retrieved *S.* Typhimurium isolates.

Quality control results of prepared vaccines

The vaccination was determined to be pure, sterile, safe, and free from adverse side effects on chickens.

Humoral immune response in the vaccinated chickens by ELISA test

Compared to prevaccination levels, GMT against *S.* Enteritidis increased from 180 to 1025 in the third week following primary immunization and 2200 in the third week post boosting (Tables 7 and 8). The level of antibodies in chickens in Group A who received the bivalent vaccination against *S.* Typhimurium rose from 176 prevaccination level to 1010 during the third week after the primary immunization and 2170 in the third week post boosting. The antibody titer against *S.* Typhimurium in the serum of chickens in Group B who received the commercial trivalent *Salmonella* vaccine (positive control group) rose from 178 prevaccination

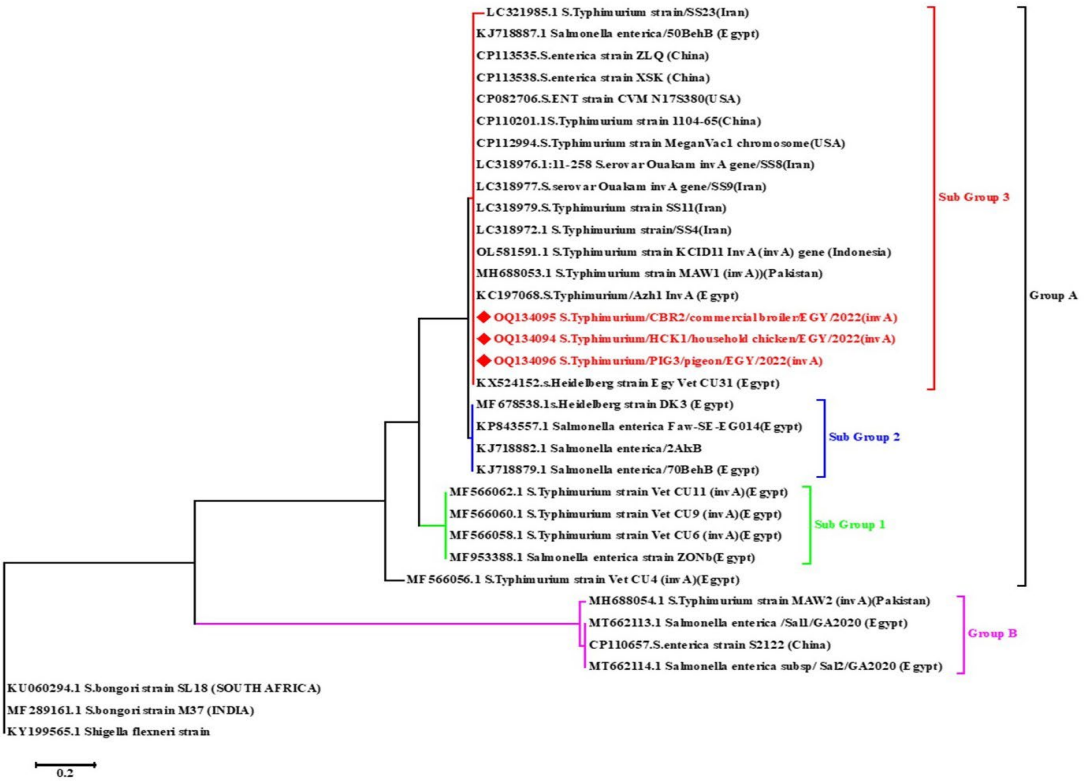

Fig.2. Phylogenetic analysis of invasion protein (*inv*A) gene, of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain. The partial nucleotide sequences from different strains of *Salmonella enterica* subsp were obtained via NCBI Resource. The phylogenetic analysis was performed using MEGA6. Construction with the maximum-likelihood (ML) analysis of evolutionary distances determined by the GTR + G + I model. NJ and ML bootstrap (1,000×) Consensus neighbor-joining trees were obtained from 1000 bootstrap replicates. The red- rhombos indicate strains under study.

Table 7. Antibody titer against *S.* Typhimurium in sera of chickens vaccinated with different inactivated *Salmonella* vaccines as measured by ELISA.

Chicken groups	Weeks post 1st vaccination			Weeks post boosting		
	1st week	2nd Week	3rd week	1st week	2nd week	3rd week
Group A	176	620	1010	1250	1420	2170
Group B	178	590	910	1150	1320	1990
Group C	175	179	182	180	184	187

Table 8. Antibody titer against *S.* Enteritidis in sera of chickens vaccinated with different inactivated *Salmonella* vaccines as measured by ELISA

Chicken groups	Weeks post 1st vaccination			Weeks post boosting		
	1st week	2nd Week	3rd week	1st week	2nd week	3rd week
Group A	180	630	1025	1270	1451	2200
Group B	179	535	890	1200	1281	1980
Group C	175	178	182	185	188	181

level to 910 in the third week after the primary immunization and 1990 at the third week following the boosting (Table 7). On the other hand, the antibody titer against *S.* Enteritidis increased from the prevaccination level of 179 to 890 and 1980, respectively, in the third weeks after the first immunization and boosting (Table 8), the ELISA antibody titer in serum from unvaccinated chicks did not exceed 188, as shown in (Tables 7 and 8).

Antibody titers against *S.* Typhimurium (Table 7) and *S.* Enteritidis (Table 8) from SPF chickens vaccinated twice (two and five weeks old) with trivalent inactivated *Salmonella* vaccine were measured. All chickens were challenged at eight weeks old. NV/Chall = nonvaccinated/challenged; V/Chall = vaccinated/challenged.

Protective efficacy of the prepared vaccine

The total number of dead and/or diseased chickens after challenge in vaccinated/challenged groups was 3 out of 20 compared to 15 out of 20 in nonvaccinated challenged groups. Consequently, (Table 9) shows that the produced vaccine had an 85% protection rate four weeks after post challenge.

Fecal shedding

Detection rates of *Salmonella* from chickens' cloacal swabs vaccinated with prepared bivalent vaccine and commercial trivalent *Salmonella* vaccine (positive control group) in 3rd, 5th, and 7th days post challenge were 10% (2/20), 5% (1/20), and 0% (0/20), respectively. On the other hand, detection rates of cloacal swabs of control nonvaccinated chickens and the reisolation rates were 75% (15/20), 50% (10/20), and 30% (6/20) in 3rd, 5th, and 7th days post challenge, respectively, as shown in (Table 10).

DISCUSSION

Salmonellosis is the most critical bacteria causing zoonotic diseases that can be transmitted by poultry interacting with humans (Hoelzer *et al*., 2011). In the surveillance of 100 cloacal swabs samples during research work, which provided proof of persistent intestinal colonization by *Salmonella*e in poultry, 31 isolates were positive. In pigeons, 35% were positive for *Salmonella* spp. as shown in Table 3; this result was greater than earlier studies from Egypt by Nasser *et al*. (2018) (4.3%), by Antonio *et al*. (2014) (0.9%), by Osman *et al*. (2013) (13.3%), and by Gonzalez-Acuna *et al*. (2007) (4%). The fact that all samples in this study came from sick birds, as opposed to other studies where this was not the case, may be responsible for the comparatively high percentage of pigeon salmonellosis. *Salmonella* spp. reservoirs in Egypt might be spread by pigeons, which could represent a major risk to both human and animal health, while Hosain *et al*. (2012) examined the incidence of salmonellosis in pigeons from the Mymensingh District and found that it was 35.71% overall, with a prevalence of 22.22%, 58.33%, and 27.50% in cloacal swabs, footpads, and feces, respectively.

In household chickens, 35% were positive for *Salmonella* spp., which was higher than that observed by Zhao *et al*. (2022) who

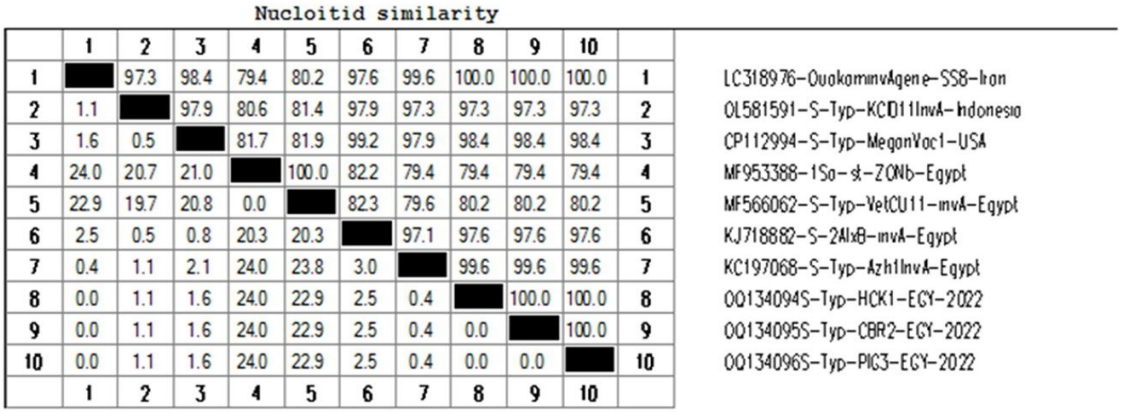

Fig. 3. Homology percentage of deduced nucleoid sequence of *inv*A Egyptian *Salmonella* Typhimurium strain in comparison with other published *Salmonella* Typhimurium strains.

Table 9. Protective efficacy of different inactivated *Salmonella* vaccines in SPF chickens challenged with virulent *S.* Typhimurium and *S.* Enteritidis strains.

Chicken groups	Total No. of birds	No. of dead and/ or diseased birds /weeks post challenge				Dead and/ or diseased/ Total	Survive/ Total	Mortality rate	Protection %*
		1st week	2nd week	3rd week	4th week				
Group A	20	2	1	0	0	20-Mar	17/20	15%	85%
Group B	20	2	2	0	0	20-Apr	16/20	20%	80%
Group C	20	8	3	2	2	15/20	20-May	75%	25%

Table 10. Results of fecal shedding of *Salmonella* from chicks after challenge.

Chicken groups	No. of birds positive for isolation/total No. of living birds		
	3rd day	5th day	7th day
Group A	2/20 (10%)	1/20 (5%)	0/20 (0%)
Group B	2/20 (10%)	1/20 (5%)	0/20 (0%)
Group C	15/20 (75%)	10/20 (50%)	6/20 (30%)

reported 12.7% of *Salmonella* spp. in free-range chickens, and lower than those reported by Alali *et al*. (2010) and Melendez *et al*. (2010), 50% and 5.6%, respectively. The recent emergence of live poultry-associated *Salmonella* (LPAS) outbreaks in humans has drawn attention to the public health risk of rearing chickens in backyards because they may harbor *Salmonella* pathogens. Backyard chickens with *Salmonella* may pose an immense risk to households and can act as environmental sources of *Salmonella* infection for nearby birds and animals. These studies shed light on the public health risk of pigeons and backyard chickens as reservoirs of zoonotic S. Enterica pathogens, which were detected in this study.

Salmonella contamination rates in chicken vary significantly between nations for various reasons, including geographic isolation, slaughter hygiene, sampling techniques, and biosecurity procedures (Chen *et al*., 2020). Additionally, *S.* Typhimurium and *S.* Enteritidis were the most prevalent serotypes discovered in Poland (Kaczorek *et al*., 2021) and China (Gong *et al*., 2014).

All isolates were confirmed by PCR detection of the conserved virulence gene (*inv*A gene) of *Salmonella*, a target gene suitable for *Salmonella* detection whose protein in the inner bacterial membrane is essential for invasion of the host epithelial cells in pathogenic *Salmonella* species. According to the latest findings, 100% of the investigated isolates tested positive for the *inv*A gene (Eid and El Oksh, 2019) discovered the *inv*A gene in all of the *Salmonella* samples (100%) examined.

Multidrug resistance poses a global threat to public health and the poultry industry, so eliminating antibiotics and finding alternatives is vital to solving this problem. The isolated *Salmonella* serovars in this study were tested against common antibiotics used in Egyptian poultry farms to establish their antibacterial susceptibility pattern. 100% of isolates indicated resistance to at least five drugs from different groups. This is hardly shocking as both people and animals frequently use these antibacterials. Poultry breeders' misuse of antibacterials involving subtherapeutic dosages and use as a preventive measure in poultry resulted in developing resistance intestinal flora, from which harmful *Salmonella* might pick up and spread resistance (Cox *et al*., 2003). The current findings closely agree with those of Abdeen *et al*. (2018), who found that all tested *Salmonella* isolates were MDR to five different types of antibiotics, and Elkenany *et al*. (2019), who found that 76.7% of isolates were MDR against three or more antibiotics. On disc diffusion, Sharma *et al*. (2019) showed that 100% of these isolates were MDR and 92.86% of the isolates were resistant to five or more antibacterial drug classes.

Due to the significant occurrence of MDR in Gram-negative bacteria, the use of polymyxins, especially polymyxin E (colistin), in the treatment of Gram-negative infections has expanded in many countries. Surprisingly, a considerable proportion of colistin resistance (93.5%) was discovered in the current investigation among *Salmonella* serovars. Uddin *et al*. (2021) obtained similar findings (92.68%), which were greater than those reported by Mir *et al*. (2015) and Waghamare *et al*. (2018) (46.87% and 4.76%, respectively).

In the current investigation as shown in Figure 1, all *Salmonella* serovars were resistant to ampicillin (83.9%), which was lower than that reported by El-Mohsen and El-Sherry (2022), who detected 100% in Egypt. The current study revealed that 100% of *Salmonella* serovars were resistant to norfloxacin. Our findings were different from those of Helal *et al*. (2019), who detected *Salmonella* serovars susceptible to doxycycline, chloramphenicol, amoxicillin, ampicillin, gentamycin, and trimethoprim-sulphamethoxazole. *Salmonella* serovars were shown to be extremely sensitive to ciprofloxacin, cefotaxime, and chloramphenicol, according to Elkenany *et al*. (2019). According to Ezzat *et al*. (2019), norfloxacin (100%) was the most efficient chemotherapeutic drug against *Salmonella* infection. In our study, 25.8% of *Salmonella* was found resistant to cefepime, a fourth-generation cephalosporin antibiotic, which is higher than the 13.3% recently detected by Mir *et al*. (2015) and Elkenany *et al*. (2019).

All *Salmonella* isolates used in this investigation (recovered from household chicken, commercial broilers, and pigeons) were MDR *Salmonella*, and 29% of the isolates were XDR *Salmonella*. The findings revealed that all *Salmonella* isolates (100%) from pigeons were MDR and 42.8% exhibited *Salmonella* as shown in Table 4 , which was more than that obtained by Abdein (2021), who reported that 16.6% of the recovered isolates showed extensive drug resistance (XDR) to several antibiotics and 42.1% of the recovered isolates showed multidrug resistance (MDR). According to the findings, 2/10 (20%) *Salmonella* isolates were XDR *Salmonella* from commercial broilers, which is in line with the findings of Asif *et al*. (2017). *Salmonella* isolates from broilers in other countries had lower MDR rates, 39.7% and 21.6% according to Alali *et al*. (2010) and Kidie *et al*. (2013), respectively, and 86.7% in Egypt by Elmonir *et al*. (2017). In Egypt, 75% of broiler farms were small-scale farms that lacked adequate biosecurity measures and the use of antibiotics. Without supervision, the management risks may promote the emergence of XDR *Salmonella* by increasing the likelihood of gene transfer in concurrent multiserovar infection and selective pressure caused by antibiotic overuse. XDR *Salmonella* will be challenging to treat, potentially increasing morbidity and mortality in the animal and human populations. The occurrence of MDR in this study could be associated with the indistinctive use of antibiotics in farms that contribute to selective pressure and the transfer of multidrug resistance genes among microbiota of humans, animals, and the environment (You and Silbergeld, 2014). In intensive livestock farming, frequent contact between animals, workers, and a contaminated environment was essential in transferring resistance traits to bacterial species that were not directly exposed to selection pressure through antimicrobial therapy (Cristobal-Azkarate *et al*., 2014).

Plasmids are pervasive mobile genetic elements in *Salmonella* spp. that help bacteria to distribute resistance genes. Also, plasmid identification is used for following the transfer between different bacterial species (Lopatkin *et al*., 2017). In our study, all 16 strains had plasmids, which can be widely dispersed and potentially contribute genes for drug resistance to help bacteria survive (Mansour *et al*., 2020).

Using the antimicrobial genotypic features of the isolates, the mobile colistin resistance gene (*mcr*1) on plasmids was studied using PCR, and the findings indicated positive detection in all 16 (100%) of the tested *Salmonella* isolates. The *mcr*1 gene was discovered in all *Salmonella* isolates from poultry. On the other hand, Uddin *et al*. (2021) recorded that 31.8% of *Salmonella* isolates had the *mcr*1 gene, and Zhang *et al*. (2018) recorded that 13.1% were in pigeons.

PCR was the ideal method for precisely detecting the quinolone resistance gene, an efflux pump-encoding (*qep*A) on plasmid from all *Salmonella* spp. isolates (100%). Nearly similar results were examined by Abd El-Aziz *et al*. (2021) (89.66%). On the other hand, low positive percentage rate (3.5%) was further obtained by Abd El- Tawab *et al*. (2015).

The current investigation as in Table 5 found a proportion of 100% (16/16 isolates) for the *flo*R gene, florfenicol resistance. These results were nearly lower that those obtained by Li *et al*. (2021), who reported that *flo*R gene has a higher incidence among resistant *Salmonella* isolates. The *sul*I gene, sulfonamide resistance, on plasmids was associated with ubiquitous and typically associated with plasmids of long-known Gram-negative bacteria (Sánchez-Osuna *et al*., 2019). In our study, the *sul*I gene was detected with a percentage of 93.8%; Pavelquesi *et al*. (2021) detected the *sul*I gene on the plasmid of 82.6% of *Salmonella* isolates.

Antibiotic efflux pump is the most common tetracycline resistance mechanism, in which *tet*A(A) gene was associated with membrane efflux proteins, which exchange a protein of a tetracycline-cation complex against a concentration gradient and export the drug to outside bacterial cells, which are associated with plasmids (Sheykhsaran *et al*., 2019). The *tet*A(A) gene was found in 16/16 (100%) of *Salmonella* spp. isolates, which agrees with the result (94.5%) of Pavelquesi *et al*. (2021).

*aad*A1 resistant gene of aminoglycoside conferred resistance

to kanamycin and streptomycin, 14 isolates (87.5%) possessed the *aad*A1 gene on a plasmid, while *aad*A1 gene was detected in 45.6% of *Salmonella* isolates (Doosti *et al*., 2016).

PGS is used now as an alternative technique, which is fast and reliable serotype information (Gymoese *et al*., 2017). This approach offers rapid identification of *Salmonella* serovars, which identified an array of single nucleotide polymorphisms (SNP) within the genome. It investigates the epidemiology of an outbreak to link poultry cases of illness to the point source of contamination and differentiates between outbreak-related and unrelated sporadic clinical cases (Lienau *et al*., 2011). PGS also has the potential to provide clinicians and researchers with additional information regarding virulence factors and antibiotic resistance markers to understand serotypes better and quickly identify and investigate outbreaks (Taylor *et al*., 2015). For identifying and monitoring foodborne epidemics, PGS is fast replacing current molecular subtyping methods (Leekitcharoenphon *et al*., 2014). In this investigation, the phylogenetic analysis of *inv*A gene of *S.* Typhimurium isolates from three isolates of household chicken, commercial broiler and pigeon demonstrated high conservation level of the gene at amino acid level as previously was proved by El-Sebay *et al*. (2017) who recorded that *inv*A gene sequence changes were an important discriminatory marker that could be used in epidemiological investigation.

The inactivated vaccine in this study was uncontaminated by germs or fungi. Additionally, it was secure when administered to particular pathogen-free chickens. At the injection site, no clinical symptoms nor responses were seen. We employed Montanide ISA 78 adjuvant in our investigation.

The inactivated bivalent vaccine in this study elicited a high antibody titer measured by ELISA. Similar results have been reported after vaccination of *Salmonella* inactivated vaccines in SPF chickens (El-Enbawaay *et al*., 2013). In our experiment, rapid and high antibody titers against *S.* Typhimurium and *S.* Enteritidis were detected in vaccinated chickens after prime and boost vaccination compared to unvaccinated chickens (Tables 7 and 8). High antibody titers after vaccination with the killed *Salmonella* vaccine could explain the significant reduction in fecal shedding and internal organ invasion (Crouch *et al*., 2020a). There was a significant reduction in fecal shedding at the 3rd and 5th days post challenge, while no *Salmonella* fecal shedding was detected on the 7th day post challenge in vaccinated chickens (Table 9). It had been previously reported that vaccination of breeder chickens with inactivated vaccines reduced the incidence of *Salmonella* in broiler progeny (Crouch *et al*., 2020b). Additionally, vaccinating the killed *S.* Enteritidis vaccine helped prevent egg contamination with *S.* Enteritidis (Cogan and Humphrey, 2003). Our inactivated bivalent vaccine induced an 85% protection rate and thus is accepted according to Egyptian Veterinary Codex (CLEVB) (2009).

CONCLUSION

There is a high incidence of multidrug resistance *Salmonella enterica* serovars in pigeon, backyard chickens, and commercial broiler in Sharkia governorate. Additionally, a bivalent killed *Salmonella* vaccine comprising two isolated serovars of *Salmonella enterica* (*S.* Typhimurium and *S.* Enteritidis) was developed and evaluated for its efficacy in SPF chickens. Vaccinated chickens developed a high humeral response measured by ELISA. Also, the protection rate of the developed vaccine in the challenge test is 85%, with a significant reduction in fecal shedding. Thus, it is recommended to raise awareness among veterinarians and farmers of the effectiveness of using customized inactivated vaccines prepared from locally isolated *Salmonella* strains with the added value of being an alternative to antibiotic treatment for salmonellosis.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

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