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Research Article

Efficacy of a Locally Prepared Inactivated Trivalent Vaccine against Salmonellosis in Poultry

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ABSTRACT

In the present study, efficacy of a locally prepared inactivated trivalent *Salmonella* vaccine consisting of *Salmonella* Typhimurium (ST), *Salmonella* Kentucky (SK) and *Salmonella* Enteritidis (SE) had been studied. A total of 120, two weeks old specific pathogen free (SPF) chicks were divided into two groups; 60 chicks each. First group was vaccinated with the prepared vaccine at the age of two weeks and boostered after three weeks, the second group was kept unvaccinated as a control group. The two groups were challenged with *Salmonella* Typhimurium, *Salmonella* Kentucky and *Salmonella* Enteritidis strains (10^8 CFU/ml of each) 1ml orally, 3 weeks post boostering of the vaccine. The degree of protection was detected according to the severity of the clinical signs, the mortality and fecal shedding of the challenged organisms. Blood samples were collected weekly after first vaccination till the third week after challenge and humoral immune response was measured against *Salmonella* strains using ELISA and microagglutination test and gives a high protective antibody titer. The prepared vaccine induced 80% protection rate in challenge test with reduced fecal shedding. These results suggest that the locally prepared inactivated trivalent *Salmonella* vaccine can be an effective tool for controlling the salmonellosis in chicken farms in Egypt.

Key words: *Salmonella*, Typhimurium, Kentucky, Enteritidis, Vaccine, Chickens

INTRODUCTION

Salmonella enterica is a gram-negative and facultative anaerobic enterobacterium that innately colonizes the human and animal gastrointestinal tract. Some serotypes of *Salmonella* enterica are the causative agents of human and animal food poisonings. *Salmonella* enterica bacteria have somatic O-antigens and flagellar H-antigens, and are classified into more than 2500 serotypes depending on the combinations of their O- and H-antigens by the Kauffmann–White scheme (Popoff *et al*., 2004).

Foodborne diseases of humans caused by *Salmonella* spp. and their contamination of chicken eggs, especially *Salmonella* Enteritidis (SE), have become major public concerns since the late 1980s (Guard-Petter, 2001) and (Cogan and Humphrey, 2003). There have been many reports regarding the detection of *Salmonella* Typhimurium (ST) in broiler chicken meat, but ST also frequently contaminates chicken eggs. So, countermeasures for the control of *Salmonella* infections in layer farms should focus not only on SE but also on ST (Leach *et al*., 1999). Recently, multidrug-resistant *Salmonella* spp. have been spreading in Western countries and Japan and the seriousness of ST DT104, which is highly resistant to various antibiotics, is especially emphasized (Helms *et al*., 2005). In some countries, bivalent vaccines consisting of SE and ST are already available commercially and are utilized for preventing SE and ST infections on farms (Okamura *et al*., 2007).

In Egypt, a significant increase in the number of *Salmonella* isolates from animal and chicken meat has been observed (Ahmed and Shimamoto, 2014). Also, Amin and Abd El-Rahman (2015) examined 200 fresh chicken meat by using standard bacteriological methods and revealed isolation of 7 S. enterica were isolated with a percentage of (3.5%), including 5 *S.* Typhimurium with a percentage of (2.5%) and 1 for each of *S.* Enteritidis and *S.* Kentucky with a percentage of (0.5%).

Vaccines for *Salmonella* are not capable of eradicating infection from flocks but can increase the threshold for infection, reduce the level of excretion of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene (OIE, 2012).

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In this study, a locally prepared inactivated trivalent *Salmonella* vaccine was evaluated and determined antibody response against *Salmonella* Typhimurium, *Salmonella* Kentucky and *Salmonella* Enteritidis by using different serological tests and challenge assay in chickens.

MATERIALS AND METHODS

Bacterial strains

Three local field isolated *Salmonella* strains (*S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis) isolated from chickens, were kindly obtained from Bacterial Sera and Antigens Research Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt and were used in vaccine preparation. All strains were confirmed as *Salmonella* different types by using both morphological and biochemical identification following the methods described by Quinn *et al*., 2002. Serological typing was performed using reference *Salmonella* antisera according to Kauffmann and Das-Kauffmann (2001).

Molecular identification of *Salmonella* strains using Multiplex PCR (Ibrahim *et al*., 2016)

DNA extraction was carried out for the three isolates of *Salmonella* (*S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis) according to the manufacturing instruction of DNA extraction kit (Qiagen Hilden, Germany). The DNA extract was stored at -20 C until use.

The DNA extract of each isolate was tested by conventional multiplex PCR assays using Biometra personal thermocycler utilizing a variety of a specific primer sets, as shown in Table (1), using standard PCR kit (iNtRON, Korea).

Experimental birds

A total number of 140 specific pathogen free (SPF) chickens of 2 weeks old were obtained from SPF poultry farm at Koom Osheem Fayuom province, Egypt. They were housed in batteries with the network floor. All birds were ascertained first to be free from *Salmonella* (antigens and antibody). They were fed on free balanced ration.

Vaccine preparation (Charles *et al*., 1994)

S. Typhimurium, *S.* Kentucky and *S.* Enteritidis were grown separately on S.S ager for 24 hrs at 37°C. Separate colonies from each type were selected and inoculated on tryptone soya broth and incubated for 24 hrs at 37°C. The bacterial suspension was adjusted to contain 10^{10} colony forming unit / ml using total colony count technique. Then each bacterial suspension was centrifuged at 5000 rpm at 4°C for 30mins to pellet the bacterial strain.

A separate final suspension from each of *S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis was prepared and the count was adjusted for each type to 10^{10} CFU/0.5ml of final product using total colony count technique. The bacteria were then inactivated by adding 0.3% formalin with agitation then Montanide ISA71 (SEPPIC®, France) was mixed with one part of bacterial suspension in a ratio of 71 adjuvant: 29 antigen.

Quality control of the prepared vaccine

Purity test

Testing of the prepared vaccine to ensure that it is free from any contamination as aerobic, anaerobic bacteria and fungi (OIE, 2012).

Safety Test (OIE, 2012).

Safety test of the prepared vaccine was monitored through injection of double field dose (1 ml) of the vaccine subcutaneously in each of 20 SPF chickens 2 weeks old. The chickens were observed daily for two weeks for any signs of local reactions, clinical signs or deaths.

Experimental design

Two groups of SPF chicks each of 60 chickens were reared separately; the first group of chickens was injected with 0.5 ml of the prepared vaccine subcutaneously at two weeks of age then boostered with another same dose and route after three weeks. The second group was used as a control (non-vaccinated). Each group was divided into three sub groups then each sub group was challenged three weeks after the booster dose by oral administration of 1ml containing 10^8 CFU of each strain (*S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis) separately (Paiva *et al*., 2009). The inoculated chickens were observed for one month. The degree of protection was assessed according to the severity of the clinical signs, the mortality and the recovery of the challenge organisms from fecal samples. Blood samples (2-5ml/bird) were collected from wing vein before immunization, weekly after each vaccination and post challenge for three weeks

Table 1: Primer sets for *Salmonella* strains PCR

Primer set	*Salmonella* strain	Target gene	Primer sequence 5..3`	Length	Amplicon fragment	Reference
S139	*Salmonella*	*invA* gene	GTGAAATTATCGCCACGTTCGGGCAA	26	284	Rabie,
S141	spp.		TCATCGCACCGTCAAAGGAACC	22		2012
ST11	*Salmonella* Spp.	Randomly cloned chromosomal fragment	AGCCAACCATTGCTAAATTGGCGCA	25	429	Soumet *et al*., 1999
*S.*Typhimurium um-STM-F	*Salmonella* Typhimurium	fliC	GGTGGCAAGGGAATGAA	24	915	Gracias and
*S.*Typhimurium um-STM-R			CGCAGCGTAAAGCAACT	22		Mckilip., 2004
Fli 15	*Salmonella*	fliC	CGGTGTTGCCCAGGTTGGTAAT		559	Soumet *et*
Tym	Kentucky		ACTCTTGCTGGCGGTGCGACTT			*al*., 1999
Tym	*Salmonella*	Sef A gen	ACT CTT GCT GGC GGT GCG ACT T	22	312	Islam,
Sef167	Enteritidis		AGG TTC AGG CAG CGG TTA CT	20		2004

(once/week) to measure and evaluate the developed humeral immune response against *S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis by ELISA test and Microagglutination test. Fecal samples were collected before the start of the experiment and after challenge for one month (once/week) using sterile swabs which were inoculated into tetrathionate broth from all chickens including the vaccinated and the control ones and examined bacteriologically for shedding of *Salmonella*e according to Cruickshank *et al.*, 1975 and Hofstad *et al.*, 1997.

RESULTS

Molecular identification was done using multiplex PCR assay along with bacteriological and biochemical characterization of *Salmonella* Spp. Before the vaccine preparation. The results obtained showed that the three used strains were positive by conventional PCR using primer sets (S139&S141) as mentioned in Table (1) and showing specific bands at 284 bp (Fig 1) for all *Salmonella* spp. Multiplex PCR could differentiate between *S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis. As two sharp specific bands were observed for each strain at the sizes of 429/312, 429 /915 and 429/559 bp, respectively (Fig. 2). While, the other *Salmonella* spp. (S. Meleagridis) showed a single band only at 429bp, using the same primer sets (Table 1).

Results of quality control of the prepared vaccines

The prepared vaccine proved to be pure, sterile, safe and free from adverse side effects on chickens.

Evaluation of humoral immune responses in the vaccinated chickens

ELISA Test

The antibody titer in sera of vaccinated group (GMT) against *S.* Typhimurium increased from 164.9 pre-vaccination level to 849.5 at the 3rd w after the primary immunization and to 2259.2 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 2275.5(Table 2). While GMT against *S.* Kentucky increased from 165.2 pre-vaccination level to 849.5 at the 3rd w after the primary immunization and to 2262.5 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 2279.7(Table 3). On the other hand, GMT against *S.* Enteritidis increased from 166.4 pre-vaccination level to 867.2 at the 3rd w after the primary immunization and to 2285.6 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 2287.3(Table 4). On the other hand, The ELISA antibody titer in sera of unvaccinated chicks was 167 Moreover, an abrupt increase of antibody titer was recorded, where the antibody titer was 895.5, 892.3 and 897.2 at 3rd week post challenge (Table 2, 3 and 4).

Microagglutination test

The antibody titer in sera of vaccinated group (GMT) against *S.* Typhimurium increased from zero pre-vaccination level to 64 at the 3rd w after the primary immunization and to 178 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 275 (Table 5). While GMT against *S.* Kentucky increased from zero pre-vaccination level to 63 at the 3rd w after the primary immunization and to 176 at the 3rd week post boostering (Table 6). At the 3rd week post challenge GMT increased to 275.On the other hand GMT against *S.* Enteritidis increased from zero pre-vaccination level to 66 at the 3rd w after the primary immunization and to 177 at the 3rd week post boostering (Table 7). At the 3rd week post challenge GMT increased to 271. On the other hand, the antibody titer in sera of unvaccinated chickens was zero. Moreover, an abrupt increase of antibody titer was recorded, where the antibody titer was 65 at the 3rd week of challenge (Table 5, 6 and 7).

Protective Efficacy of the prepared vaccine

The protection rate of the prepared vaccine was 80% after 4 weeks post challenge (Table 8).

Fecal Shedding of *Salmonella*e from challenged chickens

The re-isolation rates of *Salmonella*e from chickens vaccinated with the inactivated trivalent *Salmonella* vaccine in the 1st, 2nd and 3rd weeks post challenge was 20.75, 12.5 and 8.33%, respectively while in the 4th week the fecal shedding disappeared. Regarding the control unvaccinated birds, the re-isolation rates were 70.8, 50, 25 and 16.66% in the 1st, 2nd, 3rd and 4th weeks post challenge, respectively (Table 3).

Table 2: Antibody titer against *S.* Typhimurium in sera of chickens vaccinated with trivalent inactivated *Salmonella* vaccine as measured by ELISA.

Intervals / Groups	Geometric mean antibody titers								
	Weeks post 1st vaccination			Weeks post boostering			Weeks post challenge		
	1st Week	2nd Week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
vaccinated group	184.3	456.2	849.5	1088.5	1143.7	2259.2	1663	2250.5	2275.5
Control non vaccinated group	167	176	155.3	180.6	193.4	206.3	774	1066	895.5

Mean antibody titer of prevaccinated chickens = 164.9

Table 3: Antibody titer against *S.* Kentucky in sera of chickens vaccinated with trivalent inactivated *Salmonella* vaccine as measured by ELISA

Intervals / Groups	Geometric mean antibody titers								
	Weeks post 1st vaccination			Weeks post boostering			Weeks post challenge		
	1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Vaccinated group	183.5	454.9	849.6	1097.2	1147.9	2262.5	1668	2255.7	2279.7
Control non vaccinated group	167	176	155.7	186.3	195.2	206.8	774	1063	892.3

Mean antibody titer of prevaccinated chickens = 165.2.

Table 4: Antibody titer against *S.* Enteritidis in sera of chickens vaccinated with trivalent inactivated *Salmonella* vaccine as measured by ELISA.

Intervals / Groups	Geometric mean antibody titers								
	Weeks post 1st vaccination			Weeks post boostering			Weeks post challenge		
	1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Vaccinated group	188.1	459.7	867.2	1083.1	1156.2	2285.6	1679	2269.2	2287.3
Control non vaccinated group	167	179	158.2	181.3	195.3	209	776.1	1065.1	897.2

Mean antibody titer of prevaccinated chickens = 166.4

Table 5: Antibody titer against *S.* Typhimurium in sera of chickens vaccinated with trivalent inactivated *Salmonella* vaccine as measured by Micro-agglutination test.

Intervals / Groups	Pre-vaccination	Geometric mean antibody titers								
		Weeks post 1st vaccination			Weeks post boostering			Weeks post challenge		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Vaccinated group	0	43	52	64	132	141	178	125	230	275
Control non vaccinated group	0	0	0	0	0	0	0	35	70	65

Table 6: Antibody titer against *S.* Kentucky in sera of chickens vaccinated with trivalent inactivated *Salmonella* vaccine as measured by Micro-agglutination test.

Intervals / Groups	Pre-vaccination	Geometric mean antibody titers								
		Weeks post 1st vaccination			Weeks post boostering			Weeks post challenge		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Vaccinated group	0	43	52	63	132	141	176	125	230	275
Control non vaccinated group	0	0	0	0	0	0	0	35	70	65

Table 7: Antibody titer against *S.* Enteritidis in sera of chickens vaccinated with trivalent inactivated *Salmonella* vaccine as measured by Micro-agglutination test.

Intervals / Groups	Pre-vaccination	Geometric mean antibody titers								
		Weeks post 1st vaccination			Weeks post boostering			Weeks post challenge		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Vaccinated group	0	43	52	66	132	141	177	125	230	271
Control non vaccinated group	0	0	0	0	0	0	0	35	70	65

Table 8: Protective Efficacy of trivalent inactivated *Salmonella* vaccine in SPF chickens challenged with virulent *S.* Typhimurium, *S.* Kentucky and *S.* Enteritidis strains.

Chicken groups	Total No. of birds		No. of dead & or diseased birds / Week post challenge				Dead & or diseased/Total		Survive/Total		Mortality rate	Protection %
			1st week	2nd week	3rd week	4th week						
Vaccinated group	60	20 (ST)	1	2	1	0	4/20	12/60	16/20	48/60	20%	80%
		20 (SK)	2	1	1	0	4/20		16/20		20%	80%
		20 (SE)	2	2	0	0	4/20		16/20		20%	80%
Control non vaccinated group		60	24	10	8	6	48/60		12/60		80%	20%

*Protection % = (Survival birds/ total number of birds) X100.

Table 9: Results of fecal shedding of *Salmonella* from chickens after challenge.

Chicken groups	No. of birds positive for isolation / total No. of living birds			
	1st week	2nd week	3rd week	4th week
Vaccinated group	11/53 (20.75%)	6/48 (12.5%)	4/48 (8.33%)	0/48 (0%)
Control non vaccinated group	17/24 (70.8%)	5/10 (50%)	2/8 (25%)	1/6 (16.66%)

Chickens in vaccinated group suffered from mild white diarrhea, with slight lesions of enteritis. Chickens in the control group were suffered from profuse white watery diarrhea, depression and the birds were reluctant to move. The PM lesions included enteritis, cecal core, swollen liver, spleen and gallbladder with small necrotic foci in the liver, in some cases the pericardium was turbid and covered with yellowish white materials.

DISCUSSION

EFSA (2010) reported that the most frequently isolated *Salmonella* serovars in broiler chickens were, respectively in decreasing order, *S.* Infantis (29.2% of the *Salmonella* positive broiler carcass samples), *S.* Enteritidis (13.6%), *S.* Kentucky (6.2%) and *S.* Typhimurium (4.4%).

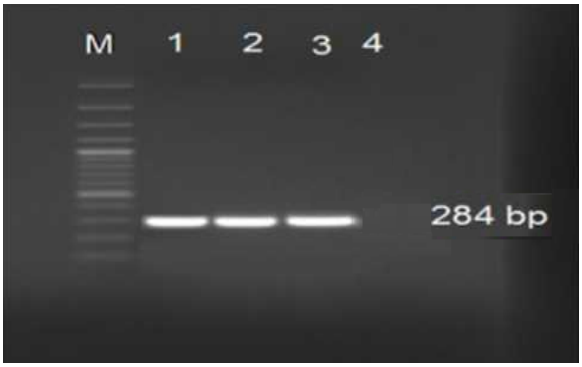

Fig 1: Identification of *Salmonella* strains by PCR. All strains shared the same band at 284 bp which is general for all *Salmonella* spp.

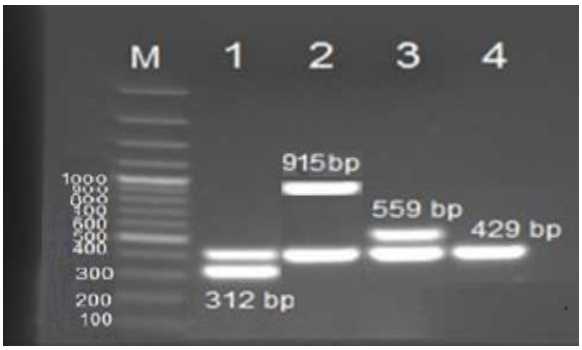

Fig 2: Genotyping of *Salmonella* strains by multiplex PCR. Lane (M): 100 bp DNA ladder (Fermentas). All strains shared the same band at 429 bp which is general for all *Salmonella* spp. Lane (1) showed band at 312 bp specific for *Salmonella* Enteritidis. Lane (2) showed band at 915 bp specific for *Salmonella* Typhimurium. Lane (3) showed band at 559 bp specific for *Salmonella* Kentucky. Lane (4) showed band at 429 bp specific for other *Salmonella* spp. (*Salmonella* Meleagridis).

In this study, a locally prepared inactivated trivalent *Salmonella* vaccine was evaluated and determined antibody response against *Salmonella* Typhimurium, *Salmonella* Kentucky and *Salmonella* Enteritidis by using different serological tests and challenge assay in chickens.

The antibody titer in sera of vaccinated group (GMT) against *S.* Typhimurium increased from 164.9 pre-vaccination level to 849.5 at the 3rd w after the primary immunization and to 2259.2 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 2275.5 (Table 2). While GMT against *S.* Kentucky increased from 165.2 pre-vaccination level to 849.5 at the 3rd w after the primary immunization and to 2262.5 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 2279.7 (Table 3). On the other hand, GMT against *S.* Enteritidis increased from 166.4 pre-vaccination level to 867.2 at the 3rd w after the primary immunization and to 2285.6 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 2287.3(Table 4). On the other hand, The ELISA antibody titer in sera of unvaccinated chicks was 167 Moreover, an abrupt increase of antibody titer was recorded, where the antibody titer was 895.5, 892.3 and 897.2 at 3rd week post challenge (Table 2, 3 and 4).

These results agree with those obtained by Okamura *et al.* (2007) and El-Enbaawy *et al.*, (2013). The antibody titer in sera of vaccinated group (GMT) against *S.* Typhimurium increased from zero pre-vaccination level to 64 at the 3rd w after the primary immunization and to 178 at the 3rd week post boostering. At the 3rd week post challenge GMT increased to 275 (Table 5). While GMT against *S.* Kentucky increased from zero pre-vaccination level to 63 at the 3rd w after the primary immunization and to 176 at the 3rd week post boostering (Table 6). At the 3rd week post challenge GMT increased to 275.On the other hand GMT against *S.* Enteritidis increased from zero pre-vaccination level to 66 at the 3rd w after the primary immunization and to 177 at the 3rd week post boostering (Table 7). At the 3rd week post challenge GMT increased to 271. On the other hand, the antibody titer in sera of unvaccinated chickens was zero. Moreover, an abrupt increase of antibody titer was recorded, where the antibody titer was 65 at the 3rd week of challenge (Table 5, 6 and 7).

These results agree with those obtained by Abd El-Ghany *et al.* (2012) and Ibrahim (2014). The protective value against *Salmonella* Typhimurium, *Salmonella* Kentucky and *Salmonella* Enteritidis; post oral challenge, in chickens vaccinated with the prepared vaccine was 80% (Table, 8). The achieved protection value by the prepared vaccine is accepted to pass the vaccine for use according to Heddleston (1975) and Egyptian Veterinary Codex- CLEVB (2009). Fecal shedding of *Salmonella* organisms in the vaccinated group of chickens reached 8.33% while the unvaccinated control group at 3 week post challenge revealed fecal shedding of 25 %. No shedding detected at the fourth week post challenge in vaccinated group, while there was 16.66% shedding in control unvaccinated group (Table, 9). Similar fecal shedding rates were reported by Sayed (2010) and Ibrahim (2014).

Conclusions

The present study shows that the locally prepared inactivated trivalent *Salmonella* vaccine is safe and effective against *Salmonella* infections in chickens in Egypt.

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