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The protective efficacy of immunoglobulin Y from immunized chickens against *Salmonella* infections in mice

Hasan Hüseyin HADİMLİ[1,*], Zafer SAYIN[1], Gökçenur SANİOĞLU GÖLEN[2]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, Selçuklu, Konya, Turkey
[2]Department of Microbiology, Faculty of Veterinary Medicine, Aksaray University, Aksaray, Turkey

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Abstract: The aim of this study was to determine the efficacy of immunoglobulin Y (IgY) obtained from chickens immunized with *Salmonella* vaccines. Chickens were vaccinated three times with inactivated monovalent, bivalent, and combined vaccines. Immunized hen eggs were collected after the third vaccination and IgYs were purified. In total, 100 mice were orally challenged with *Salmonella* serotypes. After the challenge, IgYs were orally administered to mice. Mice were observed for morbidity and mortality. Fecal samples from the mice were also cultured for the reisolation of *Salmonella* serotypes. The antibody titers in the serum samples of vaccinated chickens were higher than those of controls ($P < 0.001$). Neither morbidity nor mortality were observed in these mice. In all of the groups the reisolation numbers of the *Salmonella* serotypes from internal organs and fecal samples were low ($P < 0.001$) In conclusion, it is suggested that IgYs from immunized chickens could be used to establish protection against *Salmonella* infections.

Key words: *Salmonella*, protection, immunoglobulin Y, chicken, mice

1. Introduction

Although passive immunity provides immediate protection, the protective immunity period is very short and ranges from a few weeks to a few months (1). As the immune system of newborns is not sufficiently developed, maternal antibodies are transferred to newborns to protect them against infections (2). In mammals, including horse, cattle, sheep, and goats, maternal antibodies are transferred to colostrum. In poultry, the antibodies are found in the egg yolk (yolk sac) (3–5).

In order to reduce the use of antibiotics and to prevent the emergence of antibiotic resistance, alternative methods have been developed for the control and eradication of infectious diseases. The use of immunoglobulin Y (IgY) has been investigated in humans (6–8) and several animals for the prevention of clinical infectious diseases (9–18). In the past few years, IgYs obtained from the egg yolk of immunized chickens have been increasingly used for the diagnosis and treatment of infectious diseases (3). Passive immunization with IgY is preferred for the neutralization of snake venom, scorpion stings, and several microorganisms (9–18). Passive protection with egg yolk antibodies can also be used against the intestinal colonization of *Salmonella* serotypes (11) and it was reported that protection by anti-*Salmonella* antibodies could be effective in the control of salmonellosis (10).

Hen eggs are not only a good source of food but also contain a high level of egg yolk antibodies. The procedure for IgY isolation is simple and quick, and it also guarantees the yield of high antibody titers. Antibodies are sustained for long periods in the egg yolk of immunized chickens (4,18). While IgY and IgG show some similarities, there are also some fundamental differences in their structure. IgY is similar to mammalian IgE in terms of its structural and functional features (3).

This study aimed to determine the efficacy of IgY obtained from immunized chicken with inactive monovalent, bivalent, and combined *Salmonella* vaccines.

2. Materials and methods

2.1. Vaccination of chickens and production of immunized eggs

The monovalent (*Salmonella enterica* subsp. *enterica* serovar Dublin (*S.* Dublin), *S.* Typhimurium, *S.* Kentucky, or *S.* Anatum), bivalent (*S.* Dublin and *S.* Typhimurium), and combined (*S.* Dublin, *S.* Typhimurium, *S.* Kentucky, and *S.* Anatum) *Salmonella* vaccines were prepared in our laboratory (19). A total of 60 chickens (30 weeks old, Lohmann Brown) were vaccinated with monovalent ($4 \times 10 = 40$), bivalent ($1 \times 10 = 10$), and combined ($1 \times 10 = 10$) *Salmonella* vaccines (5). In addition, 10 chickens

* Correspondence: hhadimli@selcuk.edu.tr

were kept as negative controls. *Salmonella* vaccines were subcutaneously administered three times (30, 33, and 36 weeks of age) at 14-day intervals at a dose of 0.25 mL in the back of the neck. Each vaccine contained $\times 10^9$ cfu/mL (19). Sterile saline (0.25 mL) alone was injected for the controls (20). The chickens were raised and vaccinated at the Research and Application Unit of the Faculty of Veterinary Medicine, Selçuk University.

2.2. Isolation of antibodies from the egg yolk

Ten eggs were collected from the immunized chickens and controls before vaccination and 10 days after the third vaccination. The egg yolks were separated for the extraction of IgYs. After separation, homogenization was performed by using a mixer. The egg yolks were diluted with 100 mL of 1% l-carrageenan and were kept at room temperature (22 °C) for 30 min. Subsequently, the homogenate was centrifuged at 10,000 × *g* for 20 min (Hettich, India), and the supernatant containing IgYs was harvested. The supernatant was filtered through a 0.22-µm membrane and was stored at –20 °C until used (5,12,21,22).

2.3. Determination of IgY levels by ELISA

Modified ELISA kits for each *Salmonella* serotype were prepared for the detection of IgYs in our laboratory. These ELISA kits were optimized and standardized. Briefly, S*almonella* strains (*S.* Dublin, *S.* Typhimurium, *S.* Kentucky, or *S.* Anatum) were separately grown in brain-heart infusion broth at 37 °C for 24–48 h. The bacteria were harvested by centrifugation at 3000 × *g* for 30 min. The bacterial concentrations were adjusted to 1.2×10^9 cfu/mL with a carbonate-bicarbonate buffer (pH 9.6). The bacteria were then inactivated with formalin (0.05%). The protein values of *Salmonella* antigens were determined using a DC protein assay kit (Bio-Rad Lab., Cat. No. 500-0116, USA). In brief, ELISA plates were coated with 100 µL/well of *Salmonella* antigens suspended in a carbonate-bicarbonate buffer (pH 9.6) at 1 mg/mL. The immunoplates were incubated at 4 °C overnight. After washing with phosphate buffer solution-Tween 20 (PBS-T; 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 7.2) 5 times, 100 µL of 2% bovine serum albumin was added to the wells and the plates were further incubated for 30 min at room temperature. The microplates were rewashed 3–5 times for 5 min with PBS-T. The supernatants of the eggs that contained IgYs were diluted sequentially from 1/10 up to 1/40,960, and 100 µL of each dilution was added to the wells. The plates were incubated at 37 °C for 1 h in incubator. After the plates were washed, 100 µL of rabbit antichicken IgY (whole molecule, Sigma A-9792, St. Louis, MO, USA) at 1:8000 was added to each well and incubated at 37 °C for 1 h. Following washing, 100 µL of substrate solution (0.4 mg/mL δ-phenylenediamine dihydrochloride, 0.05 M phosphate-citrate buffer, and δ-phenylenediamine tablets (Sigma P 8287, St Louis, MO, USA)) was added, and the plates were reincubated for 20 min at room temperature. Finally, 50 µL of 2 M H_2SO_4 was added to all wells as a stop solution and the plates were immediately read in a microplate autoreader (Anthos Labtec Instruments, A 5022, Salzburg, Austria) at 450 nm (20,22).

2.4. Challenge trials in mice

The oral lethal dose (LD) 50% values of live *Salmonella* serotypes (*S.* Dublin, *S.* Typhimurium, *S.* Kentucky, and *S.* Anatum) were orally administered to mice (10 × 10 = 100) (23). The LD50 values were 5×10^8 cfu/mL for *S.* Dublin, 4×10^8 cfu/mL for *S.* Typhimurium, and 1×10^8 cfu/mL for *S.* Kentucky and *S.* Anatum. Next, the titers of IgYs were adjusted at 0.600 nm. IgYs were orally administered to 10 mice (separately for each group) at 2, 6, 12, 24, and 48 h after challenge. IgYs were given as 2 mL (5 µg/mL) for each mouse at each dispensing time in the first 24 h. At 48 h, IgYs were administered as 4 mL. In addition, 10 mice (separately for each group) that received live *Salmonella* strains alone were kept as controls. All mice were observed for morbidity and mortality for 20 days. While necropsy of dead mice was done immediately, the mice that did not die for 20 days were euthanized and necropsy was performed. The internal organs (i.e. liver, spleen, lungs, heart, and kidneys) and intestine were used for the reisolation of *Salmonella* serotypes (23).

2.5. Detection of *Salmonella* from fecal samples

For the reisolation of *Salmonella* strains, fecal samples were collected at 2-day intervals from both the mice immunized with IgYs and challenged with live *Salmonella* strains and from the controls. The reisolation of *Salmonella* strains was performed according to ISO 6579 (11,20,24).

2.6. Statistical analysis

Statistical differences among the groups were assessed by the chi-square test and variance analyses. The differences between the groups were also analyzed by Duncan's and Dunnett's test using SPSS 22.00.

3. Results

When compared to the controls, the antibody titers in the serum samples of the vaccinated chickens (immunized with monovalent, bivalent, and combined vaccine) were determined to be higher by ELISA (Table 1, $P < 0.001$).

Morbidity and mortality were not observed in any of the mice challenged with *Salmonella* serotypes and passively immunized with IgYs (Table 2). On the other hand, morbidity and mortality were observed in almost half of the control mice challenged with *Salmonella* serotypes ($P < 0.001$).

Salmonella serotypes could not be reisolated from the internal organs of the mice challenged with *Salmonella* and administrated IgYs from chickens ($P < 0.001$). Only *S.* Kentucky was reisolated from the liver, spleen, and kidney samples of a mouse administered IgY obtained

Table 1. Titers of IgY from chickens and their eggs.

Vaccine	Serotypes	Chicken F = 19.554 (P < 0.001)	Egg F = 6.12 P < 0.001
Combined	Dublin	1.260[a]*	1.157[a]
	Typhimurium	1.736[a]	1.347[a]
	Anatum	2.260[a]	1.988[a]
	Kentucky	1.550[a]	1.454[a]
Bivalent	Dublin	1.401[a]	1.343[a]
	Typhimurium	1.887[a]	1.535[a]
Monovalent	Dublin	0.989[a]	0.973[a]
Monovalent	Typhimurium	1.531[a]	1.486[a]
Monovalent	Anatum	1.950[a]	1.932[a]
Monovalent	Kentucky	1.409[a]	1.523[a]
Control	Dublin	0.065[b]	0.032[b]
	Typhimurium	0.098[b]	0.051[b]
	Anatum	0.101[b]	0.112[b]
	Kentucky	0.113[b]	0.116[b]
Blind		0.049[b]	0.049[b]
Before vaccination		0.063[b]	0.058[b]

*a, b: Differences among groups shown with different superscripts in the same column are significant (P < 0.05).

Table 2. Morbidity and mortality in mice immunized with different vaccines and challenged with different *Salmonella* serotypes.

Vaccine	Serotypes	Morbidity		χ^2	Mortality		χ^2
		No.	% of protection		No.	% of protection	
Combined	Dublin	0/8	100	$\chi^2 = 119.56$ P < 0.001	0/8	100	$\chi^2 = 117.91$ P < 0.001
	Typhimurium	0/9	100		0/9	100	
	Anatum	0/9	100		0/9	100	
	Kentucky	0/8	100		0/8	100	
Bivalent	Dublin	0/8	100		0/8	100	
	Typhimurium	0/9	100		0/9	100	
Monovalent	Dublin	0/8	100		0/8	100	
Monovalent	Typhimurium	0/9	100		0/9	100	
Monovalent	Anatum	0/8	100		0/8	100	
Monovalent	Kentucky	0/8	100		0/8	100	
Control	Dublin	6/10	40		6/10	40	
	Typhimurium	5/10	50		5/10	50	
	Anatum	5/10	50		4/10	60	
	Kentucky	5/10	50		4/10	60	

from chickens immunized with the monovalent vaccine. *Salmonella* was also reisolated from some intestinal samples of the mice excluding the controls. However, the number of reisolations from internal organs of the control mice that were only challenged with *Salmonella* serotypes was found to be high (Table 3).

The number of reisolations or the spread of *Salmonella* serotypes in fecal samples of the mice that were challenged with *Salmonella* strains and then administered IgYs obtained from vaccinated chickens was low ($P < 0.001$). However, the number of reisolations from fecal samples of the control mice challenged with *Salmonella* strains was found to be higher than in the trial groups (Table 4).

4. Discussion

Chicken egg yolk is an alternative source of immunoglobulins. IgYs are obtained from the eggs of chickens immunized with various antigens. Unlike the different classes of antibodies (e.g., IgG, IgM, IgE) found in mammalian serum, there is only one kind of antibody (IgY) in the egg yolk, which can be easily isolated by precipitation (12,21). The use of chicken antibodies is more hygienic, cost-effective, and convenient, and chicken antibodies can be obtained in higher volumes when compared to mammalian antibodies (3). The affinity of IgY is 3 to 5 times higher than that of IgG (9). IgY maintains its activity within a wide temperature range (0–70 °C) and pH range (3.5–11) (12), and it is also resistant to the degradation effects of pepsin, 4000 kg/cm^2 pressure, and chymotrypsin (13). Furthermore, purified IgY remains stable for years at 4 °C (5).

When compared to mammals, a high antibody response can be obtained with a much lower antigen dose in chickens (3,4). In the present study, the antibody titers in the blood sera of the chickens immunized with monovalent, bivalent, and combined *Salmonella* vaccines were determined to be higher than those of the controls. The differences between the antibody titers of the controls and vaccinated chickens were statistically significant ($P < 0.001$). The highest antibody titers were detected in the chickens immunized with monovalent and combined vaccines against *S.* Anatum. On the other hand, the lowest antibody titers were determined in the chickens immunized with a monovalent vaccine against *S.* Dublin. The differences observed between the vaccinated chickens for antibody titers were statistically insignificant ($P > 0.001$). Although not within the scope of this study, the differences in titers of antibodies to *Salmonella* serotypes indicate that the immunogenicity of *Salmonella* serotypes may be different.

Immunotherapy can be used against diseases that are difficult to treat with antibiotics (4). IgYs can be used to

Table 3. Reisolation of *Salmonella* serotypes from internal organs of the mice.

Vaccine	Serotypes	Liver		Spleen		Kidneys		Heart		Lungs		Intestine	
		No.	%*	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*
		$\chi^2 = 105.09$ $P < 0.001$		$\chi^2 = 75.08$ $P < 0.001$		$\chi^2 = 105.61$ $P < 0.001$		$\chi^2 = 117.91$ $P < 0.001$		$\chi^2 = 105.59$ $P < 0.001$		$\chi^2 = 88.61$ $P < 0.001$	
Combined	Dublin	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	2/8	75
	Typhimurium	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100
	Anatum	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100
	Kentucky	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100
Bivalent	Dublin	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100
	Typhimurium	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	3/9	66.7
Monovalent	Dublin	0/8	100	1/8	87.5	0/8	100	0/8	100	1/8	87.5	1/8	87.5
Monovalent	Typhimurium	0/8	100	0/8	100	0/8	100	0/8	100	1/8	87.5	1/8	87,5
Monovalent	Anatum	1/9	88.9	1/9	88.9	0/9	100	0/9	100	0/9	100	1/9	88,9
Monovalent	Kentucky	1/8	87.5	1/8	87.5	1/8	87.5	0/8	100	0/8	100	5/8	37.5
Control	Dublin	7/10	30	7/10	30	7/10	30	7/10	30	7/10	30	8/10	20
	Typhimurium	9/10	10	7/10	30	9/10	10	9/10	10	7/10	30	10/10	0
	Anatum	5/10	50	5/10	50	5/10	50	5/10	50	5/10	50	6/10	40
	Kentucky	5/10	50	4/10	60	5/10	50	5/10	50	5/10	50	7/10	30

* Protection ratio: Prevention of reisolation by % (calculated by subtracting % of samples that are positive from 100%).

Table 4. Reisolation of *Salmonella* serotypes from the fecal samples of the mice.

Vaccine	Serotypes	Sampling (day)																	
		4		6		8		10		12		14		16		18		20	
		No.	%*	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*
		F = 741.53 P < 0.001		F = 505.94 P < 0.001		F = 830.25 P < 0.001		F = 591.43 P < 0.001		F = 2329.38 P < 0.001		F = 954.35 P < 0.001		F = 717.31 P < 0.001		F = 1438.96 P < 0.001		F = 332.75 P < 0.001	
Combined	Dublin	0/8	100	0/8	100	0/8	100	0/8	100	1/8	87.5	1/8	87.5	0/8	100	1/8	87.5	1/8	87.5
	Typhimurium	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100
	Anatum	0/9	100	1/9	88.9	1/9	88.9	2/9	77.2	0/9	100	1/9	88.9	2/9	77.8	0/9	100	0/9	100
	Kentucky	0/8	100	0/8	100	0/8	100	0/8	100	1/8	87.5	0/8	100	0/8	100	1/8	87.5	1/8	87.5
Bivalent	Dublin	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100
	Typhimurium	0/9	100	1/9	88.9	1/9	88.9	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100
Monovalent	Dublin	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	1/8	87.5	0/8	100	0/8	100	1/8	87.5
Monovalent	Typhimurium	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100	0/9	100
Monovalent	Anatum	0/8	100	1/8	87.5	1/8	87.5	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100
Monovalent	Kentucky	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	0/8	100	1/8	87.5	0/8	100	0/8	100
Control	Dublin	4/5	20	5/5	0	4/4	0	4/4	0	3/4	25	3/4	25	4/4	0	4/4	0	4/4	0
	Typhimurium	9/9	0	5/8	37.5	5/7	28.6	4/6	33.4	4/5	20	4/5	20	4/5	20	4/5	20	4/5	20
	Anatum	6/10	40	6/8	25	7/8	12.5	5/7	28.6	6/7	14.3	5/6	16.7	5/6	16.7	5/6	16.7	5/6	16.7
	Kentucky	9/10	10	9/9	0	8/8	0	7/8	12.5	6/7	14.3	6/6	0	6/6	0	6/6	0	6/6	0

* Protection ratio: Prevention of reisolation by % (calculated by subtracting % of samples that are positive from 100%).

prevent bacterial and viral infections. IgYs are also known to have no toxic side effects (3). The therapeutic and prophylactic efficacy of the IgYs of immunized chickens against various pathogens has been investigated in humans (7). Hirai et al. (7) used anti-V cholera IgYs to prevent cholera in mice against challenges with inactivated *Vibrio cholerae* O1 and O139 and B subunits of recombinant cholera toxin vaccinated mice.

IgY activity in animals has been investigated in several studies (9–18). Ikemori et al. (9) determined that the survival rate of calves fed with milk containing anti-ETEC-IgY was 100%. Peralta et al. (10) immunized chickens with the purified 14-kDa fimbriae of *S.* Enteritidis and achieved a protection level of 77.8% with specific egg yolk antibodies in mice orally challenged with *S.* Enteritidis, while only 32% of the controls survived. Yokoyama et al. (11) used purified specific egg yolk antibodies (IgYs) to outer membrane proteins (OMPs), lipopolysaccharide (LPS), and flagellar proteins (FLA) of *Salmonella* spp. to determine the most protective antigen. They infected mice with *S.* Enteritidis or *S.* Typhimurium and anti-OMP, anti-LPS, and anti-FLA antibodies administered orally 3 times a day. Although a low survival rate (20%) was determined in the control mice, they achieved protection levels of 80%, 47%, and 60% with specific OMP, LPS, and FLA antibodies, respectively, in mice challenged with *S.* Enteritidis. Although all of the control mice died, protection levels of 40%, 30%, and 20% were achieved with the OMP, LPS, and FLA antibodies, respectively, in mice challenged with *S.* Typhimurium. Dahlen et al. (1) reported the intranasal administration of IgYs produced against a variety of cattle pathogens. Zhen et al. (16) immunized chickens with inactivated *E. coli* O111 isolated from cases of mastitis and purified IgY from the egg yolks of these chicken. They detected that IgYs restricted proliferation against *E. coli* O111 and other *E. coli* strains causing mastitis, and increased phagocytic activity of milk macrophages and neutrophils with purified IgY from the egg yolks of these chickens. Zhen et al. (15) also reported that anti-*S. aureus* IgYs have the potential to treat *S. aureus* mastitis in dairy cows. Similarly, anti-*E. coli* O157:H7 IgYs reduced the fecal shedding of *E. coli* O157:H7 in beef cattle (14). The intramammary administration of anti-*S. aureus* IgYs at 12-h intervals for 6 days reduced the number of somatic cells and bacteria in milk. Protection rates of 83.3% and 50% were obtained in experimental and clinical mastitis, respectively. It has been reported that IgY administration may be used as an alternative treatment for *S. aureus* mastitis (17). Guimarase et al. (25) immunized chickens with inactive *S. aureus* and purified IgYs from their egg yolk. The concentration of IgYs was high and inhibited the proliferation of *S. aureus*. IgYs obtained from chickens immunized with the neurotoxin of *Clostridium botulinum* type A provided protection against botulinum toxin type A (26). Meenatchisundaram et al. (18) reported that IgY could be used for the treatment of bovine mastitis. Vega et al. (27) indicated that protection could be established against rotavirus by passive immunization with IgYs and reported to have achieved a protection level over 80% in immunized animals when compared to the controls. Anti-Shiga toxin 1 IgY provided protection against a challenge with *E. coli* O157:H7 (28). It has been reported that IgY inhibits the proliferation of *S. aureus* causing mastitis and has a low bacteriostatic activity against *S. aureus* (29). Rofaiil and Germin (30) showed that anti-*S.* Typhimurium IgY protection was 92% in challenged mice.

In the present study, neither morbidity nor mortality was observed in the challenged mice administered *Salmonella*-specific IgYs. However, both morbidity and mortality were detected in half of the controls. The differences observed between the controls and challenged mice that were administered IgYs for morbidity and mortality were found to be statistically significant ($P < 0.001$). The number of reisolations of *Salmonella* from the challenged mice that were given IgYs was lower. Only *S.* Kentucky was reisolated from the liver, spleen, and kidneys of a mouse administered IgYs obtained from chickens immunized with the monovalent vaccine. On the other hand, the number of reisolations of *Salmonella* from the internal organs of the controls was found to be higher. The number of reisolations of *Salmonella* from fecal samples of challenged mice that were administered IgYs was very low. The differences observed for the number of reisolations between the controls and challenged mice given IgYs were found to be statistically significant ($P < 0.001$). IgYs prevented invasion of *Salmonella* serotypes by either binding to them or causing immune clearance by opsonization.

In this study, morbidity and mortality were not observed in the mice challenged with *Salmonella* strains (*S.* Dublin, *S.* Typhimurium, *S.* Anatum, *S.* Kentucky) and administered IgYs. The number of reisolations of *Salmonella* strains from the internal organs and fecal samples of these animals was low. As a result of this study, it was determined that the immunoprotective role of IgY prevented establishment of *Salmonella* infections.

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