Induction of Early Immunopotentiality to Fimbriae of *Salmonella* Enteritidis (SE) by Administering Thymulin and Zinc to SE-Vaccinated Chicken Breeders: Relationship to Protection

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ABSTRACT. The purpose of this study is to attempt the induction of early immunopotentiality of antibodies specific to fimbriae of *Salmonella enterica* serovar Enteritidis (SE), by administering thymulin and zinc to SE-vaccinated chicken breeders, and the improvement of protection against a controlled-live challenge by SE. The first two groups of breeders were administered subcutaneously at 15 and 19 weeks of age a killed SE vaccine. Breeders of the third and fourth groups were left unvaccinated. Breeders of the first group, immunopotentialized by thymulin and zinc, were able to induce the earliest antibodies in their pooled sera at 2 weeks post the first SE-vaccination, specific to fimbriae (~21 kDa) of SE. However, the second group that was only vaccinated with the same SE-vaccine produced specific antibodies to fimbriae at 3 weeks following the second vaccination (22 weeks of age). Breeders of the third group, that were neither SE-vaccinated nor immunopotentialized by thymulin and zinc, but were challenged by live SE at 22 weeks of age, were able to show specific antibodies to fimbriae at 3 weeks post challenge (25 weeks of age). The fourth group that was deprived of SE-vaccination, immunopotentializers, and challenge didn't show any background antibodies specific to SE-fimbriae. The presence of the earliest antibody-immunopotentialization to fimbriae of SE in breeders of the first group, administered thymulin and zinc, was associated with the lowest frequency of SE-infected ceca (10%) among the challenged groups. In addition, breeders of the first group were the only challenged birds resulting in absence of SE infection in their cecal tonsils. The first group-vaccinated, immunopotentialized, and challenged, and the second group-vaccinated and challenged only resulted in breeders with absence of SE infection in their oviducts and spleens. In conclusion, immunopotentialization of chicken breeders by thymulin and zinc induces the earliest specific antibodies to fimbriae of SE associated with the lowest frequency of SE-infected ceca, and absence of SE infection from cecal tonsils, oviducts and spleens.

KEY WORDS: chicken breeder, immunopotentialization, *Salmonella* Enteritidis, thymulin, vaccine, zinc.

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Salmonella enterica serovar Enteritidis (*S. Enteritidis*) is a major cause of gastroenteritis in humans and is associated with consumption of contaminated, raw, or undercooked food products [11, 16].

The fimbriae are rod-shaped structures on the surface of SE cells that are involved in adhesion of the organism to the eukaryotic cell's surface and may thus contribute to disease pathogenesis [14, 23]. The type-1 fimbriae or SEF21 (*S. Enteritidis* fimbriae 21) or what is known as the common pilli have sub-unit molecular weight of around 21 kDa that enable adhesion to a wide variety of eukaryotic cells *in vitro* [9]. A previous work showed that mice can be passively immunized against SE infection by the administration of egg – yolk antibodies specific to a certain type of fimbrial protein of SE used in the challenge [27], suggesting the important role of fimbriae in the disease process. Recently, it was shown that the early immune response in chicken to SE is not specific to any of the reported fimbriae proteins, which could explain the low immunogenicity of the fimbriae protein leading to failure of protection in chicken against SE infection [3].

Concerns about the safety of live SE vaccines have induced more research towards development of SE bacterins [5]. Experimentally acetone-killed SE oil-emulsion vaccines

have been examined in chickens [5, 15]. In both studies vaccines were able to reduce significantly the infection in the hen but were not able to eliminate it. Others have showed the failure of inactivated *Salmonella* vaccines in reaching to a complete protection against SE infection in chickens [2, 8, 34].

The incomplete protection by oil-emulsion killed vaccines against highly virulent and invasive SE organisms demands attempts to evaluate immunopotentializers that could augment the efficacy of such vaccines. Most of the research done on immunopotentialization by zinc and thymulin was in humans, animals, and rarely in avian species. A recent work on the use of thymulin and zinc proved the consistent immunopotentializing activity of these agents, resulting in augmentation of immune responses to antigens of Newcastle Disease virus, Infectious Bronchitis virus, and Infectious Bursal Disease virus contained in a trivalent killed vaccine administered to chicken layers [6]. Other works have proven the ability of thymulin and zinc to augment the immune system in mice [12, 25], and humans [18, 30] and to help in protection against different infections [21, 22, 32].

To our knowledge, this is the first work that attempts the induction of an early specific antibody response to low

immunogenic-fimbriae of SE by thymulin and zinc, and to study the relationship of this augmentation to protection in SE-vaccinated meat chicken breeders against a controlled-challenge by a highly virulent SE possessing 14.1 and ~50 kb plasmids [10].

MATERIALS AND METHODS

Breeders: Fifteen-week-old *Salmonella* Enteritidis (SE)-free meat chicken breeders, reared on the floor, were wing-banded and divided into four groups of ten breeders per group. The breeders were confirmed free of SE infection by taking an individual cloacal swab from each breeder at 15 weeks of age and culturing for SE isolation. The first three groups were each put in a separate room, within the same isolation unit. Breeders of the fourth group were left on the main farm of origin, to avoid contact with SE organisms used later in a challenge on breeders of groups 1–3. All breeders of the four groups were receiving the same feed and water as recommended by the National Research Council [24].

Vaccination: Breeders in groups 1–2 were administered an experimental acetone-killed SE oil-emulsion vaccine [5]. Briefly, the SE strain used in preparing the antigen of the aqueous phase in the vaccine possessed 14.1 and ~50 kb plasmids. The SE strain originated from liver of a chicken involved in severe salmonellosis outbreaks associated with high virulence and invasiveness of SE to internal visceral organs [4]. The SE cells were grown aerobically in tryptose phosphate broth at a temperature of 37°C for a period of 24 hr. The SE cells were precipitated by centrifugation at $2,000 \times g$ for a period of 15 min, followed by discarding of the supernatant. The SE pellets were washed with sterile saline, stirred with a vortex for 1 min. The process of centrifugation and washing was repeated 3 times, then the pellets were suspended in acetone for 24 hr, washed three times with sterile saline, and then reconstituted in sterile saline to an optical density of 0.908 at a wave length of 540 nm. Incomplete Freund's adjuvant containing non metabolizable mineral oil was used in a mix with the aqueous phase in 1:1 ratio. Each breeder in the first two groups was given 0.5 ml of the experimental vaccine, subcutaneously in the neck, at fifteen weeks of age. This vaccination was repeated four weeks later at 19 weeks of age. Breeders in the third and fourth group were left unvaccinated.

Thymulin and zinc administration: The carboxymethyl cellulose (CMC, Sigma, St Louis, MO, U.S.A.) was used as a carrier in which zinc and thymulin hormone were administered. The carrier contained 1% CMC and 0.9% NaCl. The concentration of thymulin and $ZnCl_2$ per 100 μ l of the CMC carrier was 10 ng and 1 μ M, respectively. One stock of the preparation was divided into individual portions and stored at -20°C. One portion is thawed at each administration time. Each breeder in the first group received 100 μ l of the preparation, intraperitoneally, at intervals of 3 days, for a period of 3 weeks, starting at 15 weeks of age. The total administered thymulin and $ZnCl_2$ per breeder of the first group was 90 ng and 9 μ M, respectively.

Immunoblot sera: All breeders in the four groups were bled from the brachial vein at 15, 17, 19, 22 and 25 weeks of age. Ten serum samples collected at the same age from each group were pooled in equal portions. Each pooled serum sample was stored with an equal volume of sterile glycerol at -20°C for use in Western Immunoblotting.

Immunoblot antigens: The antigens of the same SE strain that was used in preparation of the experimental vaccine were used also in the Western immunoblotting technique.

SDS-PAGE and Western immunoblotting: The banding of the polypeptides of the SE strain (possessing 14.1 and ~50 kb plasmids) was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using the discontinuous buffer system [20]. The weight of protein in the SE strain applied per lane of the SDS-PAGE was 25 μ g, contained in 30 μ l.

The polypeptides of SE resolved on SDS-PAGE gels were electrophoretically transferred onto a nitrocellulose sheet (Bio Rad Laboratory) for performing the Western Immunoblotting [35]. Briefly, each pooled chicken serum sample was diluted 1/250 before adding to the banded polypeptides of the SE strain, present on nitrocellulose strips. Horseradish-peroxidase-labeled antichickens IgG (Nordic Immunological Laboratories Drawer, CA, U.S.A.) was diluted 1/1000 in 1% gelatin TTBS diluent and applied to the cellulose strips for a period of 1 hr at 37°C. The color development reagent 4, chloro-1-naphtol (0.06 g in 20 ml of cold methanol plus 30% H_2O_2 in 100 ml of TBS) was used as substrate to obtain bands of purple color on the nitrocellulose strips within 30 min.

Protection: All birds in groups 1–3 were challenged at 22 weeks of age (three weeks post the second vaccination). The challenge inoculum was prepared by culturing SE cells possessing plasmids 14.1 and ~50 kb from brilliant green agar-seed culture into brain heart infusion broth for 4 hr at 37°C. The culture was adjusted, by sterile saline diluent to a 0.434% optical density at a wave length of 540 nm. Count of the SE live cells was established by making a serial dilution in sterile saline solution with a dilution factor of 1:10. Each dilution was plated onto brilliant green agar plates, using an inoculum of 0.1 ml per plate. Brilliant green agar plates were incubated at 37°C for 48 hr. Plates with colony forming units (cfu) between 30 and 300 colonies were counted to calculate the count in the original challenge-inoculum. Each challenged breeder in the first three groups received 1 ml in the mouth of the brain heart infusion culture, containing 6.7×10^6 cfu/ml, using a 15 cm length pipet. Infection by SE in ceca (cecal content and wall), cecal tonsils, oviducts, and spleens was evaluated at 3 weeks after challenge (25 weeks of age). A piece of about one cm² was cut aseptically from each organ, cut into fine pieces by a sterile scissor, and cultured in 5 ml of tetrathionate broth for 24 hr at 37°C. A loopful of the tetrathionate was subcultured onto brilliant green agar plate and kept at 37°C for 48 hr. One randomly chosen *Salmonella*-suspected colony from each brilliant green agar plate was subcultured individually into triple sugar iron agar slants and onto urea agar plates. Cultures matching *Salmonella*-biochemical characters were subjected to somatic and

flagellar typing [1] to confirm the identity of SE.

Statistics: The Chi square was used to compare among treatments the frequencies of breeders with SE infectivity in different visceral organs. This procedure was accomplished using statistical computing (MStat Computer Program, Michigan State University, Michigan, U.S.A.)

RESULTS

Table 1 shows the specific recognition by antibodies to fimbriae protein of SE in the different breeder groups. SE-vaccinated breeders in groups 1, treated with thymulin and zinc, showed the earliest immunopotentiality to fimbriae of SE, in which antibodies were able to recognize the fimbriae, just two weeks following the first SE-vaccination (at 17 weeks of age); however, in SE-vaccinated breeders of group 2, deprived of thymulin and zinc treatment, the recognition came late, three weeks post the second SE-vaccination (at 22 weeks of age). In the unvaccinated challenged breeders of group 3 that were also deprived of thymulin and zinc, recognition of fimbriae was the latest, occurring at three weeks post challenge (25 weeks of age). In addition, only breeders of group 4 that were deprived of vaccination, thymulin and zinc, and of challenge, didn't show any recognition to fimbriae protein, a sign of absence of false binding between non-specific antibodies and banded fimbriae of SE on the cellulose membrane.

Table 2 shows protection against SE infection in different internal organs of different breeder groups at 3 weeks post challenge. Challenged SE-vaccinated breeders, immunopotentialized by thymulin and zinc (group 1), showed the lowest infection in their ceca (1/10) (10%) in comparison to challenged SE-vaccinated breeders in group 2 (3/9) (33.3%), and challenged unvaccinated breeders in group 3 (2/9) (22.2%) ($P>0.05$). In addition, among challenged breeders, only the thymulin and zinc immunopotentialized breeders of group 1 showed an absence of infection in their cecal tonsils (0/10)

(0%). The SE-vaccinated breeders of groups 1 and 2 were the only breeders among challenged ones showing no invasiveness of the SE organisms to oviducts and spleens (0%).

DISCUSSION

The earliest specific immunopotentiality to fimbriae of SE occurred in breeders administered thymulin and zinc (Table 1); this is most likely due to the effect of the immunopotentialization on thymic cells [30], thus inducing the thymus dependent humoral antibody responses [25]. Previous works showed that zinc supplementation results in significant induction of antibodies specific to bovine herpes virus-1 (BHV-1) in feed calves [32] and in increasing lymphocyte proliferation and polymorphonuclear activity in Down's syndrome subjects [21]. Zinc unavailability diminished the induction of immune response to vaccination with *Mycobacterium bovis* BCG [22]. In other works, thymulin has proven to have the ability to induce the expression of a variety of T-cell differentiation markers and to enhance T-cell functional activities *in vivo* and *in vitro* [18, 19], to increase mature T lymphocytes [26], and to function as an immunomodulator by exerting control on cytokine production by peripheral blood mononuclear cells [29]. The immunomodulation in animals or birds by the use of killed vaccines containing weak immunogens could be the reason of their failure to induce decent immunological responses [5, 15], especially the failure in immune responses targeted against important immunogens needed in interception with the pathogenesis of this infection, such as the adhesion protein(s) or fimbriae in case of SE [3, 7].

This early and consistent presence of antibodies specific to adhesion protein of SE (fimbriae of ~21 kDa) in thymulin and zinc-immunopotentialized breeders of group 1 at 17, 19, 22 and 25 weeks of age (Table 1) could have resulted in an appropriate protection against adhesion of SE organisms targeting host cells, thus intercepting with pathogenesis of this

Table 1. Induction of early immunopotentiality to fimbriae of *Salmonella* Enteritidis^{a)} (SE) by administering thymulin and zinc^{b)} to SE-vaccinated^{c)} chicken breeders

Breeder Group	Treatment	SE-vaccination	SE-challenge ^{d)}	Recognition ^{e)} of SE-fimbriae (21.5 kDa) at different ages in weeks				
				15 (1 st SE-dose)	17	19 (2 nd SE-dose)	22 (SE-challenge)	25
1	Thymulin-Zn	Yes	Yes	—	+	+	+	+
2	NA ^{f)}	Yes	Yes	—	—	—	+	+
3	NA	NA	Yes	—	—	—	—	+
4	NA	NA	NA	ND ^{g)}	—	—	—	—

a) Antibodies specific to fimbriae of SE are detected by western immunoblotting of pooled sera from each group collected at a specific time. The pooled sera is reacted against banded polypeptides of SE acquiring 14.1 and ~50 kb plasmids.

b) Each breeder received 10 ng – 1 µM of thymulin – ZnCl₂ per 100 µl injection given intraperitoneally, at intervals of 3 days, for a period of 3 weeks starting at 15 weeks of age.

c) Each SE vaccinated breeder received 0.5 ml of the killed bacterin, subcutaneously in the neck, at 15 and 19 weeks of age.

d) Each SE challenged bird received in the mouth 1 ml of a 4 hr SE culture containing 6.7×10^6 cfu/ml. The challenge was done at 22 weeks of age.

e) The recognition of fimbriae protein (21.5 kDa) by the specific antibodies in the pooled sera collected at a specific age from the respective group of breeder is designated by +, while the absence of antibodies specific to fimbriae or the absence of recognition is designated by —.

f) NA=Not applicable.

g) ND=Not done.

Table 2. Protection against infection by SE in visceral organs at 3 weeks (25 weeks of age) post challenge of the different breeder groups

Breeder Group ^{a)}	Frequency ^{b)} (%) of breeders with SE infection in different organs			
	Ceca	Cecal tonsils	Oviduct	Spleen
1	1/10 (10.0) ^{c)}	0/10 (0.0) ^{c)}	0/10 (0.0) ^{c)}	0/10 (0.0) ^{c)}
2	3/9 (33.3) ^{c)}	1/9 (11.1) ^{c)}	0/9 (0.0) ^{c)}	0/9 (0.0) ^{c)}
3	2/9 (22.2) ^{c)}	2/9 (22.2) ^{c)}	1/9 (11.1) ^{c)}	1/9 (11.1) ^{c)}
4	0/10 (0.0) ^{c)}	0/10 (0.0) ^{c)}	0/10 (0.0) ^{c)}	0/10 (0.0) ^{c)}

a) The nature of the treatment in each of the four groups of breeders is defined in footnotes of Table 1.

b) Frequency is the number of breeders with SE recovery from the specific organ divided by the number of tested birds in a treatment.

c) Frequencies in a column followed by the same superscript c) are insignificantly different ($P < 0.05$).

SE-strain. As a result, such breeders resulted in the lowest infection of ceca, and absence of cecal tonsil's infection by SE (Table 2), in spite of the fact that in literature it is proven that the highest recovery of SE from challenge birds will be from the cecal tonsils [13]. The cecal tonsils are known to have aggregates of lymphocytes [28] that could have been immunopotentiated by thymulin and zinc in SE-vaccinated breeders. In spite of the delayed immunopotentialization of antibodies specific to fimbriae in breeders of group 2, that were only vaccinated, the recognition at 22 weeks of age, time for challenge (Table 1), was enough to provide complete protection against invasiveness of the SE organisms to oviducts and spleens (Table 2), a situation similar to the complete protection occurring in SE-vaccinated breeders of group 1, given thymulin and zinc. However, the advantage in breeders of group 1 over those in group 2, is that cecal infection is less, and that could lessen the infective dose and the chance of contamination of the egg shell that gets in contact with fecal material present in cloaca of these breeders [17, 33]. The fact that the rate of cecal infection in group 2 (vaccinated and challenged) was higher than that in group 3 (unvaccinated and challenged) ($P > 0.05$) could indicate that SE vaccination without immunopotentiators is not able to raise the local immunity or protection in the ceca.

In conclusion, immunopotentialization of chicken breeders by thymulin and zinc induces the earliest specific antibodies to fimbriae of SE associated with the lowest frequency of SE-infected ceca, and absence of SE infection from cecal tonsils, oviducts and spleens. Future investigation will include the use of continuous delivery system of immunopotentiators on a larger sample size, such as liposome, to reach to a better practical administration procedure.

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