Evaluation of protection induced by *Riemerella anatipestifer-E. coli O78* bacterin in white pekin ducks

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Primary Audience: Duck Producers, Biologics Producers, Veterinarians, Researchers

SUMMARY

Riemerella anatipestifer infection accounts for the most significant economic losses due to infectious disease in commercial White Pekin ducks in the United States. An inactivated bacterin and a live attenuated vaccine are approved for use against *R. anatipestifer* in the United States. This study aimed to evaluate the duration of protection induced by the commercial bacterin containing formalin-inactivated strains of *R. anatipestifer* serotype 1, 2, 5 (RA1, RA2, RA5) combined with *E. coli* 078 (RA-EC). Pekin ducks were vaccinated twice at 2 and 3 wk of age with 0.5 mL of RA-EC bacterin containing approximately 10^8 colony forming units (CFU) of *E. coli* and 10^9 CFU of RA1, RA2 and RA5 per dose (0.5 mL). Four-, 6-, and 8-week-old vaccinated and non-vaccinated control ducks were subsequently challenged with virulent strains of either: *E. coli* 078, RA1, RA2 and RA5. Evaluation of the vaccine protective index, histopathology, and bacteriology indicate RA-EC bacterin provides protection against virulent stains of RA1, RA2, RA5, and *E. coli* O78 throughout the 8-wk commercial Pekin grow-out period.

Key words: *Riemerella anatipestifer*, *E. coli*, vaccination, serotype, bacterin, Pekin duck

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DESCRIPTION OF PROBLEM

Riemerella anatipestifer (RA), previously referred to as *Pasteurella anatipestifer*, was first recognized in 1932 and is currently the most economically significant infectious disease affecting commercial White Pekin ducks (*Anas platyrhynchos domestica*) used in the United States [1]. Acute RA infection primarily affects ducks between 2 to 8 wk of age. Acute infection typically presents as a septicemia characterized by listlessness, respiratory signs, greenish diarrhea, and tremors of the head and neck. The disease typically results in economic losses due to high mortality, poor feed conversion rate, and increased condemnations from polyserositis lesions. Mortality is highly variable, ranging between 1to 75% and is dependent on factors such as the age and level of protective immunity in the flock and serotype of RA [2]. Management

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of the disease is complicated by limited cross protection between serotypes, high occurrence of antibiotic resistance in the field, persistence of chronic subclinical infection in flocks and by prolonged survival of the agent in the environment.

The etiological agent, *R. anatipestifer* is a gram-negative, non-motile, non-spore-forming rod. To date, 21 serotypes designated numerically have been identified worldwide by agglutination reactions [2]. RA serotypes 1, 2, and 5 are the most common serotypes identified in commercial ducks in the United States [2, 3]. RA serotypes exhibit type-specific agglutination with homologous antisera, with the exception of serotype 5, which exhibits minor cross reaction with serotypes 2 and 9 [3, 4].

A commercial bacterin with a combination of *R. anatipestifer* 1, 2, 5 (**RA1**, **RA2**, **RA5**) and *Escherichia coli* O78 (**RA-EC**) [5] was developed in the early 1980s. The *E. coli* serotype O78 was determined to be the predominant serotype (59/100) isolated on commercial duck farms in the US in the 1980s [6]. In field trials, the RA-EC bacterin was more effective at reducing RA mortality in White Pekins compared with a bacterin containing RA1, RA2, and RA5 without the *E. coli* component [1, 6]. Hence an *E. coli* serotype O78:K80:NM was selected for inclusion in the RA-EC bacterin. Previously published laboratory trials indicated that vaccination of ducks with RA-EC bacterin at 2 and 3 wk of age provided significant protection of ducks challenged with either *E. coli* O78, RA1, RA2, or RA5 at 4 wk of age [6]. However, there are no published reports indicating if vaccination with RA-EC bacterin induces adequate protection that persists throughout the grow-out period in commercial Pekin ducks. Most commercial White Pekin ducks are marketed between 7 to 8 wk of age. The aim of this study was to determine if protection induced by RA-EC vaccination extends throughout the market age of ducks.

MATERIALS AND METHODS

Experimental Ducks

The procedures used for the evaluation of RA-EC bacterin efficacy in Pekins were approved by the Institutional Animal Care and Use Committee of Cornell University. The 240 White Pekin ducks for this experiment were obtained from the Cornell University Duck Research Laboratory (DRL) which is a closed, unvaccinated RA free flock. From day of age and throughout the duration of the experiment, ducks were housed in environmentally controlled, positive pressure isolation at the DRL. Ducks were provided with commercial duck feed [7] and water ad libitum. Each experimental group of 10 was kept separately in isolation facilities.

Production of RA-EC Bacterin

All bacterial cultures used in the production of formalin inactivated RA-EC bacterin originated from field isolates from naturally occurring disease outbreaks on commercial Pekin duck farms in the United States. The *E. coli* isolate was a pathogenic 1980 strain of serotype O78:K80:NM originally isolated at the DRL from an *E. coli* outbreak in 2-week-old commercial White Pekin ducks. RA1 and RA2 were originally isolated in 1976 from two separate flocks of 2-week-old White Pekins. The RA5 was originally isolated in 1976 from 3.5-week-old Pekins. The serotyping of RA isolates was done by plate agglutination and gel diffusion methods previous described [3]. The cultures for the RA-EC bacterin were initially propagated by harvesting the cultures of RA1, RA2, RA5 and *E. coli* O78 on trypticase soy agar (TSA) with 0.05% yeast extract [8] incubated at 37°C for 24 hr. The methodology for the RA-EC bacterin production from the TSA plated cultures, bacterin safety and protective index 2 wk post vaccination have been previously described [6, 9]. The RA-EC formalin inactivated bacterin contains approximately 10^8 cells of *E. coli* and 10^9 cells of RA1, RA2, and RA5 per dose (0.5 mL).

Production of Challenge Cultures

Bacterial cultures used in challenge experiments were virulent strains of *E. coli* O78:K80, RA1, RA2, and RA5. These strains were originally isolated at the DRL from commercial White Pekin duck farms in the US. Lyophilized challenge cultures were suspended in TSB, grown separately in TSA for 24 hr and harvested

Table 1. Comparison of protective index in bacterin immunized Pekin ducks challenged with RA1, RA2, RA5, or *E. coli*.

Challenge[1]		Age of Pekins[3]					
		4 wk		6 wk		8 wk	
	Exp group[2]	Mortality	PI[4]	Mortality	PI	Mortality	PI
RA1	vaccinates	3/10	70*	0/10	100*	0/10	100*
RA 1	controls	10/10		5/10		5/10	
RA2	vaccinates	1/10	90*	0/10	100*	1/10	80
RA 2	controls	10/10		8/10		5/10	
RA5	vaccinates	0/10	100*	0/10	100*	0/10	100*
RA 5	controls	10/10		10/10		7/10	
E. coli	vaccinates	1/10	89*	0/10	100*	0/10	100*
E. coli	controls	9/10		6/10		6/10	

[1]Challenge cultures RA1, RA2, and RA5 refer to pathogenic strains of *Riemerella anatipestifer* serotype 1, serotype 2 and serotype 5.

[2]Groups consist of either vaccinates or non-vaccinated control groups. The vaccinated groups were inoculated subcutaneously with 0.5 mL of RA-EC bacterin at 2 and 3 weeks of age.

[3]Both vaccinates and controls were challenged with pathogenic strains of either *E. coli* O78:80, RA1, RA2, and RA5 at 4, 6, and 8 wk of age. These ages correspond to 2, 4 and 6 wk after the administration of the first bacterin.

[4]Protective Index (PI) was calculated as [(% mortality in controls –% mortality in vaccinates)/(% mortality in controls)] × 100.

*Protective indices delineated with an (∗) indicated statistically significant mortality rates between vaccinates and control groups at $P < 0.05$.

in normal saline solution. Plate count titrations of harvested bacterial cultures were performed to obtain 6.5×10^9 CFU/mL *E. coli*, 4.8×10^9 CFU/mL RA1, 6.5×10^9 CFU/mL RA2, and 3.0×10^9 CFU/mL RA5 based on the previously determined median lethal dose for each strain (LD_{50}).

Experimental Design

A total of n = 240 White Pekins were required for all experiments. Bacterin inoculations were performed in 120 of the white Pekins twice at 2 and 3 wk of age with 0.5 mL of RA-EC bacterin subcutaneously (**s.c.**) in the dorsal neck. The other 120 Pekins were unvaccinated and raised separately from the vaccinated birds. Four experimental groups, each consisting of 10 vaccinated and 10 unvaccinated controls were challenged with 0.5 mL of either RA1, RA2, RA5 or *E. coli* cultures administered s.c. in the medial thigh. These challenge experiments were conducted three times in 4-, 6-, and 8-week-old ducks (Table 1). Ducks were observed twice daily for mortality and morbidity. The vaccine Protective Index (PI) was calculated as [(% mortality in controls – % mortality in vaccinates)/(% mortality in controls)] × 100 (Table 1).

At the end of 7 d post challenge, surviving ducks were humanely euthanized with CO_2 gas and necropsied. At necropsy, heart and brain swabs were taken from each duck in each experimental group for bacteriology. Swabs were streaked on TSA with 0.05% yeast extract incubated in 5% CO_2 for 24 to 48 hr at 37°C. Number of samples positive for *E. coli* and RA were recorded. Serotyping of RA positive cultures recovered from necropsy was performed using the plate agglutination test as previously described [3]. Evaluation of bacterin efficacy was based on the vaccine PI, re-isolation of *E. coli* or RA serotypes post challenge, evaluation of histopathology post challenge and the serological response to vaccination.

Serology

Sera were collected from 40 vaccinates (4 replicate pens with 10 ducks/pen) and 10 controls at 4, 6, and 8 wk of age for tube agglutination tests. The geometric mean titers (**GMT**)

Table 2. Geometric Mean Titers (GMT) against RA1, RA2, RA5, and *E. coli* antigens in Pekin ducks vaccinated with RA-EC bacterin at 2 and 3 wk of age.[1]

	GMT ± SD post vaccination[3]		
Antigen[2]	4 wk	6 wk	8 wk
RA1	0.40 ± 0.00[a]	0.47 ± 0.13[a]	2.20 ± 0.19[d]
RA2	0.40 ± 0.00[a]	0.48 ± 0.14[a]	2.18 ± 0.20[d]
RA5	0.40 ± 0.00[a]	0.48 ± 0.14[a]	2.25 ± 0.18[d]
E. coli	0.46 ± 0.13[b]	1.30 ± 0.00[c]	2.03 ± 0.15[d]

[1]GMT values based on n = 40; four replicate pens with 10 ducks/pen.

[2]Whole cell cultures of RA1, RA2, and RA5 and *E. coli* used as antigen in the tube agglutination test.

[3]Geometric mean titers (GMT) ± standard deviation (SD) measured in 4-, 6-, and 8-week-old ducks.

[a–d]Values with different superscripts differ significantly by ANOVA and Tukey's HSD ($P < 0.01$).

against RA1, RA2, RA5, and *E. coli* antigens was calculated at 2, 4, and 6 wk post vaccination (corresponds to 4, 6 and 8 wk of age). Lyophilized cultures [5] of RA1, RA2 and RA5 and *E. coli* were used as whole cell antigen. The cultures were reconstituted separately in 9 mL trypticase soy broth [7] and 0.1 mL was spread onto TSA containing 0.05% yeast extract [7]. Cultures on TSA were incubated at 37°C in 5% CO_2 for 24 to 48 hr and harvested in 0.2% formalinized physiological saline (FPSS). The cells were centrifuged, washed with FPSS and resuspended in FPSS to an optical density of 0.4 at 525 nm with a spectrophotometer [10]. Tube agglutination tests were conducted using two-fold serial dilutions of sera from 1:5- 1:640. Equal volumes (0.5 mL) of whole cell antigen and diluted antisera were mixed and incubated and read at 24 and 48 hr. Reference positive and negative sera were run with each test serum. Titers were recorded as the highest serum dilution with visible agglutination [11]. Titers were expressed as the GMT ± standard deviation (SD) (Table 2).

Histopathology

Heart, brain, and liver tissue sections were taken at necropsy from each duck in each experimental group. Tissues were fixed in 10% buffered formalin, sectioned at 4 μm, stained with hematoxylin and eosin and examined by light microscopy.

Bacteriology

Hearts and brain swabs were taken from each duck in each experimental group. Swabs were streaked on TSA with 0.05% yeast extract incubated in 5% CO_2 at 24 to 48 hr at 37°C.

Statistics

A one-sided Fisher's Exact Test was used to compare the difference in mortality rates between vaccinates and controls. Table 1 shows which mortality rates were statistically significant ($P < 0.05$). Analysis of variance (ANOVA) and post-hoc Tukey's Honest Significant Difference (HSD) was used to determine statistically significant differences ($P < 0.01$) in GMT against RA1, RA2, RA5 and *E. coli* antigens (Table 2). All analyses were conducted using SPSS V22 [12].

RESULTS AND DISCUSSION

The RA-EC bacterin induced a high protective index (70 to 100%) in experimental groups challenged with pathogenic strains of RA1, RA2, RA5, and *E. coli* 4, 6, and 8 wk of age (Table 1). There was no mortality in any of the vaccinates challenged with RA5 at 4, 6, and 8 wk of age (Table 1). Results suggest that the RA-EC bacterin was most efficacious against RA5 (Table 1). This result is also supported by observation in the field [13] and prior unpublished DRL challenge experiments. There was no mortality in any of the vaccinates challenged at 6 and 8 wk of age except for the RA2 group at 8 wk of age. In 8-week-old ducks, the mortality induced by RA2 was not statistically different between the vaccinates and controls in spite of the high protective index of 80%. In all other experiments, the mortality rate in vaccinates was statistically lower than the mortality rates in controls at 4, 6, and 8 wk of age. The highest overall mortality for vaccinates occurred in the 4 wk of age challenge experiments, and this age also correlates with the lowest GMT titers calculated. This indicates that vaccination-induced immunity offered less protection 1 wk post vaccination (4 wk of age) compared to protection at 6 and 8 wk of age.

The tube agglutination test was used to monitor the post-vaccination serological responses in RA-EC vaccinated ducks. All non-vaccinated controls were negative for agglutination when tested against RA1, RA2, RA5, and *E. coli* antigens at 4, 6, and 8 wk of age. Antibody titers against RA1, RA2, and RA5 antigens did not show a significant increase at 4 and 6 wk of age compared to controls. Despite the low GMT in these vaccinates, the PI was high (70 to 100%) at the 4- and 6-wk time period. By 8 wk of age, the antibody titers against RA1, RA2, and RA5 were significantly higher than titers at 4 and 6 wk of age. This also correlates with the lowest overall mortality rates being observed at the 8 wk of age challenge period. Results suggest that seroconversion in ducks vaccinated with RA-EC occurs between 3 to 5 wk post vaccination. At 4 wk of age, the antibody titers against *E. coli* was significantly higher compared to titers against RA1, RA2, and RA5. The titers against *E. coli* antigen also increased significantly between 4 and 6 wk of age and again between 6 and 8 wk of age time points. In evaluating statistical differences in GMT within age groups there was no significant difference among the different antigens (RA1, RA2, RA5, and *E. coli*) at 8 wk of age, but at 4 and 6 wk of age, the titer against *E. coli* was statistically higher than the titers elicited against the other antigens, RA1, RA2, and RA5.

In spite of the high PI in vaccinates, the GMT against RA1, RA2, RA5 antigens was generally low (0.40) 1 wk post vaccination. Haiwen et al. reported antibody titers (>2.5) one week after the second vaccination with a trivalent bacterin containing RA serotypes 1, 2, and 10 using ELISA [14]. For interpretation of antibody titers in ducks, the functional inadequacies of duck antibodies and the lack of effectiveness of duck antibodies in precipitin and agglutination tests need be considered [15]. Ducks frequently elicit a weak antibody response to antigenic stimulation from bacterial pathogens, and antibody response alone cannot be used a measure of vaccine efficacy and protection. In addition to antibody immunity, efficacy of RA vaccination is also attributable to the additional protective effect of complement and phagocytic cells, which are activated against extracellular bacteria in septicemic and tissue stages of acute infection [16].

RA is a facultative intracellular pathogen. Cell-mediated immunity induced by antigen-specific T lymphocytes is a significant mechanism of protection against intracellular bacteria. In vitro assessment using the lymphocyte transformation and ELISA tests indicates that the reduction in immune protection against RA is due to the transient nature of the cell-mediated immune response. Due to the difficulties in interpretation of protection based solely on the immune response, the protective index (PI) is typically used to assess the efficacy of RA vaccination for licensed RA bacterins in the United States [13].

In spite of the high protective index and an overall statistically significant decrease in mortality in vaccinated groups challenged at 4, 6, and 8 wk of age, some low-level mortality and morbidity did occur in some of the vaccinated groups. All unvaccinated control groups had more severe septicemic lesions than their vaccinated counterparts. In RA and *E. coli* challenge control groups, the polyserositis lesions were characterized by fibrinous pericarditis and myocardial hemorrhages (Figure 1), hepatitis, airsacculitis, pneumonia, and swollen, mottled spleens. Diffuse lymphocytic meningitis was also a significant finding in many RA-challenged controls (Figure 2). In the *E. coli*-challenge controls, livers were generally discolored dark green. This discoloration was generally not observed in the RA-challenge groups. There were no other macroscopic or microscopic lesions that facilitated differentiation of the different serotypes of RA from each other or from *E. coli*.

In the vaccinated RA1, RA2, and *E. coli* groups that died post challenge, petechial hemorrhages in the heart were the main lesions observed. *Staphylococcus aureus* was isolated from the hearts of the ducks that did not survive the challenge from the vaccinated groups. Challenge bacteria was re-isolated from all of the unvaccinated controls from RA1, RA2, RA5, *E. coli* at 4, 6, and 8 wk of age. Challenge bacteria was not re-isolated from the vaccinated groups except for RA2 at 8 wk of age, where RA2 was

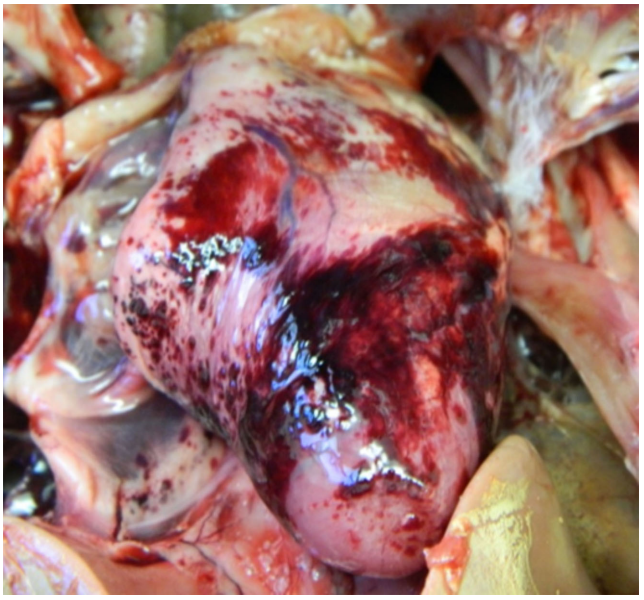

Figure 1. Petechial and ecchymotic myocardial hemorrhages in the heart of a 6-week-old control Pekin duck that died 2 d post challenge with RA5.

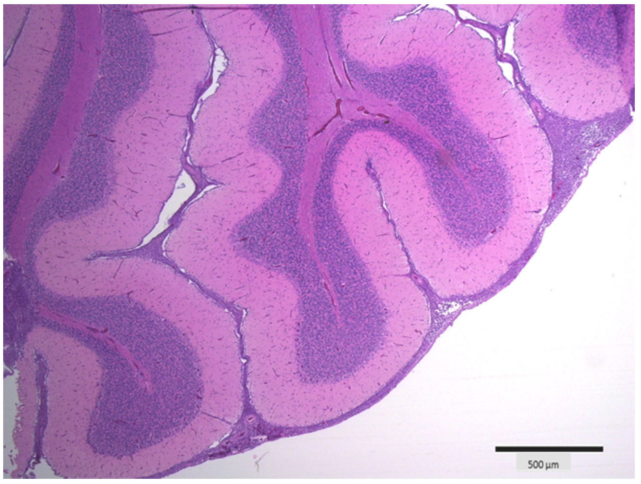

Figure 2. Photomicrograph of lymphocytic meningitis from the brain from 4-week-old Pekin control duck challenged with RA2. Hematoxylin and eosin stain. Bar = 500 μm.

isolated from the heart of the one bird that did not survive challenge.

CONCLUSIONS AND APPLICATIONS

1. Based on the protective index (70 to 100%) and post mortem evaluation of challenged Pekins, RA-EC bacterin provides adequate protection against RA serotypes 1, 2, and 5 and *E. coli* O78 in ducks up to 8 wk of age.
2. The protective index calculated from challenge studies gave a more accurate indication of efficacy of RA-EC vaccination compared to the measurement of GMT titers.
3. Experiments were conducted up to 8 wk of age since ducks exhibit an age

resistance to acute RA infection. Further research is needed to indicate the age that immunity in RA-EC vaccinates decreases to non-protective levels.

4. Further research is needed to determine the effect of RA-EC vaccination on cell-mediated immunity.

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