ABSTRACT  Recent studies have proven that Enterococcus faecalis (1.5 × 10⁷ live bacteria from a tryptic broth culture given s.c. or intra-abdominally (IA) to 5-wk-old broilers) caused pulmonary hypertension syndrome (PHS) in 97% of the birds within 48 h. Definitive diagnosis of PHS was made at necropsy by observing a cavity on the surface of the right ventricular wall and by increased ratio of left ventricular weight to total ventricular weight. A nonlethal method of diagnosing PHS would enhance the study of PHS and alert production poultrymen to the onset of ascites (waterbelly), which is the culminating event of PHS. In the present study, serum hemoglobin, glucose, protein, cholesterol, aspartate amino transferase and creatine kinase-MB (myocardial in origin) enzymes, differential leukocyte numbers, and specific antibody levels against Ent. faecalis were evaluated as nonlethal diagnostic indicators of PHS. Decreases in serum protein and cholesterol of 3 and 10%, respectively, plus increases in percentages of basophils and monocytes of 18 and 40%, respectively, appear to indicate that PHS has been initiated. An agglutinating antibody, specific against Ent. faecalis, but not against other closely related bacteria, has been developed. Presence of this antibody in a bird means that the bird has previously encountered Ent. faecalis. Thus, this antibody may become a diagnostic for PHS in fast-growing chickens.

(Key words: pulmonary hypertension syndrome, serum, leukocyte, Enterococcus faecalis, immunology)

INTRODUCTION

Pulmonary hypertension syndrome (PHS) in fast-growing chickens is thought to result from hypoxia brought on by increased blood pressure in the pulmonary tree. A number of environmental, nutritional, genetic, and pathological factors have been implicated as causes of PHS in broilers (Julian, 1990). The initial sign of PHS is formation of a cavity on the exterior surface of the right ventricular wall (RVW). As the condition progresses, right ventricular dilation and hypertrophy occur, followed by increased blood viscosity, reduced oxygen availability, congestive heart failure, and finally, ascites fluid filling the abdominal cavity, that is, waterbelly (Julian, 1985, 1993; Maxwell et al., 1992; Mirsalimi et al., 1992, 1993; Julian and Squires, 1994; Beker et al., 1995; Fedde and Wideman, 1996).

The incidence of ascites has increased over the past several years (Julian et al., 1986). In fact, Maxwell and Robertson (1997) presented a survey of the worldwide incidence of ascites. They reported information from 18 broiler-producing countries showing that ascites affected 4.7% of broilers worldwide. They estimated that losses attributable to PHS approximated $500 million annually.

Even though there are many physiological explanations as to why PHS occurs, Tankson et al. (2001, 2002a, 2002b, 2002c) have added Enterococcus faecalis as another causative agent. They showed that at 48 h after injecting chickens with 1.5 × 10⁷ Ent. faecalis either i.v. or intra-abdominally (IA), the initial symptom of PHS, that is, cavity formation on the RVW, occurred. Additional morphological symptoms were assessed that indicated the ratio of right ventricular weight to total ventricular weight in both wet (tissue weighted directly after removal) and dry (dried until all water was removed) organs, as well as wet right ventricular weight, were increased in Ent. faecalis-challenged birds. These findings suggest that the birds were experiencing PHS.

At present, to determine if chickens are expressing the pre-ascitic signs of PHS, they must be killed and necropsied. Various hematological assays have been evaluated as a means of detecting PHS (Maxwell et al., 1986, 1987;
field isolate were streaked onto separate tryptic-soy-agar plates and cultured for 24 h (at 35 to 37 C). After sufficient bacterial growth (24 h), gram stains were performed, and the Crystal Identification System was used to identify each bacterium. This procedure confirmed that the field strain and the ATCC strain were both *Ent. faecalis*. The Crystal Identification System was chosen because of its consistent qualitative results (Holmes et al., 1994; Wauters et al., 1995; Peele et al., 1997).

**Trial 1**

A total of 252 male chicks were used in this trial. Forty-two chicks were placed in each of six floor pens. At 21 d of age, each of the birds in three of the pens received 0.5 mL of sterile tryptic soy broth IA. These birds served as controls. The birds in the other three pens received 0.5 mL of tryptic soy broth, which contained 1.5 × 10^7 *Ent. faecalis* IA and were termed challenged birds. This dose level of *Ent. faecalis* has been shown to be the optimal inoculum dose to cause PHS in growing broilers with 48 h (Tankson et al., 2002b).

At 48 h post-injections, 34 birds from each pen were selected at random, and they were bled by cardiac stab, killed by cervical dislocation, and the heart was inspected for the presence or absence of a cavity on the RVW. The remaining eight birds in each pen were nonexperimental birds which were maintained to insure density of 0.07 m^2^-bird^-1.

Blood samples were immediately aliquoted into each of two vacutaner tubes. One contained EDTA as an anticoagulant, and the other did not contain any anticoagulant. The nonanticoagulated tubes were allowed to clot for 2 h (at 37 C), then serum was decanted and stored at −20 C for later analysis. Anticoagulated blood samples were gently rotated mechanically for 10 min and then stained using Wright’s stain (Schalm, 1965). Differential leukocyte numbers were determined microscopically according to criteria established by Lucas and Jamroz (1961). Hemoglobin levels were determined with standard laboratory procedures (Tietz, 1976). Serum samples were thawed, and then biochemical analyses were performed with enzymatic methods incorporated in an autoanalyzer (Elliott, 1984). Serum glucose, total protein, cholesterol, aspartate amino transferase (AST) enzyme, and creatine kinase –MB enzyme were conducted on all serum samples.

**Trial 2**

In Trial 2, 192 male chicks were placed at random in 4 floor pens, thus each pen housed 48 chicks. On Day 1 (day of placement), 24 chicks from each pen were sensitized to *Ent. Faecalis*, and the other 24 birds in each pen received an equal volume of saline. This was accomplished by injecting each chick i.p. with 0.5 mL of sterile saline containing 200 to 300 killed *Ent. faecalis* or with 0.5 mL of sterile saline. The *Ent. faecalis* used in this trial were sub-

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1Fisher Scientific Company LLC, Houston, TX.
2Becton Dickinson and Co., Cockeysville, MD.
ject to autoclave conditions (120 C for 20 min). At the end the autoclave period, tryptic soy agar plates were streaked with putative dead bacteria and colony growth did not occur. Thus, autoclaving was lethal to Ent. faecalis in this study, as shown in previous studies (Tankson et al., 2002c)

On Day 35, 24 chicks (i.e., 12 chicks that had been sensitized with Ent. faecalis on Day 1, as well as 12 chicks that had only received saline on Day 1) were challenged i.p. with 0.5 mL of 1.7 x 10^5 autoclaved Ent. faecalis. The other 24 birds in each pen received 0.5 mL sterile saline i.p. This protocol insured four replications of a 2 x 2 factorial design for the presence and absence of sensitization at Day 1 and presence and absence of challenge with Ent. faecalis on Day 35.

Immediately after challenge and on Day 42, that is, 7 d after challenge, each bird was bled via venipuncture, and approximately 2 mL of blood was allowed to clot in sterile vacutainer tubes. Clotted samples were incubated at 37 C for 2 h, antisera were decanted into sterile vials, and frozen at –20 C for later analyses.

Test Antigens

Four test antigens were prepared to assess agglutination levels in each antiserum sample. These antigens were as follows: 1) the field strain of Ent. faecalis, which was used both to sensitize and to challenge birds; 2) the ATCC strain of Ent. faecalis, which was used to verify the field strain; 3) a field strain of Staphylococcus lentus that had been isolated from chickens and identified by using the crystal identification system; and 4) an ATCC strain of Staph. aureus. Agglutination of specific Ent. faecalis antigens and failure to agglutinate the other bacterial antigens were proof that an antiserum sample was specific for Ent. faecalis.

The test antigens were prepared using cultures of each of the four bacterial strains, which had been grown on tryptic soy agar slants for 24 h in a 35 C incubator. Each strain was then cultured in tryptic soy broth for 24 h (35 C). The broth cultures were autoclaved at 120 C for 20 min. After cooling, each culture was centrifuged and the pelleted bacteria were resuspended in sterile saline. Crystal violet (1:100 vol/vol) was added to stain the bacteria, and phenol (0.01/100 vol/vol) was added as a preservative. After 24 h refrigeration, each antigen was diluted with saline to a final concentration of approximately 1 x 10^8 bacteria/mL.

Microagglutination Procedure

Standard microagglutination assays (Witlin, 1967) were performed. Each antiserum was serially diluted with saline (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512) so that four separate but identical sets of dilutions were formed. Twenty-five uL of stained antigen was added to each dilution in one set. Thus, each sample was tested against all four antigens.

After the dilutions were made and the antigens were added, microtiter plates were sealed and incubated at 37 C for 24 h. Each plate was evaluated for the presence of agglutinated bacteria. Obvious agglutination appeared as a light blue, spider-web-like layer over all or most of the bottom surface of each well in the microtiter plate. Negative and suspected negative samples exhibited a bright, blue dot on the bottom and at the center of each v-shaped well. Each plate was placed at a 45° angle for approximately 5 min. This allowed the non-agglutinated bacteria, that is, the blue dot in the bottom of a well, to form a straight-line streak on the well surface. Therefore, the last dilution to show positive agglutination (i.e., to exhibit the spider-web-like layer and not form a straight-line streak) was recorded as the titer of the antiserum in log_2 units (e.g., a dilution of 1:2 is log_2 = 1).

Statistical Procedure

In Trial 1, serum biochemical levels were evaluated statistically using a two-way ANOVA within a completely randomized design. Main effects were treatment (n = 2) and replication (n = 3). Effects attributable to replication, as well as the interaction of treatment × replication, were not found. Treatment means were separated using Bonferroni’s multiple range test. In Trial 2, agglutinin levels were analyzed by general ANOVA with main effects of sensitization, challenge, and replication. Differences attributed to replication, as well as replication × sensitization and replication × challenge, were not found. Thus, data were pooled over replications and re-analyzed as a 2 × 2 factorial design. Means were separated using least significant difference. All statements of difference are based on P ≤ 0.05. All statistical procedures were conducted using Statistix 7 Analytical Software (Statistix, 2001).

RESULTS

Biochemical assays for blood chemistries in birds of Trial 1 that were challenged with Ent. faecalis, plus non-challenged controls, are presented in Table 1. Hemoglobin, serum glucose, AST, and creatine kinase-MB were not affected by challenge with Ent. faecalis. However, serum cholesterol and total protein levels decreased in challenged birds.

Differential percentages of leukocytes, as affected by challenge with Ent. faecalis of the birds in Trial 1 are presented in Table 2. Heterophil, eosinophil, and lymphocyte numbers were unaffected by challenge with Ent. faecalis. However, basophil and monocyte numbers in challenged birds were elevated at 48 h post-challenge.

Scoring of hearts of the birds of Trial 1 was not made, but the heart in each bird was visually inspected. All of the birds challenged with Ent. faecalis exhibited a cavity on the RVW, while only two nonchallenged birds possessed this cavity.

Agglutination levels in chicks at 7 d post-challenge with Ent. faecalis are presented in Table 3. Challenge with the field strain increased the levels of anti-Ent. faecalis antibodies, regardless of whether or not the chicks had been...
sensitized at hatching, when compared with chicks that were not challenged. This effect was found when antisera were evaluated serologically with both the field strain and ATCC strain of \textit{Ent. faecalis}.

Sensitization caused a numerical, but not significant, elevation in agglutinin levels against both strains of \textit{Ent. faecalis}, as compared with the chicks that were not sensitized. The mean agglutinin level for all antisera that were tested with the field strain of \textit{Ent. faecalis} was 3.74, while that for antisera tested against the ATCC strain was 3.51. Thus, antisera reacted equally well against both strains of \textit{Ent. faecalis}. It should be noted, however, that sensitization without challenge against both the field strain and ATCC strain resulted in only marginal agglutinin levels of 0.64 and 0.51, respectively. However, this is expected, because titers that may have developed after the challenge on Day 1 would have dissipated by Day 42 when antisera samples were collected.

The antisera raised against \textit{Ent. faecalis} did not react with either of the \textit{Staphylococcus} antigens, with the exception of one bird. This particular bird had been sensitized but not challenged with \textit{Ent. faecalis}, and the titer was only 1:2. Antisera were specific for \textit{Ent. faecalis} and not for \textit{Staph. lentus} or \textit{Staph. aureus}.

**DISCUSSION**

The findings that both serum protein and cholesterol were decreased in \textit{Ent. Faecalis}-challenged birds is interesting. Julian (1990) indicated that fluid pressure in the liver is the major cause of fluid accumulation in the peritoneal cavity. When fast-growing chickens are experiencing ascites, serum protein decreases (Cardenas et al., 1985; Biswas et al., 1995). Therefore, the decrease in serum protein in chicks challenged with \textit{Ent. faecalis} support the findings of previous authors.

At 48 h after challenge with \textit{Ent. faecalis}, fluid accumulation in the peritoneal cavity is not readily apparent; however, many birds exhibit increased fluid accumulation in the pericardial sac, extra hepatoperitoneal space, and abdominal cavity. Reductions in both serum protein and cholesterol may well be physiological adaptive responses to the impending loss of extracellular fluids via ascites.

Hemoglobin and hematocrit levels are usually elevated in chicks experiencing ascites (Maxwell et al., 1986, 1987; Niazi et al., 1989; Odom et al., 1989; Witzel et al., 1990; Beker et al., 1995). If PHS had been monitored as it progressed in birds of this study, it is likely that hemoglobin levels would have increased due to fluid loss.

Blood levels of AST and creatine-kinase MB are early indicators of myocardial damage in humans (Wirz et al., 1990; Tanasijevic et al., 1997; Falahati et al., 1999) and in

![TABLE 1. Mean ± SEM of blood chemical parameters in fast-growing chickens at 48 h postchallenge with \textit{Enterococcus faecalis}](https://academic.oup.com/ps/article-abstract/81/12/1826/1544354)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Ent. faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>6.19 ± 0.12</td>
<td>6.24 ± 0.16</td>
</tr>
<tr>
<td>Serum glucose (mg/dL)</td>
<td>251.50 ± 4.66</td>
<td>252.00 ± 5.49</td>
</tr>
<tr>
<td>Serum protein (g/dL)</td>
<td>92.39 ± 2.93</td>
<td>82.64 ± 3.11*</td>
</tr>
<tr>
<td>AST1 (U/L)</td>
<td>3.03 ± 0.05</td>
<td>2.93 ± 0.06**</td>
</tr>
<tr>
<td>AST2 (U/L)</td>
<td>158.48 ± 3.89</td>
<td>177.67 ± 9.90</td>
</tr>
<tr>
<td>CK-MB1 (U/L)</td>
<td>2748.00 ± 112.14</td>
<td>2574.16 ± 143.24</td>
</tr>
</tbody>
</table>

**Means within a parameter differ significantly from the control (P < 0.01).**

1 Aspartate aminotransferase enzyme.
2 Units of enzyme.
3 Creatine kinase (myocardial in origin).
4 Means within each parameter differ significantly from control (P < 0.05).

![TABLE 2. Mean ± SEM of differential leukocyte counts in fast-growing chickens challenged with \textit{Enterococcus faecalis}](https://academic.oup.com/ps/article-abstract/81/12/1826/1544354)

<table>
<thead>
<tr>
<th>Leukocyte (%)</th>
<th>Samples (n)</th>
<th>Control</th>
<th>Ent. faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterophile</td>
<td>204</td>
<td>33.61 ± 2.06</td>
<td>32.77 ± 1.93</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>204</td>
<td>3.06 ± 0.44</td>
<td>3.94 ± 0.35</td>
</tr>
<tr>
<td>Basophil</td>
<td>204</td>
<td>0.06 ± 0.04</td>
<td>0.71 ± 0.25*</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>204</td>
<td>58.39 ± 2.16</td>
<td>57.87 ± 1.72</td>
</tr>
<tr>
<td>Monocyte</td>
<td>204</td>
<td>4.00 ± 0.77</td>
<td>5.60 ± 0.64*</td>
</tr>
</tbody>
</table>

**Pooled means within each parameter differ significantly from the control (P < 0.05).**

**DISCUSSION**

The findings that both serum protein and cholesterol were decreased in \textit{Ent. Faecalis}-challenged birds is interesting. Julian (1990) indicated that fluid pressure in the liver is the major cause of fluid accumulation in the peritoneal cavity. When fast-growing chickens are experiencing ascites, serum protein decreases (Cardenas et al., 1985; Biswas et al., 1995). Therefore, the decrease in serum protein in chicks challenged with \textit{Ent. faecalis} support the findings of previous authors.

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Blood levels of AST and creatine-kinase MB are early indicators of myocardial damage in humans (Wirz et al., 1990; Tanasijevic et al., 1997; Falahati et al., 1999) and in

![TABLE 3. Agglutination levels in chicks at 7 d postchallenge with \textit{Enterococcus faecalis}](https://academic.oup.com/ps/article-abstract/81/12/1826/1544354)

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Sensitization</th>
<th>Pooled sensitization X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field Strain Ent. faecalis</td>
<td>Yes</td>
<td>8.66 ± 0.12</td>
</tr>
<tr>
<td>No</td>
<td>0.64 ± 0.02</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>ATCC strains of Ent. faecalis</td>
<td>Pooled sensitization X</td>
<td>4.65</td>
</tr>
<tr>
<td>Field Strain Staph. lentus</td>
<td>Yes</td>
<td>8.35 ± 0.13</td>
</tr>
<tr>
<td>No</td>
<td>0.51 ± 0.02</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>ATCC strain of Staph. aureus</td>
<td>Pooled sensitization X</td>
<td>4.43</td>
</tr>
</tbody>
</table>

**Means within a parameter differ significantly from the control (P < 0.05).**
chickens (Eppenberger et al., 1967). However, results of this study indicated that these two enzymes are probably not early indicators of PHS caused by Ent. faecalis in chickens as they are in humans.

In a previous report, we documented inflammatory changes in both the lungs and heart of fast-growing chicks (Tankson et al., 2002b). In this study, the percentages of all leukocytes remained within the physiological range (Lucas and Jamroz, 1961); however, increases in both monocytes and basophils were found. Changes in percentages of these leukocytes are expected during inflammatory responses.

The agglutination responses indicate that Ent. faecalis is an effective antigen in chickens. Sensitization at hatching immunized the chicks; however, at 5 wk the primary response was marginal. Yet, challenge at 5 wk resulted in a primary humoral titer that was comparable to other bacteria, for example, Salmonella pullorum and Sal. typhimurium (Buxton, 1954; Williams and Whittemore, 1975). Finally, the interaction of sensitization and challenge activated a pronounced anamnestic response. This response indicated that it is possible to produce protective levels of antibodies against Ent. faecalis.

Challenge studies of birds that have been exposed to Ent. faecalis are mandatory to determine if immunity, as evidenced by the antibody titers in this study, will protect birds from PHS. Envisioned are studies involving sensitization alone, sensitization followed by priming (vaccination), and a single priming dose followed by a challenge dose of Ent. faecalis. Regardless of whether vaccination will ensure protection against PHS, it may be feasible to monitor flocks of birds for previous exposure to Ent. faecalis. Antisera from a representative portion of flocks would be subjected to an agglutination test using Ent. faecalis antigen. As indicated previously, Tankson et al. (2002a) found that approximately 10% of broilers had Ent. faecalis in their heart and lungs at some time during their late embryonic and early post-embryonic periods. Moreover, Maxwell and Robertson (1987) estimated that the incidence of ascites was about 5% on a worldwide basis and about 10% in United States.

In summary, decreased serum levels of protein and cholesterol, along with elevated circulating basophil and monocyte numbers, may be early biochemical and hematological indicators of PHS in fast-growing chickens. Additionally, results herein show that Ent. faecalis is an effective antigen in broilers. It is possible that this antigen can be used to develop a vaccine that will protect chicks from ascites caused by Ent. faecalis. Equally, this antigen may serve as an effective diagnostic to monitor if birds have been exposed to Ent. faecalis.

REFERENCES


