Pulmonary Hypertension Syndrome in Young Chickens Challenged With Frozen and Autoclaved Cultures of *Enterococcus faecalis*[1]

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***Enterococcus faecalis*, when administered in a growth medium or sterile saline, will cause pulmonary hypertension syndrome (PHS) in chickens. The objective of this study was to determine if frozen and/or autoclaved cultures of *E. faecalis* retain ability to evoke PHS. In Trial 1, chicks were inoculated with 3.6×10^7 *E. faecalis* (IA) in tryptic soy broth (TSB) from either a live culture or one that had been autoclaved (120°C for 20 min). Controls received TSB. Autoclaved and live cultures produced the same degree of PHS in a majority of the birds. Trial 2 used the same protocol, except a frozen (–70°C for 60 min) culture of *E. faecalis* was compared with the control. The results agreed with those of Trial 1, i.e., the frozen culture also produced PHS. Trial 3 was conducted to determine if *E. faecalis* caused PHS by producing and releasing some unknown substance into the supernatant. Incidence of PHS was based on percentage of birds exhibiting ascites fluid at 24 hr after challenge. Controls received sterile, frozen, or autoclaved TSB. As compared with controls, those birds that received challenge with *E. faecalis* alone, supernatant alone, and *E. faecalis* plus supernatant from live cultures exhibited similar incidence of ascites, whereas birds that received *E. faecalis* plus supernatant and supernatant alone from cultures that had been either frozen or autoclaved exhibited elevated incidence of ascites as compared with controls. Also, with frozen and autoclaved cultures, those birds that received only pelleted *E. faecalis* exhibited incidence of ascites that did not differ from controls. Apparently, *E. faecalis* produces PHS in chicks by producing and releasing an unknown toxin.** Exp Biol Med 227:812–816, 2002

Key words: *E. faecalis*; chicken; pulmonary; hypertension; supernatant.

Pulmonary hypertension syndrome (PHS), also known as ascites, is a major cardiopulmonary condition in young chickens that causes economic losses by increased incidence of mortality, as well as carcass condemnation at the processing plant. PHS is caused by increased pulmonary vascular pressure, resulting in an overworked right ventricular wall (RVW). The RVW usually undergoes hypertrophy and dilation, eventually leading to right ventricular failure, and finally to accumulation of ascitic fluid in the pericardial sac and/or abdominal cavity (1–15). Many explanations suggest that hypoxia results from a series of physiological agents, which ultimately cause PHS (1, 2, 4, 6, 9).

Enterococcus faecalis (E. faecalis) has recently been added to the list of agents and conditions that cause PHS (16). *E. faecalis* is a gram-positive, facultative anaerobe (17). It will grow in 6.5% NaCl, between 10° and 45°C, at a pH of 9.6, and can survive heating at 60°C for 30 min (18, 19). It is part of the normal flora of the intestinal tract in humans (17) and chickens (20). In humans, this bacterium can cause endocarditis, as well as urinary, intraabdominal, and pelvic infections under appropriate conditions (17).

An early sign of PHS in chickens is a cavity or depression on the external surface of the right ventricular wall, similar to that caused by hypoxia (1, 2, 4, 6–9). One study by Tankson *et al.* (16) showed that this depression occurred on the external surface of the RVW when chickens were administered *E. faecalis*. In this study, a heart scoring system was based upon this visible external cavity on the RVW, and then flaccidity and thinning of the RVW. In another study, Tankson (21) evaluated different morphological characteristics in the heart after administration of *E. faecalis*. These results agreed with literature that measuring the ratio of the RVW to the total ventricular mass (RV:TV) was an indicator of PHS in chickens (8, 23, 24). However, when PHS is caused by *E. faecalis*, the RV:TV ratio has not proven to be as reliable as when PHS is caused by other factors (22). Biochemical and immunological assays also have been conducted after administration of *E. faecalis* (21). Results indicated that decreased blood levels of protein and

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cholesterol, concomitant with increased circulating percentages of monocytes and basophils, were indicators of PHS in its earliest stage. Finally, anti-*E. faecalis* antibody levels may also be of diagnostic value in determining the onset and severity of PHS (21).

In all of these reports, live *E. faecalis* was administered in the media in which it was propagated. Many bacteria are known to produce endotoxins, exotoxins, or both (25). *E. faecalis* has not been shown to release such toxins. However, plasmids from *E. faecalis* are known to release a toxin, cytolysin (26–28). The purpose of this study was to determine if live cultures of *E. faecalis,* as well as cultures that had been frozen (injured) or autoclaved (killed), produce PHS in fast-growing chickens.

Methods

Husbandry. In all trials, male chicks were obtained from a commercial hatchery in Alabama. These chicks were raised using standard broiler management guidelines in a curtain-sided grow-out house on floor pens covered with pine shavings litter. Feed and water were available *ad libitum.* All birds received a diet containing 23% protein and 3194 kcal ME/kg for the first 2 weeks; thereafter, they received a diet containing 20% protein and 3194 kcal ME/kg. These diets have previously been described (21).

Microbiology. *E. faecalis* was obtained from a field strain and was then propagated in the laboratory by reculturing it every 7 days on tryptic soy agar (TSA) slants. An American Type Culture Collection (ATCC) strain of *E. faecalis* was used to confirm the identity of the field strain. Confirmation was accomplished by growing both the ATCC and field strains in tryptic soy broth (TSB) for 24 hr at 35°-37°C and then subjecting samples to a gram stain. Both cultures stained positive and were then identified as *E. faecalis* using the BBL Crystal Identification System (Becton Dickson, Fullerton, CA). The use and accuracy of this system has been verified previously (29–31).

Challenge. Challenge preparations were prepared by serially diluting a concentrated 7-day-old TSB culture of field strain *E. faecalis*. These dilutions facilitated enumeration of bacteria on a volume basis (31). One milliliter of stock culture was diluted so that it contained 1×10^6 bacteria. This volume was confirmed using the conventional plate count methodology. Final preparation of the bacteria occurred within 1 hr of challenging the chickens.

In all three trials, the challenge dose of *E. faecalis* was 3.6×10^7 in 0.5 ml of diluent. This dose was shown to cause PHS in over 90% of fast-growing chickens within 48 hr. In all cases, whether *E. faecalis* was injected in a diluent or the diluent was injected alone, injections were made by the intraabdominal route. Previous studies demonstrated no significant difference between intraabdominal or intravenous injection (21).

Necropsies. Birds were sacrificed by cervical dislocation at 48 hr after challenge. Within 5 min of death, the heart for each bird was removed. Each heart was evaluated by a subjective scoring system (21) or by the presence or absence of ascites fluid. The scoring method involved examining the heart. A normal appearing heart with normal muscular tone was given a score of 1. If a depression or cavity was seen on the external surface of the RVW, the heart received a score of 2. A score of 3 was recorded if the heart possessed a cavity on the RVW, as well as exhibited flaccidity of the musculature of the RVW. Finally, a score of 4 represented the presence of a cavity and flaccidity of the RVW, as well as presence of a thinned section of the RVW.

Trial 1. Ninety male chicks were used in Trial 1. Ten birds were placed in each of nine pens. At 6 weeks of age, three treatments were administered to birds in each of three pens. These treatments were controls, live *E. faecalis*, and *E. faecalis* autoclaved at 120°C for 20 min and then cooled to room temperature. A 1-ml sample of the autoclaved culture was pour plated on a TSA plate, incubated at 35°-37°C for 24 hr and bacterial growth did not occur. Necropsies were performed at 48 hr postchallenge, and hearts were scored according to the scoring system of Tankson (21) within 5 min after death.

Trial 2. Sixty male chicks were allocated to six pens and each pen housed 10 birds. At 5 weeks of age, birds in each of three pens received one of the following treatments: challenge with sterile TSB and challenged with a culture of *E. faecalis* grown in TSB that had been frozen at –70°C for 60 min, then thawed, and allowed to equilibrate to room temperature. A 1-ml sample of this culture was also pour plated on a TSA plate and incubated at 35°-37°C for 24 hr. The organisms were viable. It is not known if freezing causes injury and subsequent release of cytolysin by its plasmids.

Trial 3. A total of 600 birds were used in Trial 3. This trial consisted of two separate replications that were initiated 1 week apart. In each replication, 12 pens were used and each pen housed 25 chicks. At 5 weeks of age, birds in each of the 12 pens in each replication received one of the treatments. At 48 hr postchallenge, each bird was sacrificed and then examined for the presence of ascitic fluid accumulation either in the pericardial sac, hepatoperitoneal area, or in the abdominal cavity. Birds possessing serous fluids in any site or all three sites were recorded as positive for ascites. Samples of serous fluid from the abdominal cavity of 10 birds that received challenge with a live culture of *E. faecalis* were smeared on microscope slides, allowed to air dry, then stained with Wright's stain and evaluated microscopically for the presence of cells.

Statistical Procedures. Results of Trials 1 and 2 were analyzed using Friedman's nonparametric two-way analysis of variance (ANOVA). Observations of heart score were used as the dependant variable, whereas treatments and pens were declared categorical variables. Data of Trial 3 were analyzed by a two-way ANOVA. A significant replication effect was not found; therefore, results were pooled over replications for convenience of presentation. Trial 3

data were then analyzed by a one-way ANOVA. Means were compared using LSD and statements of significance are based on $P < 0.05$ (33).

Results

Figure 1 illustrates heart scores in control birds of Trial 1, as well as those challenged with live and autoclaved cultures of *E. faecalis*. Control heart scores ranked lower than birds challenged with live or dead cultures. In addition, birds challenged with a dead *E. faecalis* culture exhibited heart scores that ranked equal to birds challenged with a live *E faecalis* culture.

Figure 2 depicts heart scores in birds of Trial 2. Chickens challenged with a frozen and then thawed culture of *E. faecalis* received a higher mean heart score ranking than control birds.

Results of Trial 3 are presented in Figure 3. With the live culture, groups that received *E. faecalis* alone, supernatant alone, or *E. faecalis* plus supernatant all expressed similar increases in incidence of ascites as compared with the live culture controls. When frozen and autoclaved cultures were used, the groups that received *E. faecalis* alone did not experience an increased incidence of ascites as compared with controls. However, the groups that received supernatant, either alone or with reconstituted pelleted *E. faecalis*, experienced increased incidence of ascites as compared with controls and birds that received *E. faecalis* alone.

The ascites fluids collected from the abdominal cavity in birds that received *E. faecalis* plus supernatant from a live culture in Trial 3 were examined microscopically. Occasional erythrocytes were noted, but these were the only cells seen. Neither leukocytes nor macrophages were observed in any of the fluid samples.

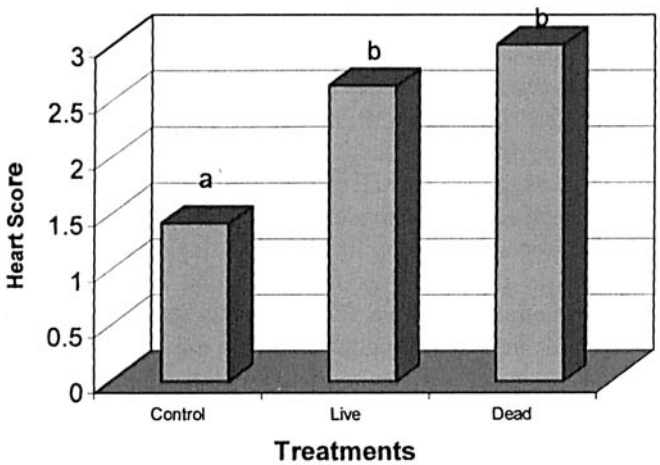

Figure 1. Heart scores in broilers challenged with live and dead cultures of *E. faecalis*. [a,b], mean with different superscripts rank differently ($P < 0.05$; SEM range = 0.00–0.16). Control, challenged with 0.5 ml of sterile TSB; Live EF, challenged with 0.5 ml of 3.6×10^7 *E. faecalis* grown in TSB; Dead EF, challenged with 0.5 ml of 3.6×10^7 *E. faecalis* that had been autoclaved at 120°C for 20 min, then cooled to room temperature. Heart scores are as follows: 1, normal heart; 2, cavity on external surface of the RVW; 3, cavity on RVW, as well as flaccid musculature of the RVW; and 4, cavity, flaccidity, and a thinned sectioned on the RVW.

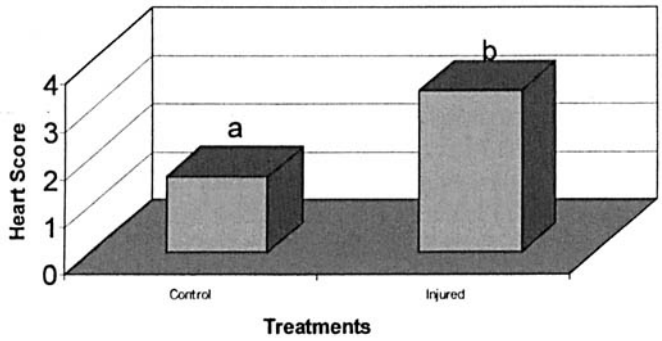

Figure 2. Heart scores of broilers challenge with frozen (injured) cultures of *E. faecalis*. [a,b], mean with different superscripts rank differently ($P < 0.05$; SEM range = 0.07–0.11). Control, challenged with 0.5 ml of sterile TSB; Frozen EF, challenged with 0.5 ml of 3.6×10^7 *E. faecalis* that had been frozen at –70°C for 60 min, then thawed to room temperature. Heart scores are as follows: 1, normal heart; 2, cavity on external surface of the RVW; 3, cavity on RVW, as well as flaccid musculature of the RVW; and 4, cavity, flaccidity, and a thinned sectioned on the RVW.

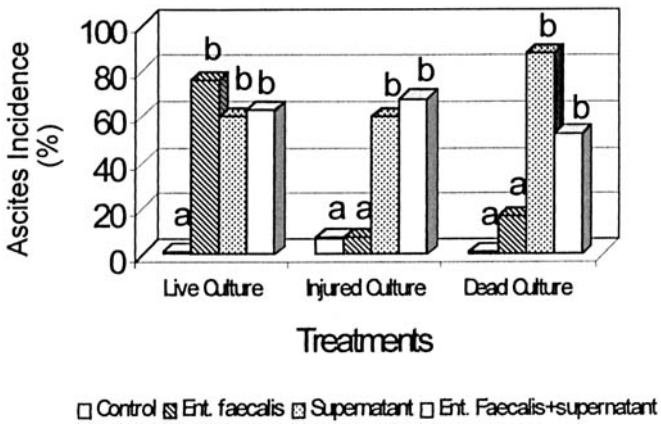

Figure 3. Incidence (%) of birds expressing ascites after challenge with live, frozen (injured), or autoclaved (dead) cultures of *E. faecalis* Control, sterile TSB (treatments 1, 5, and 9); *E. faecalis*, pellet after centrifugation was resuspended in TSB (treatments 2, 6, and 10); supernatant, obtained by pelleting *E. faecalis Treats* (treatments 3, 7, and 11); and *Ent faecalis* + supernatant, whole culture (treatments 4, 8, and 12). The symbols a,b indicate significance of means at $P < 0.05$.

Collectively, the data of these three trials indicate that *E. faecalis* releases a toxin-like substance that is both heat and cold stable. This substance will cause PHS within 48 hr after healthy chicks are challenged.

Discussion

Trials 1 and 2 showed that *E. faecalis* cultures, which had been either frozen or autoclaved, retained the ability to cause PHS in broilers, as indicated by elevated heart scores. However, these results did not partition the causative effect. That is, from the results of Trials 1 and 2, it was impossible to state that PHS was caused by a direct effect of *E. faecalis* or that a toxin or toxin-like substance caused the syndrome. Finally, it is possible that *E. faecalis* acted synergistically in the presence of its secreted toxin to evoke PHS.

Thus, Trial 3 was designed to understand the causative

relationship of *E. faecalis* to PHS in chickens. Results indicate that a live culture of *E. faecalis* did produce a toxin or toxin-like substance that was released into the supernatant. When *E. faecalis* was live, injured (frozen), or dead (autoclaved), the putative toxin was released into the supernatant at levels sufficient to cause expression of ascites fluid in a majority of the birds.

Many gram-positive bacteria, whether alive or dead, produce exotoxin (25). *Clostridium botulinium, Bacillus cereus*, and *Staphylococcus aureus* are examples of disease-producing bacteria that release toxins (34, 35). *Listeria monocytogenes*, another pathogen, also produces an exotoxin called listerolysin that protects phagocytic vesicles and facilitates continued replication (36, 37). Some streptococcal species cause inflammation via teichoic acid and by peptidoglycan fragments located on the cell wall that are similar to lipopolysaccharides located on gram-negative cell walls (38). *Streptococcus pneumoniae* produces pneumolysin, a cytoplasmic protein, which is released from the bacterium only when lysis of the bacterium occurs. Pneumolysin causes inflammatory responses in host tissues, as do other hemolysins such as streptolysin and listeriolysin, the only difference is that the latter two proteins are secreted from live bacteria (39).

Live *E. faecalis* has been reported to produce a toxin termed cytolysin, which is associated with plasmids (26–28). Cytolysin causes lysis of both eukaryotic (hemolysin) and prokaryotic cells (bacteriocin). Even though much is known about the cytotoxic effects of *E. faecalis*, there is no established animal model for assessing the role of cytolysin in enterococcal infections. Freezing and autoclaving live cultures of *E. faecalis* have not been shown to cause the release of cytolysin by plasmids.

Previous reports show that when carbon particles (40) or Sephadex beads (41) were injected into chickens, ascites fluid was expressed. This fluid accumulated in the hepatoperitoneal and abdominal areas within 4 hr after carbon particles were injected, and in the abdominal area, within 40 hr after Sephadex was introduced. In both cases, ascites fluids contained large numbers of all the leukocytic cells, as well as a plethora of macrophages. These responses were described as immune driven hypersensitivity responses. Because reticuloendothelial (RES) cells were not found in abdominal ascites fluids of the birds inoculated with live culture of *E. faecalis* (treatment 4), the classic delayed-type hypersensitivity response (42) to Freud's complete adjuvant in laboratory rodents that ensures accumulation of a large quantity of abdominal ascites did not occur in the chickens of this study.

It is possible, however, that RES cells attacked *E. faecalis* or its toxin-like product in the heart or lungs (or both), resulting in inflammatory by-products that blocked air passageways in the pulmonary tree. Thus, PHS resulted, first with damage to the RVW, then with expression of ascitic fluids. The pattern of leakage from the circulatory system was into the pericardial space, then into the hepatoperitoneal area, culminating with sustained collection in the abdominal area. This condition could also have resulted from a direct intravascular reaction in the pulmonary arteries.

Regardless of the mechanism of action, the present results show clearly that *E. faecalis*-induced PHS appears to constitute an excellent model of bacterial involvement in cardiorespiratory diseases.

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