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Initial evaluation of novel preparations of *Bordetella avium* by determination of antibody-response titers

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ABSTRACT

The efficacy of killed vaccines generally is not equal to live vaccines. However, due to safety and ease of production, they remain a vital part of controlling and preventing diseases. In this study, the immune response to four different vaccination preparation techniques for the agent of bordetellosis of turkeys, *Bordetella avium* (BA), was compared. Preparation/inactivation techniques included (1) formalin inactivation, (2) opsonization of formalin-inactivated BA, (3) buffered acetic-acid BA inactivation, or (4) opsonization of buffered acetic-acid-inactivated BA. Non-adjuvated suspensions containing equal antigen mass were administered subcutaneously (0.2 mL) at day-of-hatch in all cases. For each treatment (N=40/treatment), plasma samples were obtained on d 6, 10, and 21. Specific antibody titer was determined by enzyme-linked immunosorbent assay (ELISA). Results were analyzed by percentage of responders, calculated by determination of sample-to-positive (S/P) ratio. At d 6, the formalin-killed vaccination caused the most rapid response with significantly higher S/P ratios than other treatments. At d 10 there were no significant differences between the treatments. By d 21, formalin-inactivated antigen produced the highest percentage of responders. In this preliminary experiment, neither buffered acetic-acid BA inactivation nor opsonization of inactivated BA antigen improved turkey poult responsiveness to this pathogen.

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INTRODUCTION

Bordetellosis is an extremely contagious poultry disease caused by the organism *Bordetella avium* (BA), a small, Gram-negative bacterium that colonizes in the ciliated epithelium of the trachea of young turkeys (poults). The disease is characterized by interference with the respiratory mucosa and symptoms include sneezing and oculonasal emissions (Skeeles and Arp, 1997). Although it is difficult to determine the precise economic loss to the poultry industry because the disease is often coupled with mortality and morbidity due to secondary infections, estimates of economic losses due to reduced body weight, reduced feed efficiency, and animal mortality are in the millions of dollars each year (USDA ARS, 2002).

Once infection has become apparent, treatments for bordetellosis have focused chiefly on antibiotic treatment rather than preventative care. This approach is slowly being phased out as the industry is moving away from using antibiotics in animals intended for human consumption (USDA ARS, 2002). Additionally, the emergence of antibiotic resistance has become a major concern worldwide. Indeed, several years ago in a document outlining strategies against microbial antibiotic resistance, the European Union labeled the situation as a "public health priority" and addressed the problem by taking steps to restrict antibiotic use to serious humans and animal health problems only (Commission of the European Communities, 2001).

Various studies have focused on treating the problem only after it arises with such treatments as the use of niacin (Yersin, 1991) and a novel oxy-halogen formula (Pardue and Luiginbuhl, 1998), both of which are administered through drinking water. Prevention in the form of vaccination is certainly the most effective as well as economic treatment, but current vaccines are not consistently protective. One research team explored the idea of passive immunity by isolating serum and tracheal remnants from convalescent poults, which resulted in a small reduction of adherence of *B. avium* to tracheal mucosa (Arp and Hellwig, 1988). A temperature-sensitive *B. avium* mutant known as ART-VAX, given via eyedrop/oral and spray cabinet protocols, showed a reduced severity of lesions although it failed to prevent infection upon challenge (Houghten and Skeeles, 1987). A formalin-inactivated bacterin also failed to produce consistent and long-lasting results under a challenge model (Hofstad and Jeska, 1985).

One promising method of vaccine production takes advantage of immune complexes, which are formed during an immune response to a foreign antigen through the binding of highly specific antibodies to a specific antigen. Introducing immune complexes to lymphoid follicles is thought to promote the creation of germinal centers, which in turn leads to the proliferation of memory B cells. This can result in increased antibody pro-

MEET THE STUDENT-AUTHOR

Joel L. Gallagher

After graduating from Ruston High School (La.) in 2002, I decided to attend the University of Arkansas and major in biology on the premedical track. Upon acceptance to the university, I was awarded the Chancellor's Scholarship, and during my junior year I was awarded the Gilbert Premedical Scholarship. I am member of the American Chemical Society, Alpha Epsilon Delta, and Golden Key International. I am also active with Catholic Campus Ministries, where I lead a student group that provides aid and advocacy for the poor.

During the spring semester of my freshman year, I began working for Dr. Hargis with his research on probiotic treatments for *Salmonella* enteriditis. I started my research project examining the immunological responses to novel *Bordetella avium* vaccines two years later, and then applied for and was awarded a State Undergraduate Research Fellowship (SURF) for two consecutive years. After graduation, I plan to pursue a career in medicine, specializing in pediatrics.

duction (Nayak et al., 1999; Nie et al., 1997; Kunkl and Klaus, 1981).

Although the precise mechanism remains uncertain, studies that examined the effects of immune complexes made with immunoglobulin-E (IgE) hypothesized that the increased antigen-specific response is due to efficient absorption of the complexes into B-cells via a low-affinity IgE receptor located on the B-cell surface. They also observed that the complexes specifically led to an increase in the number of immunoglobulin-G (IgG)-secreting cells, leading to the possibility of the existence of a secondary mode of antibody response that occurs without the need for priming (Westman et al., 1997). Complexes composed of equal amounts of antigen and antibody or a slight surplus of antigen had greater success at generating memory B-cells than antigen alone. The constant portion of the antibody appeared to be the key; the variable portion of the antibody protein proved to be less effective (Klaus and Humphrey, 1978). Immune complexes may also play a role in controlling the release of antigens by increasing the amount of time that immune cells are exposed to the antigen (Sah and Chien, 1996). Additional studies have shown that immune complexes resulted in a stronger localized response compared to the uncomplexed controls (Levy et al., 2001).

This concept of immune complexes has been incorporated into the development of a vaccine for infectious bursal disease virus (IBDV). Field trials have proven somewhat successful. Jeurissen et al. (1998) found that virus detection in the IBDV/complex group was delayed for 5 d compared to the IBDV group, and that germinal centers were much more prevalent in the spleens of the IBDV/complex chickens, demonstrating higher activity of B-lymphocytes in the IBDV/complex group. Although both treatments lead to depletion of B-cells in the bursal follicles, the reduction was less severe at all time points in the IBDV/complex group (Jeurissen et al., 1998). Using reverse-transcriptase PCR to detect the presence of IBDV in trials with the vaccine, it was found that the viral load peaked at d 17 for the SPF chickens and d 21 for the maternally immune broilers, eventually disappearing altogether as seroconversion occurred (Ivan et al., 2005). Another research team concluded that the IBDV/complex vaccine can be administered quite successfully as early as d 1 despite the presence of variable levels of maternal antibodies (Haddad et al., 1997).

Another area of vaccine production that can be improved upon is the inactivation method of killed vaccines. They are usually created by growing the organism of interest, and then adding formalin to ensure the organism is killed prior to administration via injection. Numerous vaccines have been created in this fashion, ranging from the Salk polio vaccine to diphtheria and tetanus vaccines. However, formalin will often cross-link proteins on the surface of cells through hydroxymethylene bridges and thus modify the three-dimensional protein structure within 24-48 hours of exposure (Metz et al., 2004; Werner et al., 2000; Rappuoli, 1997). Therefore, the animals might produce antibodies to artificial epitopes formed by the crosslinks rather than antibodies that are protective against live organisms during an actual infection. It is necessary to preserve antigenic structure in order to allow an adequate amount of antigen to be present for an extended period of time on the surface of dendritic follicular cells. This leads to the synthesizing of a higher number of B-memory cells specific to that antigen and hence a stronger immune response (Regenmortel, 1992).

The tendency of formalin to alter antigenic epitopes has been shown in several studies (Metz et al., 2003; Nencioni et al., 1991), especially with pertussis vaccines for humans. Clearly, formalin has the potential to alter the epitopes in a detrimental fashion. A more natural deactivation method is needed in order to avoid the potential outcomes associated with the use of formalin. An ideal deactivating agent would retain intact bacterial epitopes while inactivating the pathogen. Therefore, the objective of this study was to manufacture a killed vaccine that could be administered with a single injection and produce a strong, long-lasting immune response.

MATERIALS AND METHODS

Production of antibodies for immune complexes

Turkey-origin antibodies (Ab) were generated for use in these studies. Briefly, turkeys were injected twice with inactivated BA at 6 (10^{10} cfu) and 9 (10^{5} cfu) weeks of age. Serum obtained 10 d after the final injection was used for this experiment. Agar gel immunodiffusion (AGID) assays were used to estimate the relative titers for standardization during the opsonization process.

Vaccine production and administration

Four groups of vaccines were created – two batches killed with 3% formalin and two additional batches killed with 10% acetic-acid. One formalin and one acetic-acid group were complexed with the harvested turkey antibodies (Ab), while the two remaining formalin and acetic-acid groups were left uncomplexed (Table 1). The acetic-acid was buffered with the addition of NaOH to a pH of 4.75, which is near the pKa of acetic-acid. To create the complexed vaccines, *B. avium* was killed using methods described above, and dilutions of Ab were combined with 100 µl of *B. avium* at a concen-

tration of 10^9 cfu/ml on slides, stained with Trypan blue, and examined in a microscope. The least Ab dilution that displayed evidence of immune complexes with some loose bacteria present was selected for opsonization. Once the dilution was determined, the formalin-complexed and acetic-acid-complexed vaccines were prepared. A fifth group consisted of negative controls, which were injected with either a formalin or acetic-acid vehicle (0.2 ml/poult).

The vaccines were administered to poults on the day of hatch by subcutaneous injection (0.2 ml/poult at 5.68 x 10^9 cfu inactivated). Each group, consisting of 40 tagged poults each, was kept in separate pens furnished with heat lamps, feed, and water arranged in similar locations in each pen. Blood samples were obtained at d 6, 10, and 21 using 22-gauge needles and 5-ml syringes. In order to minimize clotting, heparin was used at a concentration of 20 units/ml. Each sample was centrifuged at 1500 RPM for 10 min and the plasma was isolated and stored in 1.5-ml microcentrifuge tubes at –20°C.

Titer determination

The antibody titer of each group at each time point was determined using an ELISA. The 96-well plates for the test were prepared and stored according to Hopkins et al. (1988), using 0.1M carbonate/bicarbonate buffer rather than 0.05M. Each plate contained triplicates of seven identical positive dilutions of serum from the hyperimmunized birds and triplicates of a negative dilution composed of pooled sera from the control group samples. These positives and negatives served as internal standards in each plate (Table 2).

A preliminary assay was conducted with selected dilutions of unknown samples to determine the appropriate dilution factor. From this preliminary assay, it was determined that a dilution of 1:25 provided the most reliable results while maintaining a feasible development time. Therefore, each unknown sample was diluted 25-fold and placed as duplicates into the remaining wells on each plate. The results from each plate were normalized using the internal standards and the absorption values of each of the unknowns were averaged. These values represented the relative titers between the samples. These relative titers provided a numerical measure of the amount of antibody present. In order to put the results into a more easily understood value, the sample-to-positive (S/P) ratio of each sample was calculated according to the below equation:

$$\frac{(Sample\ mean - NC\bar{x})}{(PC\bar{x} - NC\bar{x})}$$

where $NC\bar{x}$ represents the negative control mean and $PC\bar{x}$ represents the positive control mean. It was decided to use the 1:16,000 positive dilutions because it gave absorption readings around 2 absorption units. Readings around 2 absorption units indicated that those samples were on a part of the standard curve graph that was less sigmoidal and more linear. Using a more linear portion of the graph is more useful for titer predication purposes. This technique is widely used in various immunological contexts (Hendrick et al., 2005; Nagy et al., 2002). S/P ratios can even act as a rough estimate of the level of antibody in the serum (Snelson, 2003).

The mean, standard deviation, standard error, and probability were calculated using SAS (SAS, Inc., Cary, N.C.) statistics software to determine significant differences among groups. Differences are reported at $P<0.05$.

RESULTS AND DISCUSSION

S/P Ratios

Those samples that displayed a positive S/P ratio were said to have had an immune reaction to the vaccines. Alternatively, those with a negative S/P ratio were said to have little or no reaction. The graphs for each day depicted both the average positive and total average S/P ratio for each group in order to compare both the averages of those that responded and the total averages of responders and non-responders alike.

Percent of Positive S/P Ratios

All groups reacted to the vaccines to some extent (Fig. 1). More birds initially reacted to the formalin-inactivated group (F) compared to the acetic-acid-inactivated group (AA). However, by d 21, 50% of the acetic-acid group had responded. No obvious differences were evident until d 21. At d 21, percentages generated by both formalin groups (F/F+Ab) were higher than those induced by both acetic-acid groups (AA/AA+Ab), reaching levels commonly believed to be necessary for flock immunity. The formalin-complexed (F) group on d 21 had statistically higher coverage than either of the acetic-acid groups (AA/AA+Ab), although results were not statistically different than formalin inactivation (F) alone.

Day 6 S/P Ratios

It is possible that the negative values displayed in the S/P ratio averages could be a result of the presence of non-specific binding sites on the *B. avium* bacteria for the constant (Fc) portion of antibodies (Fig 2). However, this possibility was not evaluated in our study. Although all groups had some negative S/P ratio averages, the formalin group (F) was significantly higher than the acetic-acid-complexed group (AA+Ab) and not significantly different from the other two groups. The acetic-acid group (AA) and formalin-complexed group (F+Ab) were not significantly different than the other

groups. Of the birds that had a positive S/P ratio and hence did respond to the vaccine, no significance was observed in any of the groups.

Day 10 S/P Ratios

The averages on d 10, where intermediate responses were anticipated, were not elucidating. No significant differences existed in either the total averages or positive averages in any of the groups (Fig. 3). Due to the similarity of the formalin/formalin-complexed and acetic-acid/acetic-acid-complexed averages, especially the positive averages, it appears that the addition of immune complexes did nothing to aid in the immunological reaction to the vaccine.

Day 21 S/P Ratios

With regard to the positive S/P ratio averages, the formalin and formalin-complexed groups exhibited significantly higher averages than the acetic-acid-inactivated group. The acetic-acid-complexed group was not significantly different from any group. Including all birds increased the interpretability of these results. The formalin-complexed and formalin groups both featured significantly higher averages than the acetic-acid-complexed and acetic-acid groups (Figure 4).

The formalin-inactivated vaccines, which approached 80% positive antibody response, were superior to the acetic-acid-inactivated vaccines by d 21. Addition of immune complexes was not of any benefit. From this experiment, it does not appear that either opsonization or the acetic-acid method for inactivation of BA for vaccine generation offer improved methods for inactivated BA vaccination of turkeys.

Development of an improved vaccine for *Bordetella avium* would have implications on all species that are susceptible to infection by Bordetella species, including canines and humans. Improvement of killed vaccines in general would have repercussions for many of our current vaccines for various animal immunizations.

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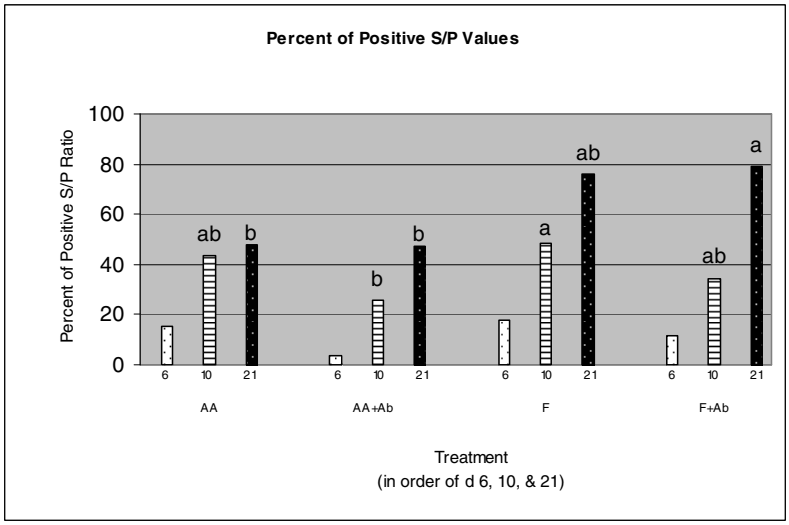

Fig 1. Percentages of each group with positive S/P ratios at day 6, 10 or 21. Histograms with different letters are significantly ($p<0.05$) different. Abbreviation of treatments are as follows: AA = acetic-acid, AA+Ab = acetic-acid-complexed, F = formalin, F+Ab = formalin-complexed.

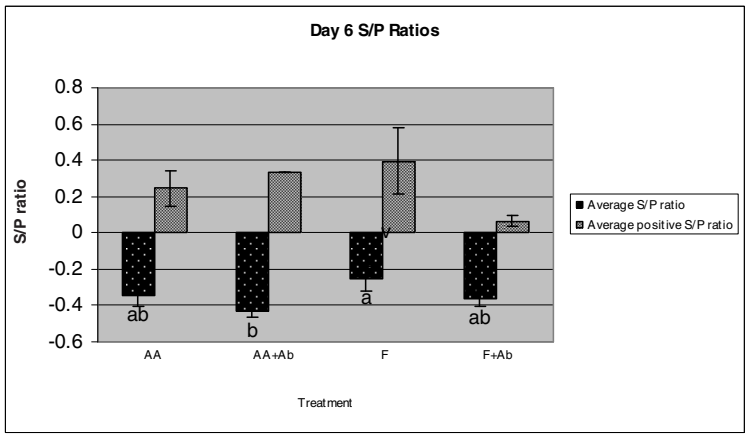

Fig 2. Averages of all S/P ratios and positive S/P ratios in each group as measured on day 6. Histograms with different letters are significantly ($p<0.05$) different. Abbreviation of treatments are as follows: AA = acetic-acid, AA+Ab = acetic-acid-complexed, F = formalin, F+Ab = formalin-complexed.

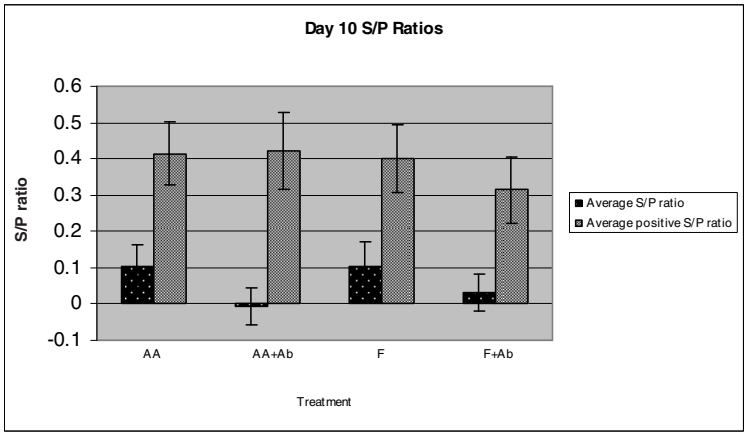

Fig 3. Averages of all S/P ratios and positive S/P ratios in each group as measured on day 10. Differences between groups were not significant ($p>0.05$). Abbreviation of treatments are as follows: AA = acetic-acid, AA+Ab = acetic-acid-complexed, F = formalin, F+Ab = formalin-complexed.

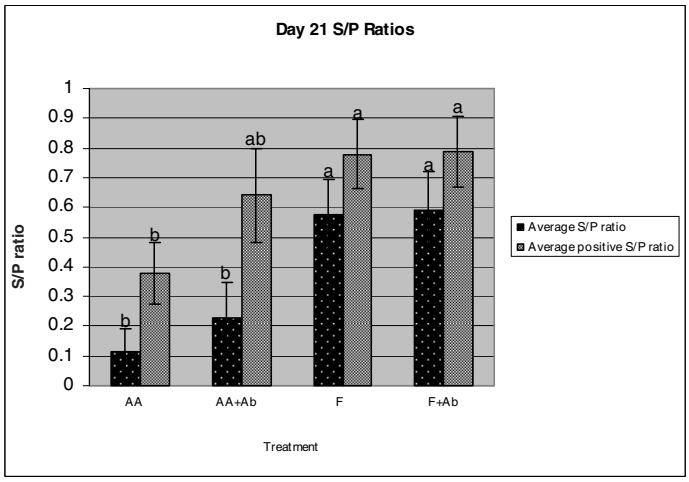

Fig 4. Averages of all S/P ratios and positive S/P ratios in each group as measured on day 21. Histograms with different letters are significantly ($p<0.05$) different. Abbreviation of treatments are as follows: AA = acetic-acid, AA+Ab = acetic-acid-complexed, F = formalin, F+Ab = formalin-complexed.

Table 1. Production process of the four vaccines and controls.

Grow *B. avium* (10^{10} CFU/ml)				0.9% Saline	
Kill using 10% AA @ pH 4.2-4.5 (AA)		Kill using 3% formalin (F)		Add 10% acetic acid @ pH 4.2-4.5 (AA)	Add 3% formalin (F)
Group 1 Complexed Acetic acid **AA+Ab** (*Ba* + Ab)	**Group 2** Uncomplexed acetic acid **AA** (*Ba* only)	**Group 3** Complexed formalin **F+Ab** (*Ba* + Ab)	**Group 4** Uncomplexed formalin **F** (*Ba* only)	**Group 5** Acetic acid negative controls (saline + AA)	**Group 6** Formalin negative controls (saline + F)