

## *Pasteurella haemolytica* Ultraviolet-Irradiated Vaccine by Parenteral and Aerosol Routes Compared in the Goat Model

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**Abstract.** Positive control 1 (PC1) (n = 9) goats were injected transthoracically into the left lung with live *Pasteurella haemolytica* biovar A, serovar 1 (PhA1) in polyacrylate (PA) beads on days 0 and 21. Positive control 2 (PC2) (n = 6) goats were nebulized with live PhA1 and PA beads on days 0 and 21. Negative control (NC) goats (n = 6) were each injected transthoracically into the left lung with PA beads alone on days 0 and 21. Four groups (n = 6) were administered PA beads mixed with ultraviolet (UV) killed PhA1 on days 0 and 21. The treatment doses of bacteria for these groups were principal group 1 (PR1) injected into the left lung ( $7.7 \times 10^{10}$  cfu); PR2,  $7.7 \times 10^{10}$  UV-killed PhA1 injected subcutaneously (SC); PR3,  $7.7 \times 10^{10}$  UV-killed PhA1 injected SC only on day 21; PR4, nebulized with PA beads mixed with  $5.6 \times 10^{10}$  cfu of UV-killed PhA1; and PR5, nebulized with PA beads mixed with  $5 \times 10^8$  cfu of UV-killed PhA1. All goats were challenged transthoracically in the right lung with  $1 \times 10^8$  cfu of live PhA1 on day 42 and necropsied on day 46. The sizes of consolidated lung lesions at the challenge site were used as a measure of immunity. The data show that the introduction of live PhA1 into the lungs of goats, either by injection or aerosolization, offers excellent protection against a subsequent homologous challenge. The data also demonstrate that two transthoracic injections (21 days apart) of UV-killed PhA1 (PR1), and subcutaneous injection of UV-killed PhA1 (PR2) also offer excellent protection against a subsequent homologous live PhA1 challenge. One SC injection of UV-killed PhA1 (PR3) appears to offer only partial protection against a subsequent homologous live PhA1 challenge. Inhalation of UV-killed PhA1 mixed with PA beads (PR4 and PR5) induced no protection in goats against a subsequent live PhA1 transthoracic challenge.

*Pasteurella haemolytica* biovar A, serotype 1 (PhA1) is the organism most often associated with the acute fibrinous bronchopneumonia found in bovine respiratory disease (BRD) in stocker and feeder calves [6]. The development of a safe and efficacious PhA1 vaccine would be of great importance to the feeder calf industry [1]. BRD most commonly develops after calves are marketed and shipped. It is thought that the complex marketing, shipping stress, and respiratory virus involvement interaction induces PhA1 infection, hence the name "shipping fever." The nasal flora of these calves has been shown to change rapidly during the time period they are marketed [4]. BRD most commonly develops during the first two weeks that the calves are in the feedyard, and PhA1 can be easily isolated from their nares during this

time [10]. Paradoxically, it is unusual to isolate PhA1 from the nares of calves while on the farm of origin or from those that have been in the feedyard for over one month.

We have recently shown that a subcutaneously (SC) administered, UV-killed PhA1 bacterin induced solid protective immunity equal to that induced by virulent live PhA1 injected into the target organ, the lung [13]. We have also shown that there is a high degree of immunity in ruminants after exposure to live PhA1 imbedded in agar beds [11]. Other workers have shown that a PhA1 aerosol containing  $1 \times 10^9$  to  $1.3 \times 10^9$  organisms/ml directed into a bag attached to the nose of a calf for 15 min, then repeated 7 days later, induced resistance to a PhA1 transthoracic challenge of  $5 \times 10^9$  colony forming units (cfu) [8]. The purpose of this study was to determine

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whether ruminants could be vaccinated by aerosolization with a UV-killed PhA1 bacterin. Obviously, introduction of live PhA1 into the lungs of ruminants, although effective, is not a safe way to immunize these animals. Using aerosolized UV-killed PhA1 would guarantee that the animals would not become infected by the bacterium used in the vaccination process. Vaccination by the intranasal route would also avoid tissue reactions, meat trim loss, and protect the consumer from broken needles and meat contamination.

The goat has been shown to be a very good model to test the efficacy of various PhA1 vaccines [9, 11]. The immunity generated in these animals by transthoracic injection of the bacteria can be measured by observing the volume of the consolidated lung tissue 96 h after challenge. If a particular vaccine preparation is capable of generating protection, the volume of the consolidated lung tissue in  $\text{cm}^3$ , after the challenge injection of live PhA1, will be larger in the negative controls than in the principal groups.

## Materials and Methods

**Animals.** Fifty-one weanling male Spanish goats were purchased from one herd in southern Texas. These animals had no history of respiratory disease or of being vaccinated with *Pasteurella* products. They were transported by truck to the United States Department of Agriculture, Agriculture Research Service, Research Laboratory located in Bushland, Texas. Upon arrival at the research facility, the goats were treated for internal parasites and coccidia (Ivomec-MSD AGVET and Amprolium respectively, Merck and Co., Inc., Rahway, NJ), and conditioned to their new surroundings for 6 weeks. They were housed in a covered, three-sided barn and were maintained on a pelleted sheep/goat ration (0.4 kg/goat/day) with ad lib access to alfalfa hay and fresh water.

**Bacteria.** PhA1 was isolated from a pneumonic calf and identified by colony morphology, Gram stain, biochemical reactions, and by use of specific serotyping antisera [5]. Live PhA1 vaccine and challenge-inoculum cultures [11] were routinely grown on nutrient agar plus 5% bovine (citrate) blood for 16 h at 37°C in 5%  $\text{CO}_2$ . Cultures were harvested in phosphate-buffered saline (PBS). Bacterial counts (cfu/ml) were determined by preparing tenfold dilutions and culturing overnight at 37°C. One hundred ml of each of the dilutions in duplicate was plated on the surface of nutrient agar containing bovine red blood cells (5%). For UV-irradiation, the bacterial suspension (in 0.15 M saline, pH 6.6) was dispensed into uncovered petri dishes to a depth of 5 mm and irradiated (Spectroline model TR-312A ultraviolet transilluminator, Spectronics Corp., Westbury, NY) at 315 nm for 60 min inside a vertical laminar flow biological hood. After irradiation, a sterile water rinse was added to the bacterial suspension. The UV-irradiated bacterial suspension was repeatedly cultured to insure complete killing.

**Experimental design.** Goats were randomly assigned to eight groups. Negative control (NC) goats ( $n = 6$ ) were each injected transthoracically in the left lung with 0.05 g of polyacrylate beads (Dow Chemical Company, Midland, MI). These beads were prepared by rehydrating dry beads in sterile saline. Positive control 1 (PC1) goats ( $n = 9$ ) were injected transthoracically into the left lung with live PhA1 on days 0 ( $6 \times 10^5$  cfu) and 21 ( $2 \times 10^6$  cfu) in 0.05 g of PA beads. PC2 group ( $n = 6$ ) were nebulized (Devilbiss Powder Insufflator #119, Devilbiss Co., Somerset, PA) with live PhA1 on days 0 ( $1.0 \times 10^{10}$  cfu plus 0.6 g

PA beads) and 21 ( $2 \times 10^{10}$  cfu plus 0.6 g PA beads). Five principal groups (PR1, PR2, PR3, PR4, and PR5,  $n = 6/\text{group}$ ) were administered PA beads plus UV-irradiated PhA1 on days 0 and 21 (except for PR3, which was injected SC on day 21 only). All goats were tranquilized (100mg xylazine, intramuscularly, Bayvet Division Cutler Laboratories, Inc., Shawnee, KS). This product was diluted 1:100 with saline and 2-3 mg given per goat, 15 min before injections were made into the lungs [11]. A local 1-ml injection of 2% Lidocaine (Professional Veterinary Pharmaceuticals, Kansas City, MO) was used to anesthetize the skin at the site of the transthoracic injection. The treatment doses of bacteria were: PR1, injected transthoracically into the left lung ( $7.7 \times 10^{10}$  cfu in 0.05 g PA beads); PR2, injected SC with  $7.7 \times 10^{10}$  cfu in 0.05-g PA beads; PR3, injected SC with  $7.7 \times 10^{10}$  cfu in 0.05-g PA beads; PR4, nebulized with  $5.6 \times 10^{10}$  cfu plus 0.6 g PA beads; and PR5, nebulized with  $5 \times 10^8$  cfu plus 0.6 g PA beads. All goats were challenged transthoracically in the right lung with  $1 \times 10^8$  live PhA1 on day 42. On day 46, goats were euthanized with an overdose of barbiturate anesthetic and immediately exsanguinated [11]. Necropsies were performed, and lungs were examined for PhA1-induced lesions. Consolidated lesions were measured (length  $\times$  width  $\times$  thickness) with calipers. Immune protection was determined on the basis of the volume of consolidated lung tissue 4 days after transthoracic challenge exposure. Any goat that became so gravely ill that it was not expected to live was immediately euthanized. Any goat that became ill, but was expected to live, was given appropriate supportive care by a veterinarian. The goats were always treated in accordance with the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Testing Guidelines [3].

**Specimen collection.** Blood samples were collected on days 0, 7, 14, 21, 28, 41, and 45. They were collected via jugular venipuncture and allowed to clot for approximately 20 min at room temperature. They were then placed on ice for 1 h and centrifuged at 4°C. The serum was decanted into cold glass vials and was frozen at  $-80^\circ\text{C}$  until they were assayed for indirect hemagglutination (IHA) titer.

**Clinical observation and serum assays.** Physical examinations were performed and rectal temperatures were recorded for all goats on days 0, 1-3, 7, 14, 21-23, and 41-45. In addition, all goats were observed twice a day for adverse clinical signs throughout the experiment. The PhA1 IHA serum titers were determined by the method of Frank and Smith [4]. All serum samples from one animal were assayed on the same day the blood was drawn and titers were determined using appropriate serum controls.

**Statistical analysis.** Mean data were compared by use of general linear models procedure for repeated measures [14]. Variables included in the model were time, treatment, and time versus treatment interaction. Differences were considered significant when  $P \leq 0.05$ . When a significant treatment differences by sample day interaction was detected, data were subsequently analyzed within days. Significant differences between treatment groups were further evaluated by use of Bonferroni's adjusted paired *t*-test [14], which allowed a pairwise comparison of all treatment group means within any sampling day.

## Results and Discussion

**Goats and rectal temperature readings.** There were significant differences in rectal temperatures among treatment groups on days 1, 22, 42, and 45 (data not shown). Rectal temperatures (RT) increased (from  $39.3^\circ\text{C}$  to  $41.0^\circ\text{C}$ ) within 24 h after the first live PhA1 injection into the lungs (PC1). They also increased (from  $39.4^\circ\text{C}$  to

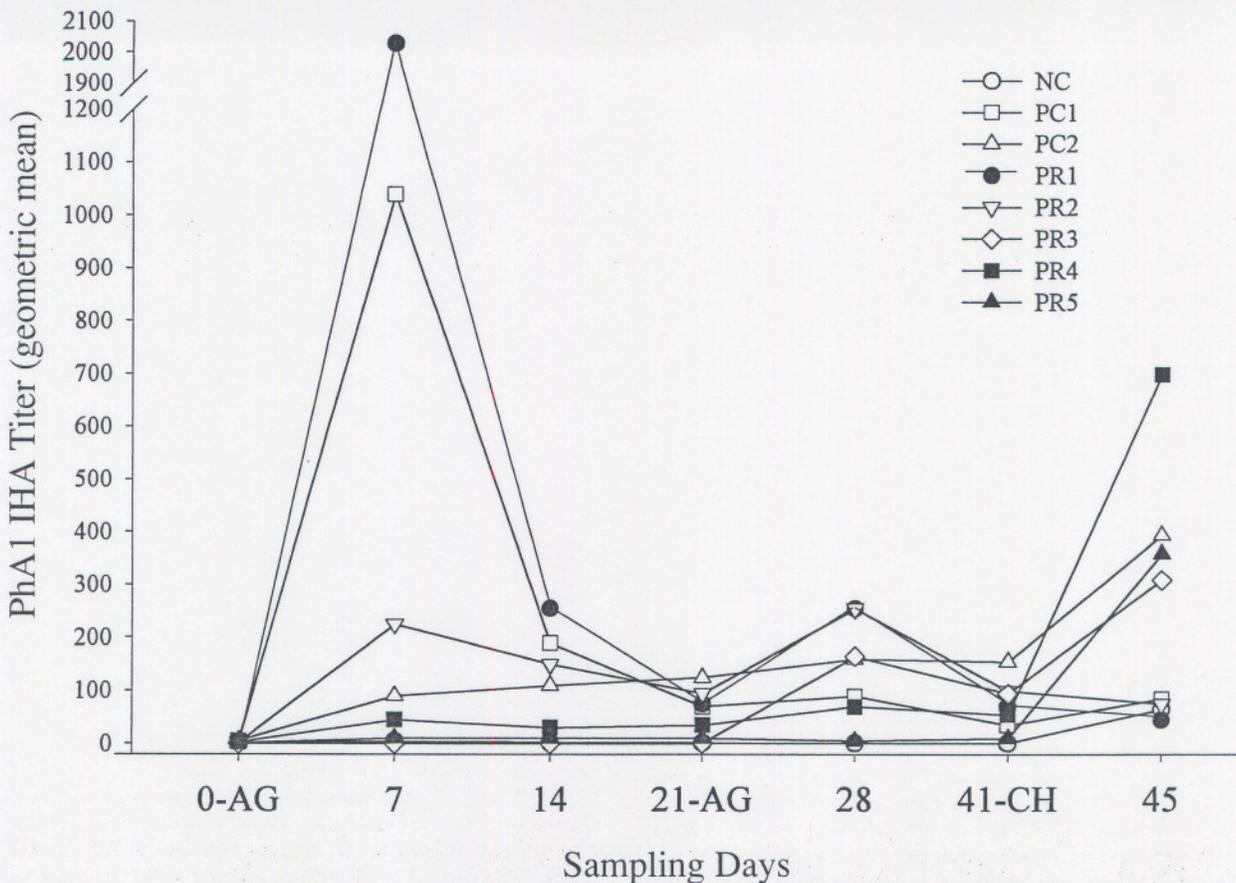


Fig. 1. Indirect hemagglutination antibody titer (geometric mean) of goats treated with live PhA1 and UV-irradiated PhA1. Negative control (NC) goats were injected transthoracically into the left lung with PA beads alone on days 0 and 21. Positive control 1 (PC1) goats were injected transthoracically into the left lung with  $6 \times 10^5$  live PhA1 plus PA beads on days 0 and 21. Positive control 2 (PC2) goats were nebulized with  $1 \times 10^{10}$  live PhA1 plus PA beads on days 0 and 21. PR1 goats were injected transthoracically into the left lung with  $1 \times 10^{10}$  UV-irradiated PhA1 plus PA beads on days 0 and 21. PR2 goats received  $1 \times 10^{10}$  UV-irradiated PhA1 SC plus PA beads on days 0 and 21. PR3 goats received  $1 \times 10^{10}$  UV-irradiated PhA1 SC plus PA beads on day 21 only. PR4 goats received  $5 \times 10^6$  UV-irradiated PhA1 plus PA beads nebulized on days 0 and 21. PR5 goats received  $5 \times 10^8$  UV-irradiated PhA1 plus PA beads nebulized on days 0 and 21. AG signifies injection or inhalation days. CH signifies challenge day.

40.3°C) 24 h after  $1 \times 10^{10}$  cfu of UV-killed PhA1 was injected transthoracically into the left lung (PR1) and 24 h after the SC injection of the same number of PhA1 (PR2; from 39.3°C to 41.0°C). The latter two groups experienced an increase in RT (from 39.0°C to 40.5°C) one day following the second UV-irradiated PhA1 injection, as did group PR3, which had received  $1 \times 10^{10}$  cfu of UV-irradiated PhA1 SC. Increases in RT were almost certainly owing to the large amount of lipopolysaccharide present in  $1 \times 10^{10}$  cfu of PhA1 [12]. After challenge exposure, the greatest increase in RT was in the NC group and all five groups receiving UV-irradiated PhA1.

**Serology.** The PhA1 geometric mean titers were examined for each animal on days 0, 7, 14, 21, 28, 41, and 45. It was established that none of the goats had significant quantities of anti-PhA1 antibodies (IHA  $\leq 4$ ) until they

were injected or nebulized with either live or UV-irradiated PhA1. Figure 1 shows the IHA titers by treatment group. The geometric mean PhA1 antibody titers were all low ( $<1:2$ ) on day 0. Seven days after the first PhA1 injection, there was a primary antibody response in all groups except NC. The response was highest in the PR1 group (1:2100) with the PC1 group (1:1050) a close second. These high values were not reached again, even after the second PhA1 inoculation at day 21. The PR3 group (which received its first injection on day 21) had a primary antibody response of (1:250) 7 days after the initial injection. The PC1 group demonstrated an increase in IHA four days after the live challenge, as did the PR3, PR4, and PR5 groups. However, the response by the PC1 group was less than that of several of the other groups.

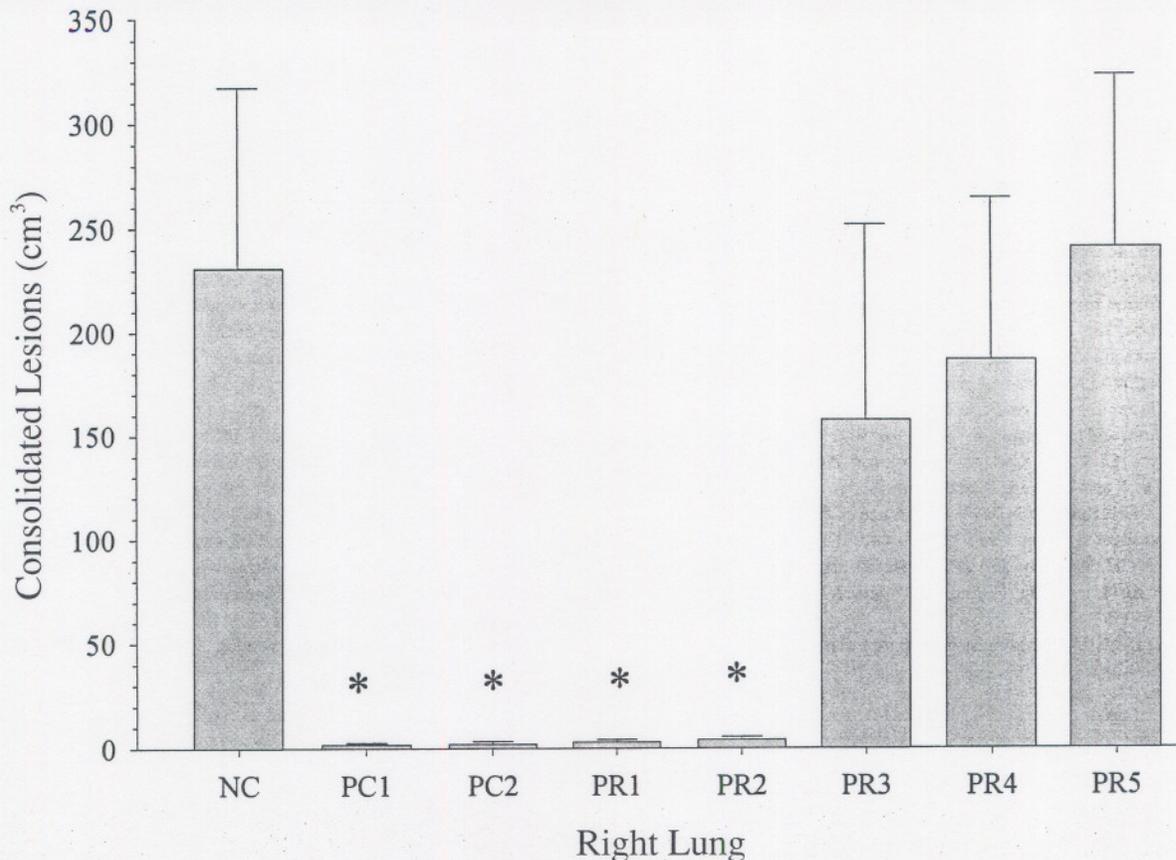


Fig. 2. Mean volume (cm<sup>3</sup>) of consolidated lung tissue of goats treated with live PhA1 or UV-irradiated PhA1. The right lung was the site of transthoracic live PhA1 challenge. Means significantly different from the NC ( $p \leq 0.05$ ) are denoted by an \*. NC, PC1, PC2, PR1–PR5 are described in the legend to Fig. 1.

**Gross and microscopic pulmonary lesions.** Mean volume  $\pm$  standard error of the mean (SEM) of consolidated lung tissue for the NC goats was 231.0 cm<sup>3</sup>  $\pm$  193.6 cm<sup>3</sup> (Fig. 2). The mean volume  $\pm$  SEM of PC1 and PC2 groups were 1.7 cm<sup>3</sup>  $\pm$  2.3 cm<sup>3</sup> and 2.0 cm<sup>3</sup>  $\pm$  2.7 cm<sup>3</sup>, respectively. The mean volumes  $\pm$  SEM of PR1, PR2, PR3, PR4, and PR5 were 2.9 cm<sup>3</sup>  $\pm$  2.1 cm<sup>3</sup>, 3.8 cm<sup>3</sup>  $\pm$  3.2 cm<sup>3</sup>, 158 cm<sup>3</sup>  $\pm$  209.5 cm<sup>3</sup>, 186.3 cm<sup>3</sup>  $\pm$  174.2 cm<sup>3</sup>, and 240.4 cm<sup>3</sup>  $\pm$  186.4 cm<sup>3</sup>. These data demonstrate that the NC goats had no protective antibody against PhA1. Likewise, groups PR3, PR4, and PR5 had no protective antibody against PhA1. Both positive controls, PC1 and PC2, showed excellent protection against a homologous live PhA1 transthoracic challenge. Groups PR1 and PR2 demonstrated excellent protection against a live PhA1 transthoracic challenge.

Ultraviolet irradiation has been shown to be a very effective way of producing a PhA1 bacterin. UV irradiation has been demonstrated to be very effective in killing PhA1 [7]. UV-irradiation is known to lead to the formation of covalent bonds between adjacent thiamine resi-

dues in the same DNA strand, thus inhibiting DNA synthesis and shutting down all multiplication. Perhaps more importantly, UV-irradiation has little or no effect on the PhA1 cell surface, where it is thought the important immunogens are located [2]. Thus UV-irradiation provides a PhA1 bacterin with its cell surface essentially intact.

Our results indicate that UV-irradiated PhA1 plus PA beads injected transthoracically into the lungs or given SC are essentially as effective at inducing immunity as the controlled live lung infection produced by transthoracic challenge or by inhalation. However, at least two SC injections are necessary to achieve this level of protection, as one SC injection (PR3) was not effective. Finally, inhalation of UV-killed PhA1 appears not to be an effective way to immunize ruminants against a live homologous, transthoracic challenge.

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