

The humoral immune response in cattle after immunization with a multivalent IBR/PI₃/*Pasteurella haemolytica* A1 leukotoxin vaccine

M.W. ODENDAAL¹, S. MORRIS², E. DU PREEZ² and H. AITCHISON³

ABSTRACT

ODENDAAL, M.W., MORRIS, S., DU PREEZ, E. & AITCHISON, H. 1997. The humoral immune response in cattle after immunization with a multivalent IBR/PI₃/*Pasteurella haemolytica* A1 leukotoxin vaccine. *Onderstepoort Journal of Veterinary Research*, 64:205–212

A multivalent vaccine consisting of inactivated bovine herpes virus-1 (BHV-1), also known as infectious bovine rhinotracheitis virus (IBR), para-influenza type-3 virus (PI₃) and the leukotoxin of *Pasteurella haemolytica* A1, were combined with the addition of aluminium hydroxide as adjuvant, and administered to post-weaned calves, and the serum tested for seroconversion to each antigen. Two groups of calves ($n = 150$ and $n = 68$) were used and were randomly divided into three subgroups. The first group of 150 calves were immunized with the multivalent vaccine (three batches) to test its first-stage stability (vaccine 3 months old) and the second group were immunized with the same vaccine 1 year later, in order to test its immunogenicity.

A significant increase in titres occurred after the day 0 and 28 immunizations, for each of the three antigens in the multivalent vaccine, as measured on days 0 and 42. Immunoconversion occurred after immunization with the 3-month- and 1-year-old vaccine. The immunogenic stability of antigens in the vaccine was demonstrated after a 1-year period when the vaccine was kept at 4–6 °C.

Keywords: BHV-1, bovine herpes virus-1, cattle, IBR, immunoconversion, immunogenicity, infectious bovine tracheitis, multivalent vaccine, para-influenza, *Pasteurella haemolytica*, PI₃

INTRODUCTION

A significant finding in the mid-1970s provided evidence that respiratory viruses interact with *P. haemolytica* in the bovine lung, to exacerbate and enhance the pathogenesis of bovine respiratory disease.

It was clearly demonstrated that exposure to para-influenza type 3 (PI₃) virus depressed the clearance of bacteria by lung macrophages (Lopez, Thomson & Savan 1976). A similar phenomenon was found

with bovine herpes virus-1 (BHV-1), when viral infection was followed by *P. haemolytica* infection (Jericho & Langford 1978).

Viral infection of the respiratory tract with BHV-1, alters the epithelial environment. Not only does it result in the death of the cells but it adversely affects the host-specific and non-specific defense mechanisms by altering the function of the macrophages and monocytes (Babiuk, Morsy, Campos & Harland 1995). This invariably leads to retardation or even inhibition of the bacteria-clearing mechanism in the lungs, resulting in increased bacterial colonization, and the production of extracellular toxins such as leukotoxin. The increased levels of cytokines resulting from the viral infection, appears to be involved in the mediation of the acute-phase reaction, an increase in leukocyte reactivity and immunopathology.

Viral infection of the epithelial cells of the respiratory tract not only results in cell death, but also in some

¹ Bacterial Vaccine Development Unit, Onderstepoort Veterinary Institute, Onderstepoort, 0110 South Africa

² Faculty of Veterinary Science, Medical University of South Africa, 0204 South Africa

³ Onderstepoort Biological Products, Onderstepoort, 0110 South Africa

instances in the alteration of the production of cell secretions. Decreased clearance of bacteria follows, allowing the establishment of microcolonies of bacteria and eventual bacterial pneumonia.

The preceding infection with respiratory viruses, such as bovine herpes virus, para-influenza 3, or bovine respiratory syncytial virus is regarded as an important predisposing cause in bovine respiratory disease, as it enhances the ability of *Pasteurella haemolytica* biotype A serotype 1 (A1) to colonize the nasal passages of the calf. Experimental procedures to prove this, have been established beyond any reasonable doubt (Frank, Briggs & Gillette 1986; Frank, Briggs & Gillette 1987; Frank 1988). This invariably leads to colonization of the lower respiratory tract resulting in severe fibrinonecrotic pneumonia associated with pneumonic pasteurellosis. The *P. haemolytica* A1 produces a leukotoxin that is undoubtedly a major virulence factor, in that it protects the bacterial organism by destroying the phagocytic cells such as the neutrophils and broncho-alveolar macrophages (Whiteley, Maheswaran, Weiss, Ames & Kannan 1992). Even at subcytolytic concentrations the leukotoxin may enhance the inflammatory response by activating cells to produce mediators and release reactive oxygen metabolites and proteases into the surrounding lung tissue.

Immunoprophylaxis with modified live viruses against these respiratory viruses, is common practice, especially in feedlots (Jericho, Loewen & Smithson 1991; Fulton, Confer, Burge, Perino, d'Offay, Payton & Mock 1995). A number of these commercial vaccines are currently available in South Africa. Onderstepoort Biological Products produces a bivalent inactivated IBR and PI₃ combination vaccine, specifically for situations where the use of the modified live virus vaccine is contra-indicated. It is also indicated for use in the feedlots.

Numerous authors have established that the traditional bacterin-based *P. haemolytica* vaccines have a poor record of protecting cattle against respiratory disease (Wilkie, Markham & Shewen 1980; Confer, Panciera, Fulton, Gentry & Rummage 1985; Shewen, Sharp & Wilkie 1988). Immunization with these bacterin vaccines induces the formation of antibodies against some antigens present on the organism, which results in their enhanced opsonization and phagocytosis. The presence of leukotoxin subsequently destroys the phagocyte, with ensuing pathologic changes in the lungs. These changes prompted us to investigate leukotoxin as an alternative subunit vaccine for *Pasteurella haemolytica* A1.

A leukotoxin-based culture supernatant vaccine has been used successfully to immunize calves, with the development of protective antibodies. These antibodies neutralize the cytotoxic effect of the leukotoxin, produced by *P. haemolytica in situ*; this has been

demonstrated in a number of clinical studies (Baluyut, Simonson, Bemrick & Maheswaran 1981; Shewen & Wilkie 1988; Shewen, Sharp & Wilkie 1988; Srinand, Hsuan, Yoo, Maheswaran, Ames & Werdin 1996b; Sreevatsan, Ames, Werdin, Yoo & Maheswaran 1996). The multicomponent vaccine used in this study was formulated to contain the three most important antigens that have been demonstrated unequivocally to play a role in the pathogenesis of BRD resulting in morbidity and mortality.

The purpose of this study was to determine the immunogenicity and the stability of the antigens incorporated into the multivalent vaccine from three different production batches, over a 1-year period. The vaccine contains inactivated bovine herpes virus-1, para-influenza-3, and leukotoxin produced by *Pasteurella haemolytica* A1. The neutralizing titres were determined from each batch for each individual antigen component and the serological response compared from day 0 to day 42.

MATERIALS AND METHODS

Preparation of cultures for leukotoxin vaccine production

Pasteurella haemolytica biotype A serotype 1 was activated from a freeze-dried vial onto blood tryptose agar and grown for 24 h at 37°C under aerobic conditions. Several single colonies were inoculated into 200 ml of brain-heart infusion broth and grown until it reached log phase on a shaking platform. It was subsequently inoculated into 1–2 l of BHI. When it reached log-phase growth, it was inoculated into 15 l of BHI in a Braun ED fermenter and grown according to established parameters for 3 h. The bacterial cells were harvested by high-speed centrifugation and used as inoculum for 15 l of RPMI 1640 medium with 3.5% foetal-calf serum. After incubation for 1 h it was centrifuged and filtrated through a 0.45 µm filter, dispensed into plastic vaccine containers, tested for sterility and stored at 4–8°C (Shewen & Wilkie 1983). Immediately after filtration a number of 1 ml leukotoxin specimens were dispensed into plastic tubes, placed on dry ice and cryopreserved at –8 to –70°C, for the subsequent determination of leukotoxic activity. Subcultures were prepared on 5% bovine-blood tryptose agar, after the completion of each stage, to test the purity and integrity of the *Pasteurella haemolytica* during the whole process and to ensure that no other contaminants occurred.

Detection of leukotoxin activity

The leukotoxin activity was determined by a microcolorimetric dye-reduction assay in high-binding 96-well flat-bottomed ELISA microtitration plates, with the use of bovine neutrophils (20 × 10⁶ cells/ml) isolated by means of Histopaque 1077 (Sigma

Chemicals) (M. Saadati, personal communication 1994). Viable neutrophils reduce the nitroblue tetrazolium dye (MTT, Sigma Chemicals) to a dark blue formazan and the number of cells are directly proportional to the optical density of the colour change. This optical density was measured at a test wavelength of 550 nm against a reference wavelength of 620 nm (SLT Easy Reader spectrophotometer, Austria). Log₂ dilutions of the leukotoxin were made and its cytotoxic activity was defined as the reciprocal of the dilution causing 50% cytolysis of the neutrophils (Vega, Maheswaran, Leiniger & Ames 1987). The 50% endpoint titration method of Reed and Muench (1938) was used to calculate the final titre.

Preparation of IBR and PI₃ virus antigens

The international prototype SF4 master-seed antigen was used for PI₃ vaccine production. It was grown on MDBK (Maden Darby Bovine Kidney) cells with Eagles medium, for 3–5 d. The virus titre before inactivation was never less than 10⁴ TCID₅₀/ml. It was then inactivated with formalin.

The master seed for the production of IBR (Infectious Bovine Rhinotracheitis) virus antigen is an Onderstepoort strain, IBR FH 335 TC 66, which is kept frozen at a temperature of –10 to –15°C. It was grown on MDBK cells in Eagles medium. The virus antigen was allowed to grow for 3–5 d. The virus titre before inactivation was never less than 10⁴ TCID₅₀/ml. It was then inactivated with formalin.

Experimental design

Three batches of the multivalent vaccine were produced under similar conditions and administered to two different groups of indigenous *Bos taurus* type Bonsmara cross-bred calves, 1 year apart. In the first stage of stability testing (vaccine 3–6 months old), 150 calves were randomly divided into three groups of 51, 50 and 49 animals, with each one receiving a different batch. There were no negative control groups. The day 0 serum of each animal served as its own negative control. One year later, the same vaccine was administered to 68 calves divided into three groups of 23, 23 and 22 animals. On both occasions the animals were immunized on days 0 and 28, with 6 ml of vaccine by subcutaneous injection and serum was collected on days 0 and 42 (pre- and post-vaccination serums). Serum was prepared by centrifugation, filtrated through a 0,45 µ filter to remove all particulate material, heated at 60°C for 10 min and stored at –20°C until use.

Final composition of vaccine

Each 5 ml dose of vaccine consisted of 50% IBR-inactivated virus, 25% PI₃ virus and 25% aluminium hydroxide to which 1 ml of leukotoxin was added.

Detection of anti-leukotoxin titres

A leukotoxin neutralization colorimetric microtitration test was used to determine the 50% anti-leukotoxin endpoint titres (Vega *et al.* 1987). In the first group of 150 calves the day 0 and 42 titres were determined separately. At least ten log₂ dilutions of each serum that was collected on days 0 and 42, were prepared and incubated with a volume of leukotoxin standard, a specific volume of bovine neutrophils, and nitroblue tetrazolium dye (MTT) giving rise to colorimetric changes in the presence of viable neutrophils and measured spectro-photometrically. This information was used to calculate the percentage of neutralization by a formula devised by Vega *et al.* (1987).

The 50% endpoint neutralization titre was subsequently determined. It was regarded as the reciprocal of the log₂ serum dilution that gave 50% neutralization (refer to Table 1, Fig. 1), by use of the formula described by Reed & Muench (1938). The differences between the day 0 and 42 titres were compared statistically.

TABLE 1 The range of day 42 *Pasteurella haemolytica* leukotoxin antibody titres detected in the serum of two groups of calves immunized with multivalent vaccine, over a 1-year period

Range of titres (u/ml)	Percentage of titres	
	First stage stability (n = 150)	One year stability (n = 68)
0–99	49	29
100–149	14	9
150–199	21	18
>200	16	44

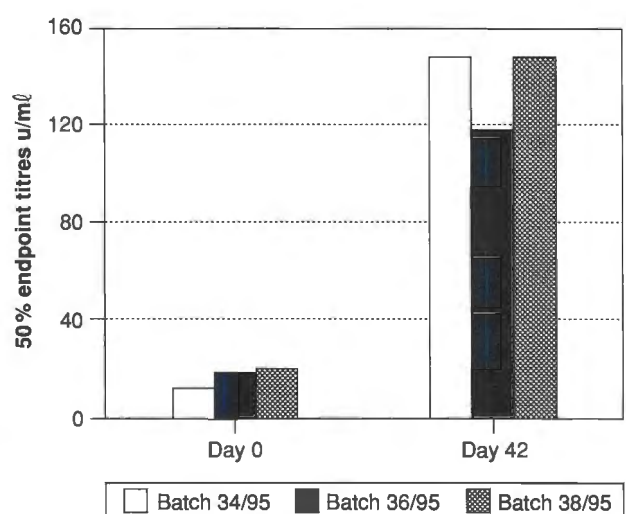


FIG. 1 Changes in the day 0 and 42 50% endpoint antibody titres (geometric mean) in calves against three different batches of *Pasteurella haemolytica* leukotoxin in multivalent vaccine, testing the first-stage stability of 3-month-old vaccine

For practical reasons this method was adapted to a single-dilution method for the detection of anti-leukotoxin antibodies for serum specimens from the second group of 69 calves, measuring only the final titre (refer to Table 1) in comparison with a positive control serum (Williams 1988). Convalescent serum obtained from a bovine in a feedlot that had recovered from *Pasteurella pneumonia*, with an antibody titre of 256 units/ml (u/ml) was used as a positive control. All the control and test serums were diluted 1/100 and the absorbance measured at a wavelength of 550 nm against a reference of 620 nm.

In this case, the increase in the absorbance of the serum collected on days 0 and 42 was used as an indication of the increase in protective antibodies (Fig. 2). These absorbances were the values consequently used in the statistical calculations.

Detection of antibodies to PI₃ and IBR viruses

Antibodies to the PI₃ virus antigen were detected by the plaque-reduction neutralization test with the use of Vero-cell culture (Onderstepoort Biological Products, Quality Assurance Standard Operating Procedures) (Table 2, Fig. 3 and 4).

Antibodies to the IBR virus antigen were determined by the serum-neutralization test in which MDBK cells were used as target cells (Onderstepoort Biological Products, Quality Assurance Standard Operating Procedures) (Table 3, Fig. 5 and 6).

Titres were determined for days 0 and 42, reflecting the pre-immunization and post-immunization immune status of the animals. Statistical analysis was not performed on these titres.

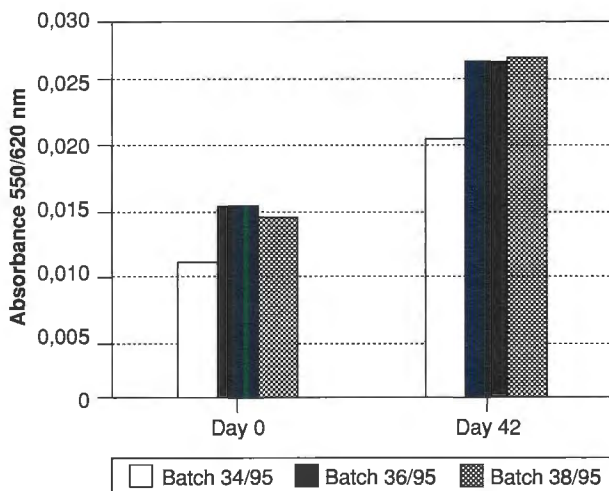


FIG. 2 Changes in the day 0 and day 42 absorbance values of antibody titres in calves against three different batches of leukotoxin in multivalent vaccine, testing its one year stability

TABLE 2 The range of day 42 PI₃ antibody titres in the serum of two groups of calves immunized with the trivalent vaccine, over a 1-year period

Range of titres (1/20 dilutions)	Percentage of titres	
	First-stage stability (n = 150)	One-year stability (n = 68)
0	46	0
20	11	12
40	11	10
80	12	15
120	3	7
160	17	56

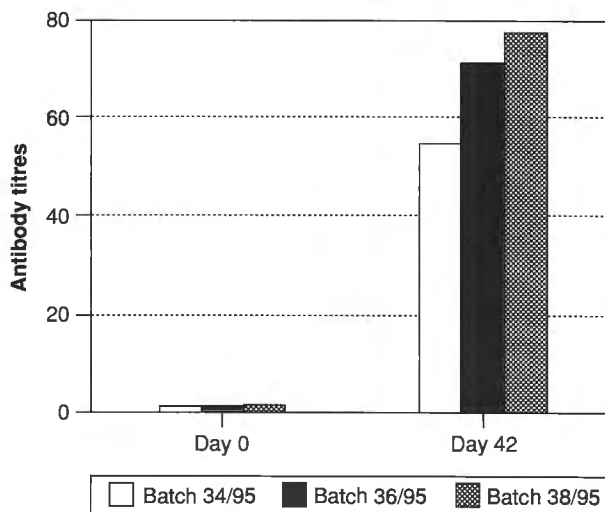


FIG. 3 Changes in the day 0 and 42 antibody titres in calves against three different batches of inactivated PI₃ virus antigen in combination vaccine, testing the first-stage stability of 3-month-old vaccine

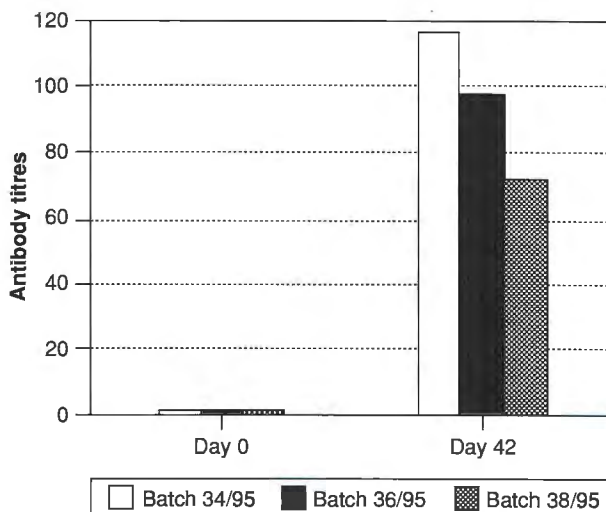


FIG. 4 Changes in the day 0 and day 42 antibody titres in calves against three different batches of inactivated PI₃ virus antigen in combination vaccine, testing its 1-year stability

TABLE 3 The range of day 42 IBR antibody titres in the serum of two groups of calves immunized with the trivalent vaccine, over a 1-year period

Range of titres (Log ₂ dilutions)	Percentage of titres	
	First-stage stability (n = 150)	One-year stability (n = 68)
0	7	6
2	0	0
4	13	9
8	17	7
16	17	9
32	46	69

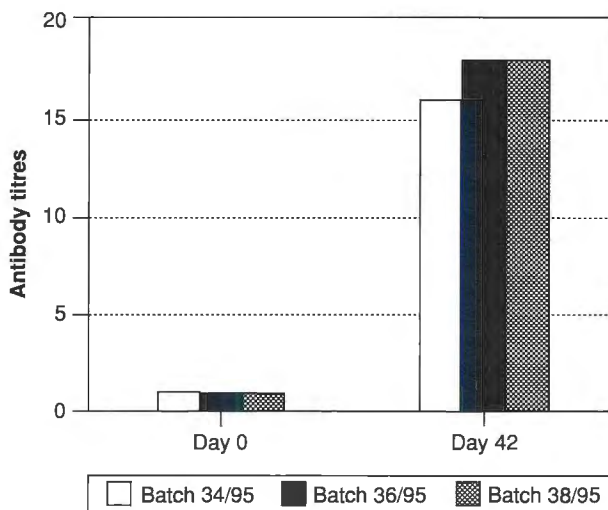


FIG. 5 Changes in the day 0 and 42 antibody titres in calves against three different batches of inactivated IBR virus antigen in multivalent vaccine, testing the first-stage stability of 3-month-old vaccine

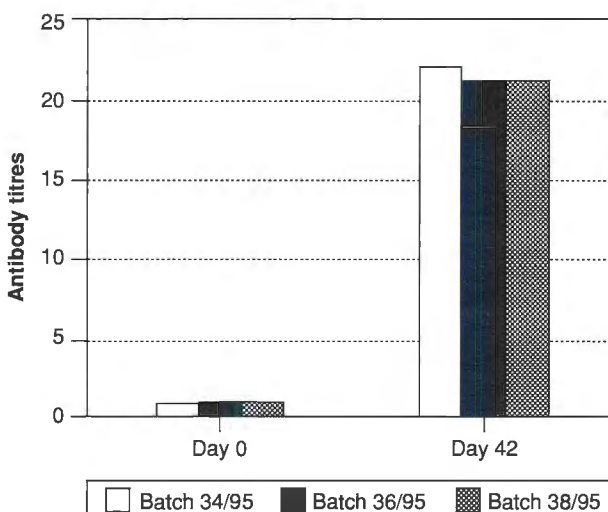


FIG. 6 Changes in the day 0 and day 42 antibody titres in calves against three different batches of inactivated IBR virus antigen in multivalent vaccine, testing its 1-year stability

Testing the stability of vaccines

Three different production batches of vaccine were used to immunize the calves. The vaccine was 3 months old when the first group of calves were immunized. It was stored at 4–6°C for 1 year and used to immunize the second group of calves. The ability of the vaccine to retain its immunogenicity after 1 year was evaluated.

Statistical analysis

The significance of the increase in leukotoxin 50% endpoint titres (Fig. 1) from days 0–42 as well as the increase in absorbance (Fig. 2) to demonstrate immunoconversion, from day 0–42 for each animal, was done by using the two-sided, paired t-test.

Analysis of variance by use of the SAS System (SAS Institute, Inc., 1989) was implemented to compare the differences between the leukotoxin serological response from each vaccine batch for the 3-month- as well as the 1-year-stability test period. It was also used to compare the differences between the day 42 final titres as reflected in Fig. 1 and 2, as well as the final titres of the individual vaccine batches 34/96, 36/96 and 38/96.

Owing to the fact that most of the day 0 titres had values of 0 and the day 42 values had increased obviously and significantly, it was not possible to perform any statistical analysis on the day 0 and day 42 titres of both the PI₃ and IBR antigens.

RESULTS

Leukotoxin activity

The leukotoxic activity for the three batches of vaccine ranged between 400 and 600 u/ml (Vega *et al.* 1987).

Detection of leukotoxin antibodies

There was a statistically significant increase in anti-leukotoxin titres from day 0–42, for both first-stage and 1-year stability tests (Fig. 1 and 2) ($P < 0,01$), for all three vaccine groups. The analyses of variance demonstrated clearly that the averages of day 0 and day 42 titres did not differ significantly ($P > 0,05$). This applies to the averages for each of the three vaccine groups testing the immunogenicity of the 3-month- and 1-year-old vaccine. By use of the same technique, it was also demonstrated that there was no deterioration in the immunogenicity when the titres of each batch of vaccine over a 1-year period ($P > 0,05$), were compared with each other.

During the first stage, the stability of 51% animals had increases of > 100 u/ml, which increased to 70% after 1 year (Table 1).

Detection of PI₃ antibodies

A large number of animals (46%) did not show any titre increases after the first-stage stability tests, in contrast with none after the 1-year stability tests. Though the titre increases from day 0–42 occurred in both cases (Table 2, Fig. 3 and 4) and differed significantly. After the first-stage stability, only 42% had PI₃ antibody titres of 40 and higher, whereas after the 1-year stability, it was 87%.

Detection of IBR antibodies

There was a general and significant increase in IBR antibody titres from day 0–42, for both first-stage and 1-year stability tests (Table 3, Fig. 5 and 6). For the first-stage stability tests, 7% of animals had no serum conversion, whereas this dropped to 5.9% after the 1-year stability tests. The majority of animals for the first-stage (79%) and 1-year stability (85%) had antibody titres of 8 and more, representing a threefold log₂ increase.

DISCUSSION

In spite of new and advanced vaccines bovine respiratory disease (BRD) is still the most important disease affecting feedlot cattle in North America, and its economic impact is underestimated (Jim, Booker, Ribble, Guichon & Thorlakson 1993). Death resulting from respiratory infections appears to be one of the major problems occurring in the feedlot. Death due to respiratory disorders ranges from 0.073–0.234% of total occupancy, and 44.1% of total death loss over a period of 1 year. This figure is much higher during the colder months of the year such as autumn and winter when it reaches 52%. During the milder and warmer months of spring and summer, this figure drops to 37.2% (Vogel & Parrott 1994).

In a large commercial feedlot with an annual turnover of 58 000 head of cattle, lower respiratory tract diseases accounted for approximately 46% of the total disease cost, and 60% of the cost of the ten most prevalent diseases (Frank, Salman & MacVean 1988). Lower respiratory tract disease was the cause of morbidity in 50% animals in a feedlot, with 58% cases occurring in the first 40 d of feeding, the incidence peaked prior to 20 d of feeding (Alexander, MacVean & Salman 1989). In a large Irish feedlot, respiratory disease was the most frequently recorded disease and was responsible for 62% morbidity and 58% mortality (Healy, Monaghan, Basset, Gunn, Markey & Collins 1993). Although the feedlot sector is a relatively new one in Australia's beef industry, respiratory diseases are already regarded as the most important problem (Watson & Fell 1993), with *Pasteurella haemolytica* and *Pasteurella multocida* being the most important isolates.

In South Africa, 66.9% mortalities in a feedlot were ascribed to BRD, with *Pasteurella* playing a dominant role in 56.8% cases (Orsmond 1988). Here BRD was responsible for 24% morbidity and 0.67% mortality. Recent figures regarding the role of BRD indicate a morbidity rate of 14.6% and a mortality rate of 0.14% (M.W. Odendaal, unpublished data 1997). Of the total annual morbidity, 67% was ascribed to respiratory disease. Twenty-two percent mortalities were associated with respiratory diseases of which 15% were a direct result of fibrinous pleuropneumonia. Although there has been a decline in the mortality rates from 0.67–0.14%, and in the morbidity rates from 24–15%, since 1988 to the present, BRD is still the most important disease in the feedlot. It is also the greatest cause of financial losses, in spite of the increased usage of local and imported vaccines. A new approach to increase the herd resistance to respiratory disease in especially feedlots, is needed, and may include new ways of stimulating the active immune response. There is also a need to investigate the practical application of passive immunity.

The level at which a serological titre to a particular antigen becomes biologically significant in terms of affording some protection from clinical disease, is difficult to establish. Seroconversion was considered to have taken place when an individual animal showed at least a fourfold increase in titre or a twofold increase in reciprocal log₂ titres (Allen, Viel, Bateman, Nagy, Rosendal & Shewen 1992).

Some of the earlier studies have revealed a strong positive correlation between the presence of leukotoxin antibodies in circulation and resistance to fibrinous pleuropneumonia lesions developing in the lung (Shewen & Wilkie 1983). This leads to the surmise that the peripheral circulating antibodies are an indication of the anti-leukotoxin protection in the lung. At this stage it is not clear what the minimum protective anti-leukotoxin level is, and neither what concentration of leukotoxin is required to elicit such an antibody response. The final anti-leukotoxin titre of each production vaccine batch is a possible indication of the protective ability of the antibodies against the leukotoxin, and is reflected in these results. This was one of the prime reasons why we did not embark on challenge tests in calves to test the efficacy of the antibodies, as was the case in numerous studies performed in the USA.

There is no quantitative measurement to indicate the titre required by an animal to ensure at least 90% protection against natural challenge by virus. The significant increase of antibody titres from day 0–42 for both the IBR- and PI₃-inactivated virus antigens, is an indication that seroconversion has taken place according to the definition of Allen *et al.* (1992). All three components of the multivalent vaccine resulted in adequate seroconversion in the animals that were

immunized. The method of antibody titre detection for each of the three antigens was that of neutralization, which means that the antibody that actually neutralizes the virus or leukotoxin, is measured. The significant increase of the neutralizing antibodies against the PI₃ and IBR antigens indicates that seroconversion has taken place and that antibodies have formed and are present in the circulation. The minimum antibody titre required to ensure adequate protection against both these viruses, has not yet been determined.

Not only did each of the three antigens demonstrate adequate immune response for each of the batches produced, but they still afforded protection after a 3-month and a 1-year stability period. For leukotoxin the titre increase for batch 1 was 15–153 u/ml, for batch 2 20–117 u/ml, and for batch 3 18–132 u/ml. The 1-year stability demonstrated similar leukotoxin end titres for each of the three batches. Active-challenge studies in calves are commonly used to test the efficacy of the *Pasteurella haemolytica* vaccines. The lung-lesion scores of immunized and non-immunized calves have been determined and the protective index calculated for each type of vaccine (Srinand, Ames, Maheswaran & King 1996). At this stage, a number of challenge methods have been described. Variations regarding the deliverance of the challenge material occur, such as the transthoracic method (Confer & Panciera 1994), endobronchial instillation by means of a fiberoptic bronchoscope (Sreevatsan *et al.* 1996) or intrabronchial instillation (Shewen *et al.* 1988). *In vivo* challenge studies were not considered for this study.

The importance of culture supernatant containing leukotoxin as a protective antigen, has been established beyond any doubt, and its inclusion in future vaccines for prophylactic immunization against *Pasteurella haemolytica* pneumonia, seems inevitable (Srinand *et al.* 1996a; Sreevatsan *et al.* 1996).

ACKNOWLEDGEMENTS

The capable technical assistance of Mrs Leanna de Jager, Mrs Rulis du Bruin, Mr Pieter Pieterse, and Mr Petrus Molotsi made this study possible, and is gratefully acknowledged. Mr Roelof Coetzer of the ARC Argimetrics Institute is thanked for performing the statistical analysis.

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