The production and evaluation of *Pasteurella haemolytica* leukotoxin in the supernatant of submerged cultures in fermenters

M.W. ODENDAAL and C.E. ELLIS

Bacterial Vaccine Development Unit, Onderstepoort Veterinary Institute
Private Bag X05, Onderstepoort, 0110 South Africa

ABSTRACT

ODENDAAL, M.W. & ELLIS, C.E. 1999. The production and evaluation of *Pasteurella haemolytica* leukotoxin in the supernatant of submerged cultures in fermenters. *Onderstepoort Journal of Veterinary Research*, 66:265–272

The optimal production of *P. haemolytica* leukotoxin in the culture supernatant of a fluid medium is dependent on a number of factors. The leukotoxin has to be produced by using a strain that is known for its ability to produce high quantities of leukotoxin, inoculated into the most suitable type of medium at the correct culture density containing the necessary supplements and harvested after a certain growth period. The volume in which it is produced may also have an influence. Two different procedures are described to produce the leukotoxin in 5 to 15-ℓ quantities in RPMI 1640 medium. The first method used to produce leukotoxin is one that has been repeatedly described since the presence of the leukotoxin was first established in 1978. Using this method seven batches of leukotoxin were produced in litre quantities with leukotoxin activity ranging from 23–67 u/mℓ. The seed culture inoculum is prepared in brain heart infusion broth, which is centrifuged before the organisms are inoculated into RPMI 1640 medium containing 3,5 % foetal calf serum and incubated for only 1 h in a fermenter, after, which the leukotoxin is harvested.

An improved alternative method was devised which yielded higher levels of leukotoxin activity by utilising the ability of the *P. haemolytica* organisms to grow and produce leukotoxin during the logarithmic growth phase in a fermenter. A seed culture harvested in the log phase was prepared in brain heart infusion broth by means of a series of cultures and inoculated into RPMI 1640 containing 3,5 % foetal calf serum. Three hours of active growth were allowed during which the leukotoxin was measured by its biological activity and an ELISA assay, and the increase in cell mass by means of the optical density every 30 min. The average leukotoxin biological activity measured 260 u/mℓ and by means of the ELISA test the leukotoxin concentration measured 315 u/ℓ which is a substantial increase in leukotoxin production. In comparison the average optical density only measured 0,469 at 650 nm. Previous findings were substantiated that the highest cell density was not reflected in the highest leukotoxin activity. It is possible to induce high levels of leukotoxin secretion in submerged cultures with RPMI 1640 medium containing foetal calf serum in the controlled environment of a fermenter in large enough quantities for use as a vaccine by the improved preparation of the seed culture inoculum.

Keywords: Antigen, cytotoxin production, ELISA, fermenter, leukotoxin production, *Pasteurella haemolytica*, toxin secretion, vaccine production

INTRODUCTION

The production of leukotoxin by *Pasteurella haemolytica* in the culture supernatant of a submerged culture has been described on numerous occasions in the literature (Baluyut, Simonson, Bemrick & Maheswaran 1981; Shewen & Wilkie 1982; Shewen & Wilkie 1985; Confer & Durham 1992; Gatewood, Fenwick & Chengappa 1994; Waurzyniak, Clinkenbeard, Confer & Srikumaran 1994). The volumes of leukotoxin rich supernatant produced in these reports were small and mostly from static cultures and was not enough to be used and evaluated for vaccine production. There is a distinct paucity on information in the literature relating to the growth aspects and the produc-

Accepted for publication 13 September 1999—Editor

tion of *P. haemolytica* leukotoxin in submerged culture in fermenters for vaccine purposes in large volumes. It is known that some pharmaceutical companies do produce *P. haemolytica* leukotoxin based vaccines (Confer & Panciera 1994; Sreevatsan, Ames, Werdin, Yoo & Maheswaran 1996) but their production methods have not been revealed. A distinct need arose in South Africa to produce leukotoxin in large quantities that could be included as a protective antigen in mono- or multivalent vaccines for use in feedlot cattle which would replace the existing and redundant *P. haemolytica* bacterin vaccine.

Two methods were employed to produce *P. haemolytica* leukotoxin in large quantities in submerged cultures in a fermenter under controlled conditions. These methods compared the production of leukotoxin from one strain of *P. haemolytica* in RPMI 1640 with the addition of 3,5% foetal calf serum and grown for either 1 or 3 h. One of the main differences between the two methods was the method of preparing the seedculture inoculum. The results in terms of leukotoxin yield and densities of cell growth obtained from a number of production batches during cultivation in large fermenters with both production methods are compared and discussed.

MATERIALS AND METHODS

P. haemolytica strain

The *Pasteurella haemolytica* biotype A serotype 1 strain designated as ph 01/10, was obtained from Dr Richard Davies, Department of Microbiology, University of Glasgow, Scotland.

Sampling method for measurement of cell growth by optical density

During the incubation period (1 or 3 h) 3 mℓ of the RPMI 1640 growth medium containing 3,5% foetal calf serum (FCS) (Highveld Biologicals, Midrand, Gauteng Province) was removed every 30 min and 1 mℓ dispensed separately into each of three 1-mℓ Eppendorf tubes and centrifuged for 2 min in a Hettich Eppendorf microcentrifuge (Labotec, Midrand, Gauteng Province). The supernatant was removed with a micropipette and immediately after placing it in a 1-mℓ Eppendorf tube which was identified, it was frozen with dry ice and stored at –70 °C until the leukotoxin activity could be determined. The remaining cell pellet was resuspended in 1 mℓ of sterile deionized water and the optical density (OD) of the suspension measured at 650 nm (Odendaal & Du Plessies 1999).

Measurement of leukotoxin activity

The method of Vega, Maheswaran, Leininger & Ames (1987) utilizing bovine neutrophils was used to quantify the amount of leukotoxin present in culture supernatant. It gives an indication of the biological activity of the leukotoxin. One unit of leukotoxin is defined as the reciprocal of the highest dilution causing 50% cytolysis of neutrophils and is calculated according to the method of Reed & Muench (1938).

Isolation of bovine neutrophils

A calcium and magnesium-free phosphate buffered saline stock solution was prepared by adding 8,0 g of NaCl, 2 g of KCl, 1,15 g of Na_2HPO_4, 0,2 g KH_2PO_4 and 1 g dextrose to 100 mℓ of deionized water. A working solution was prepared by diluting this in a ratio of 1:9 with deionized water. Blood from a 6-month-old calf was collected in heparin vacuum tubes and centrifuged. Approximately 12–15 mℓ of red blood cells were added to an equal volume of the working solution of the phosphate buffer. This was carefully layered on top of 7 mℓ of Histopaque 1077 (Sigma catalogue no. 1077, Labretoria, Pretoria, Gauteng Province) in a sterile, 50-mℓ plastic centrifuge tube and centrifuged at 830 *g* for 30 min. The plasma fraction containing the mononuclear cells were siphoned off and discarded. Lysis of the red blood cells in the sediment was effected with ice-cold ammonium chloride solution and again centrifuged at 400 *g* for 5 min. This procedure was repeated twice. The neutrophils were subsequently suspended in 2–10 mℓ of the working solution of the phosphate buffer and counted in an Improved Neubauer counting chamber [Resistance, LW Germany, Labotec (Pty) Ltd, Halfway House, Gauteng Province].

Leukotoxin assay with ELISA

A coating buffer was prepared by dissolving one 50-mM carbonate tablet (pH 9,6) in 100 mℓ deionized water, and a lactogen/casein/PBS Tween (PBS/T) buffer by adding 3,5 g lactogen, 0,5 g casein hydrolysate, 0,5 mℓ Tween 20–50 mℓ of the working phosphate buffer. A citrate (4,2 g sodium citrate/200 mℓ) and phosphate buffer (5,68 g Na_2HPO_4/200 mℓ) was prepared separately and dispensed in small volumes and stored at 4 °C. A substrate was prepared by adding 4 mg o-phenylenediamine (OPD) to 5 mℓ citrate buffer and 5 mℓ phosphate buffer to which was added 0,5 mℓ of a 1/100 dilution of H_2O_2. A 4N H_2SO_4 solution was used to stop the enzyme reaction. Primary anti-leukotoxin antibodies used were prepared by injecting recombinant leukotoxin in Complete and Incomplete Freunds Adjuvant, (obtained from Dr Robert Davies, Moredun Research Institute, Edinburgh, Scotland) intradermally and intramuscularly, respectively in White New Zealand rabbits. Reference leukotoxin was prepared in a fermenter with high leukotoxin values, filtered through a 0,2-µ filter, evaluated according to the method of Vega *et al.* (1987) and dispensed into 1-mℓ volumes and frozen at –70 °C.

An ELISA plate was subdivided into sections coated with a 1/20 dilution of reference leukotoxin, 1/10 dilution of test specimens, and 1/10 leukotoxin controls. The leukotoxin control was used as an internal quality control to evaluate the standard deviation of the day-to-day variation. The plate was well sealed and incubated overnight at 4 °C, after which it was washed five times with PBS/T and shaken dry. Thereafter 300 mℓ of the usual blocking buffer was added to each well and the plate incubated at 37 °C for 1 h and rinsed once with PBS/T. The primary rabbit anti-leukotoxin antibodies were diluted 1/500 with blocking buffer (24 µℓ/12 mℓ). This solution was mixed well and 100 mℓ of it were pipetted into each well and the plate was incubated for 1 h at 37 °C whilst shaking. The plate was washed five times with PBS/T and shaken dry.

The secondary anti-rabbit peroxidase conjugate (Sigma Immunochemicals, Atlasville, South Africa) was diluted 1/2000 in blocking buffer (6 µℓ/12 mℓ), mixed well and 100 mℓ pipetted into each well, incubated for 1 h at 37 °C on a shaking platform. The plate was again washed five times with PBS/T and shaken dry. The substrate solution was prepared and 100 µℓ were added per well and the reaction allowed developing at room temperature for 5 min. The reaction was stopped with the addition of 50 µℓ 4N H_2SO_4 per well.

Finally the absorbance was read at 492 nm on a Titertek plate reader.

First leukotoxin production method

The *P. haemolytica* organism was taken from the master seed freeze-dried collection, activated on blood tryptose agar (BTA) and grown for 18–20 h at 35–37 °C. On day 1, 5–6 single colonies were inoculated into 100 mℓ brain heart infusion broth (BHI) and grown on a shaking platform for 12–14 h at 35–37 °C. After this an additional volume of 100 mℓ pre-incubated BHI was added to the 100 mℓ culture and shaken for another 1–2 h at 35–37 °C. This culture was subsequently inoculated into 5 ℓ of BHI in fermenter and grown for 3 h at a temperature of 35–37 °C. This culture was centrifuged in 200-mℓ quantities at 10 000 rpm in a Beckman J2-21 centrifuge [Beckman Instruments (Pty) Ltd, Halfway House 1685, Gauteng Province], the cell sediment was collected under aseptic conditions in a biohazard laminar flow cabinet and the supernatant discarded.

The cells were inoculated into 5 ℓ RPMI 1640 medium with 3,5% foetal calf serum in a Braun ED 10 ℓ fermenter [Labotec (Pty) Ltd, Halfway House 1685, Gauteng Province]. This was grown for 1 h at 35–37 °C, before it was siphoned off and centrifuged at 6 °C in 200-mℓ volumes, and the culture supernatant filtered through filters ranging from a series of pre-filters (AP 25, AP 20 and AP 15 pre-filters, Millipore 142 mm diameter [Microsept (Pty) Ltd, Bramley 2018, Gauteng Province] and microfilters to a final filter size of 0,45 µ. The final product was collected in sterile 10-ℓ glass containers and stored at 4–8 °C. Three 5-mℓ samples were taken from the final filtrated product of which 1 mℓ was inoculated into 5 mℓ of BHI and grown overnight at 35–37 °C to ensure the sterility of the filtration procedure. This is the final product and is dispensed into 12 mℓ plastic vaccine containers in 10 mℓ quantities.

Second leukotoxin vaccine production method

Frozen *Pasteurella haemolytica* biotype A serotype 1 master seed cultures were prepared by selecting five colonies from a pure blood agar culture and inoculating it into 150 mℓ BHI broth and shaken mildly for 2 h at 37 °C. Sterile glycerol (15%) was added to the cells in the BHI, and 1-mℓ volumes dispensed into sterile Eppendorf tubes and stored at –70 °C.

The seed culture inoculum for the fermenter was prepared by removing and thawing the frozen culture at room temperature. The 1-mℓ cell volume was added to 250 mℓ BHI and incubated at 37 °C for 4 h while shaking. A Gram stained smear of the culture was made to ensure its purity. One litre of this BHI was added to 250 mℓ prewarmed BHI culture in a sterile 2-ℓ container and incubated a further 2 h, after which an additional 750 mℓ BHI was added and incubated for another 1 h. Gram smears and subcultures on three BTA plates were made after each stage as a control measure to check for any contamination. The absence of any contamination in the seed culture was regarded as an indication that the fermenter was ready for inoculation.

The 30-ℓ model Braun Biostat 421 fermenter was prepared by thoroughly rinsing it with deionized water, filling it with 30 ℓ deionized water and steam sterilizing it for 30 min at 121 °C, after which it was allowed to cool down. The water was siphoned off and replaced with 15 ℓ of sterile RPMI 1640 medium that was prepared by pouring 15 ℓ deionized water into a Cornelius stainless steel container, with the required mass of RPMI 1640 medium and with the addition of 2 g/ℓ sodium bicarbonate. It was shaken until it had dissolved. The pH was set at 7,2 with aeration of carbon dioxide gas. The required volume of FCS was removed from the –20 °C freezer and allowed to thaw overnight at room temperature. This was added to the RPMI 1640 medium immediately before filtration. The RPMI 1640 medium with FCS was filtered into the fermenter by means of a sterile 10-inch stainless steel 0,22-µ Fluorordyne filter [Deomed (Pty) Ltd, Halfway House 1685, Gauteng Province]. After filtration, a specimen of the medium was collected aseptically and cultured aerobically at 35–37 °C on BTA for a 3-d period. The pH of the medium measured between 7,0 and 7,4 but was not controlled during the growth phase, the pO_2 supply was set at 100%

during the length of the growth phase. The maximum airflow of 50 ℓ/min was allowed. The stirrer speed was set to between 300 and 400 rpm and the temperature was kept constant at 37 °C. All the above parameters were activated after inoculation of the fermenter with the seed culture. The airflow was switched off during inoculation.

Samples from the fermenter culture were removed every 30 min to measure the microbial growth curve and to determine the leukotoxin activity. After centrifugation of 1 mℓ of the culture, the supernatant was siphoned off and used for the detection of leukotoxin activity (Vega *et al.* 1987) and 1 mℓ of deionized water was added to the cells and resuspended, and its OD measured at 650 nm. Deionized water was used as the standard reference. The first sample was collected directly after inoculation had taken place to establish a starting point. The culture was grown for between 3 and 5 h (depending on the spectrophotometric cell density) after which a sample was collected to prepare a Gram smear and check for signs of contamination. The contents of the fermenter were siphoned off in 10-ℓ sterile glass containers and centrifuged in 1-ℓ volumes (Beckman J2-21 from Beckman Instruments, Halfway House, Gauteng Province) at a speed of 4 500 rpm for 35 min. The supernatant was collected in sterile 10-ℓ glass Schott containers and kept in a large room refrigerator at 4–7 °C. The remaining cells were inactivated by means of steam sterilization and discarded.

Microfiltration of leukotoxin was done by positive air pressure from Cornelius stainless steel canisters to ensure its sterility. These canisters were steam sterilized before use. A 0,5-µ Profile II depth filter and a 10-inch 0,22-µ Fluorodene II filter were used, of which both had to be steam sterilized. If during filtration the 0,5-µ filter became blocked it was replaced and after filtration discarded. After the 0,22-µ filtration, the Fluorodyne filter was regenerated by placing it in an ultrasonic bath followed by a thorough rinse in clean water and allowed to dry. The whole filtration process took place under laminar flow filtration in a class II biosafety cabinet. Three leukotoxin filtrate samples were collected after the final filtration process. The first one was to determine the sterility of the final product on BTA under aerobic and anaerobic growth conditions, the second sample was sent to the Quality Assurance Section for sterility and safety and the third sample was used to determine its leukotoxin activity.

RESULTS

First production method

Seven batches were produced of which the highest leukotoxin activity detected was 67 u/mℓ and the lowest 23 u/mℓ, with a mean of 40 u/mℓ and standard deviation of 18,9. There was no increase in the OD as the culture appeared to remain static for the 1-h period.

Second method of production

Fourteen batches of leukotoxin were produced that could be used as antigen in mono- or multivalent vaccines. In some batches only the ELISA measurements of the leukotoxin activity were determined and in some only the biological activity. Though more batches were produced, some were discarded due to either no leukotoxin yields, contamination with bacteria and spillage. The cytotoxic activity of the leukotoxin ranged from 44 u/mℓ (batch 270597) to 512 u/mℓ (batch 240697) after 3 h logarithmic growth. The mean for the nine batches was 260 u/mℓ after 3 h with a standard deviation of 199 (Table 1).

TABLE 1 The leukotoxin biological activity produced every 30 min by *Pasteurella haemolytica* strain 1/10 for 3 h grown in fermenter in RPMI 1640 medium with 3,5 % FCS ($n = 9$)

Batch no.	Leukotoxin u/mℓ produced every 30 min						
	t0	t1	t2	t3	t4	t5	t6
080497	4	3	7	5	14	49	121
200597	4	18	18	27	50	112	319
270597	3	6	10	8	15	21	44
120697	0	20	33	40	150	330	490
190697	0	12	3	54	89	512	–
240697	0	14	96	454	192	400	512
260697	0	4	41	220	402	446	427
150797	0	0	0	15	32	57	66
170797	0	2	11	88	36	196	103
Ave ($n = 9$)	1	9	24	101	109	236	260
SD	2	7	30	148	126	189	199

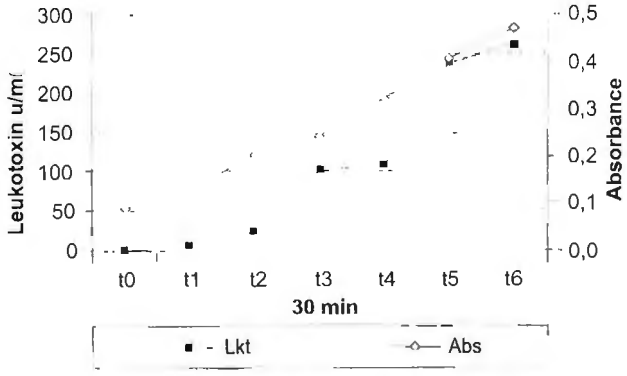

FIG. 1 A graphical comparison between the average cytotoxic activity of the leukotoxin vaccine produced by the second method and the average concomitant cell density measured at 650 nm ($n = 9$, $r = 0,97$, $P < 0,001$)

There was a slow and consistent increase in the production of the leukotoxin every 30 min during the production cycle of 3 h. This was associated with the concomitant increase in the optical density measured at 650 nm. A strong correlation was demonstrated between these two variables (r = 0,97, P < 0,001) (Fig. 1). The changes in absorbance during the logarithmic growth cycle of 14 leukotoxin batches are shown in Table 2 as measured every 30 min over a 3-h period.

After 3 h the highest OD was 0,73 and the lowest 0,275 for two different batches. The leukotoxin vaccine batch (batch 270597) that produced the lowest concentration of leukotoxin (44 u/mℓ), also had a low OD peak of 0,27, whereas the highest peak of 0,72 produced in batch 120897 only produced 161 u/mℓ as measured with the ELISA (cf. Table 3 for the ELISA values).

The ELISA test showed an average leukotoxin activity of 315 u/mℓ (n = 11) with a SD of 157. The maximum/minimum values achieved after 3 h growth (t6) ranged between 604 and 106 u/mℓ. There was a strong correlation between the OD attained and the ELISA measurement (r = 0,97, P < 0,001) (Fig. 2).

The correlation between the leukotoxin activity measured with ELISA and the biological activity was 0,98 (P < 0,001) (Fig. 3).

TABLE 2 The optical density measured at 650 nm every 30 min of *P. haemolytica strain* 1/10 for 3 h during cell growth in fermenter in RPMI 1640 medium with 3,5 % FCS (n = 14) for production of leukotoxin vaccine

Batch no.	Optical density at 650 nm measured at 30 min intervals						
	t0	t1	t2	t3	t4	t5	t6
080497	0,063	0,079	0,17	0,168	0,192	0,237	0,275
200597	0,077	0,120	0,15	0,184	0,243	0,361	0,416
270597	0,081	0,110	0,14	0,181	0,207	0,234	0,270
120697	0,104	0,091	0,16	0,141	0,203	0,254	0,302
190697	0,126	0,108	0,18	0,282	0,408	0,549	0,632
240697	0,079	0,119	0,17	0,241	0,261	0,310	0,399
260697	0,086	0,109	0,17	0,226	0,319	0,358	0,452
150797	0,096	0,124	0,15	0,210	0,275	0,400	0,473
170797	0,058	0,076	0,22	0,114	0,201	0,269	0,418
050897	0,139	0,194	0,26	0,364	0,502	0,594	0,720
120897	0,078	0,150	0,25	0,370	0,530	0,690	0,730
130897	0,108	0,177	0,29	0,330	0,430	0,540	0,570
260897	0,053	0,061	0,16	0,240	0,290	0,340	0,420
270897	0,083	0,140	0,29	0,290	0,370	0,440	0,490
Ave (n = 14)	0,0879	0,118	0,20	0,239	0,317	0,398	0,469
SD	0,0246	0,038	0,05	0,08	0,114	0,145	0,148

TABLE 3 The leukotoxin activity produced every 30 min by *P. haemolytica* strain 1/10 for 3 h grown in fermenter in RPMI 1640 medium with 3,5 % FCS (n = 11) measured with ELISA (u/mℓ)

Batch no.	ELISA u/mℓ measured every 30 min						
	t0	t1	t2	t3	t4	t5	t6
170797	33	33	36	36	50	70	106
050897	15	19	40	94	193	216	219
120897	19	29	34	67	132	133	161
130897	61	69	80	148	172	239	250
260897	0	72	93	202	285	418	484
270897	0	83	95	240	442	560	604
060597	15	32	44	56	93	125	124
200597	26	24	55	46	93	156	362
240697	31	33	45	97	195	240	423
260697	17	19	30	79	199	305	362
150797	23	23	27	52	122	186	376
Ave (n = 11)	21	38	50	95	167	224	315
SD	16	23	25	68	113	148	157

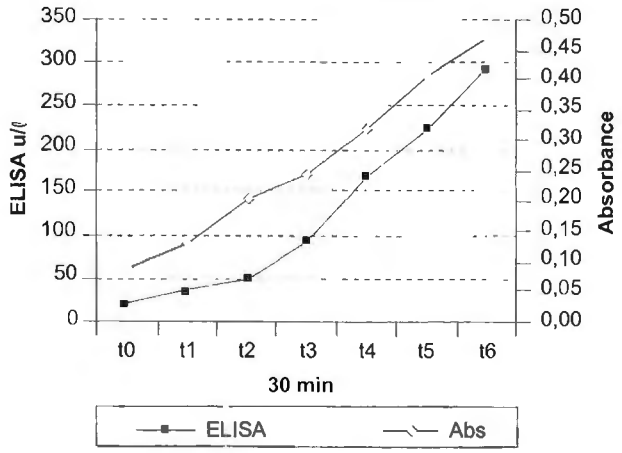

FIG. 2 A graphical comparison between the average ELISA values of the leukotoxin vaccine produced by the second method and the average concomitant cell density measured at 650 nm ($n = 14$, $r = 0,97$, $P < 0,001$)

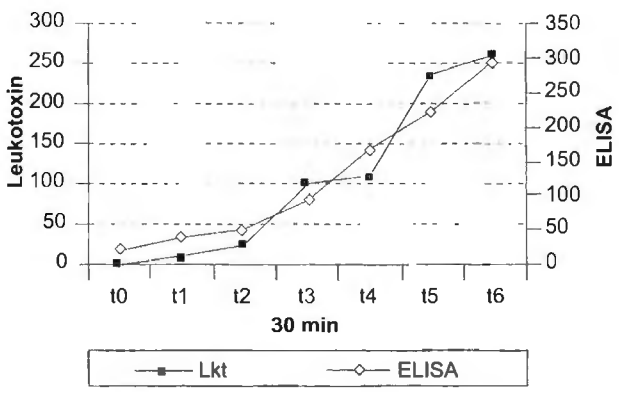

FIG. 3 A graphical comparison between the average ($n = 9$) leukotoxin cytotoxic values (u/mℓ) and the average ($n = 11$) ELISA values of the leukotoxin vaccine produced by the second method ($r = 0,98$, $P < 0,001$)

DISCUSSION

Casein hydrolysate was one of the first media that had been described to define the optimal growth conditions of *P. haemolytica* (Wessman 1965). At that stage the presence of leukotoxin was not yet known. It was only later that it was established that the leukotoxin is produced in culture supernatant by actively growing *Pasteurella haemolytica* type 1 in RPMI 1640 medium with and without foetal calf serum (FCS) (Baluyut *et al.* 1981; Shewen & Wilkie 1982; Shewen & Wilkie 1985). Metabolic active cells produce the leukotoxin during the final stages of the lag phase and most of the logarithmic growth phase of *P. haemolytica,* with a decline in production during the stationary phase (Shewen & Wilkie 1985). Knowledge of the growth curve and leukotoxin production yields of the *P. haemolytica* during this growth phase is important if optimal yields of leukotoxin are to be produced in small or large volumes of medium. The production of *P. haemolytica* leukotoxin in small quantities in various types of media has largely been utilized to study its biochemical and physical aspects (Mosier, Lessly, Confer, Antone & Gentry 1986: Clinkenbeard, Mosier & Confer 1989) and to produce a leukotoxin that could be free of any extraneous proteins that may interfere with the purification thereof (Sun & Clinkenbeard 1998). In other studies, semi-defined media was developed that supported the growth of the organism that was associated with the production of leukotoxin and in the process revealing the potential role of nitrogen availability in the regulation of leukotoxin expression (Highlander 1997). But as in previous studies this was also done in small quantities. The type of medium in which the leukotoxin is produced is an important consideration if it is to be used as a vaccine in ruminants. It should be compatible to the recipient animal and should not have any negative influence on the host. The production of leukotoxin in RPMI 1640 medium containing additives or without it has been successfully evaluated as a vaccine in calves by Shewen & Wilkie (1988a; 1988b; 1988c), Sreevatsan *et al.* (1996), Odendaal *et al.* (1997) and Odendaal (1999).

The leukotoxin producing abilities of a medium, the strain of *P. haemolytica* used, the inoculum density required as well as the supplements or additives involved, and the final volume in which it is produced may play a role in the final production yield of leukotoxin. It appears that not all strains of *P. haemolytica* have the ability to produce high yields of leukotoxin (Saadati, Gibbs, Parton & Coote 1997; Odendaal & Du Plessies, submitted for publication). An attempt should first be made to produce and evaluate the leukotoxin yields in small volumes testing all the possible variables before scaling up to the required production volumes. Sutherland, Donachie, Jones & Quirie (1989) successfully produced and evaluated a leukotoxin vaccine under experimental conditions for use in sheep with nutrient broth in 500 mℓ quantities in dialysis bags. Though this type of technology may be expected to have certain limitations when it is to be upscaled and used for commercial purposes.

The production of leukotoxin in large quantities for use as a vaccine is best performed in fermenters under controlled conditions. The first production method described in this article simulated the efforts of Baluyut *et al.* (1981), and Shewen & Wilkie (1982; 1985). The biological activity of the leukotoxin was measured at between 23 and 67 u/mℓ with an average of 40 u/mℓ. During this process there was no increase in the OD of the culture, indicating that the culture remained static for that period and that no active cell growth had taken place. The leukotoxin activity produced during this method was evaluated by its

biological activity on bovine polymorphonuclear leukocytes (Vega *et al.* 1987). The leukotoxin produced by this method, was evaluated as a vaccine and proved to be antigenic and protective when tested in cattle under intensive and extensive conditions (Odendaal *et al.* 1997; Odendaal 1999). Because the leukotoxin yields were not satisfactory a second production method was developed utilizing the fact that leukotoxin is secreted into the culture supernatant during the logarithmic growth phase of *P. haemolytica*.

The second method proved more efficient and increased the production yield of leukotoxin considerably to attain an arithmetic mean of 260 u/mℓ ($n = 9$) after 3 h growth, assayed with the biological method of Vega *et al.* (1987) (Table 1), the range of leukotoxin yield produced after 3 h growth varied from 44–512 u/mℓ (Table 1). With the immunochemical ELISA assay this increase in leukotoxin production was measured at 315 u/mℓ after 3 h growth ($n = 11$) (Table 3) ranging from 106–604 u/mℓ. The leukotoxin produced by this method was also immunogenic and protective when tested extensively in calves in feedlot field trials (Odendaal 1999). The reason why two batches produced low levels of leukotoxin during the 3-h growth cycle, reaching a maximum of 44 and 66 u/mℓ (Table 1) is difficult to explain. It may be related to certain shortcomings in the preparation and growth of the seedculture and after inoculation of the fermenter or perhaps even in the leukotoxin assay method as it was determined by the biological method of Vega *et al.* (1987). When the 3 h leukotoxin yield of the batch that was tested at 66 u/mℓ, was repeated with the ELISA assay, a value of 376 u/mℓ was obtained. The reason for this may be speculative, but it has been recorded that the *P. haemolytica* leukotoxin is temperature labile and that it may undergo natural toxoiding when it is held at room temperature for a short while, even during refrigeration or when kept at temperatures of –70 °C over extended periods (Chang, Renshaw & Richhards 1986). The immunogenicity of the leukotoxin may remain intact, but not the biological activity as measured with bovine polymorphonuclear leukocytes, enabling the immunochemical detection assay to be more sensitive when the leukotoxin detection assay is not performed immediately.

With the second leukotoxin production method, both the biological cytotoxic activity (Table 1) and the immunochemical assay (Table 3) methods were used to evaluate the leukotoxin produced during the active growth cycle of 3 h. Both leukotoxin detection methods demonstrated an increase in leukotoxin secretion rate during the logarithmic growth cycle of 3 h. A concomitant increase in the cell growth was also demonstrated by an increase in OD (Table 2). This increase in OD compares significantly with the increase in leukotoxin biological activity in Fig. 1 ($r = 0,97$, $P < 0,001$) as well as with the ELISA immunochemical assay of leukotoxin in Fig. 2 ($r = 0,97$, $P < 0,001$). These findings support the notion that the secretion of leukotoxin is an active process, dependent on the metabolic growth of *P. haemolytica* in a specific growth medium that is able to support and sustain its metabolic needs (Shewan & Wilkie 1985). This is an aspect which has to be investigated under local conditions when producing leukotoxin in large quantities for use as a vaccine.

The correlation between the two leukotoxin assays was positive and statistically significant ($r = 0,98$, $P < 0,001$) as shown in Fig. 3. In this trial the leukotoxin concentration in u/mℓ determined for each time period (t0–t6) the values were slightly higher with the ELISA method than the biological method for all measurements except for those of t3 and t5 (Tables 1 and 3, Fig. 3). This may indicate a higher immunogenic value obtained with the ELISA than the biological test. Another aspect regarding the large scale production of leukotoxin which confirms the earlier findings of Gentry, Confer, Weinberg & Homer (1986), is that the amount of leukotoxin produced in a culture supernatant is not necessarily correlated with the OD of the organisms in the culture.

From the results obtained it is evident that the second production method produces higher yields of *P. haemolytica* leukotoxin than the first, and would be regarded as the method of choice for its production in quantities ranging from 5–15 ℓ. It is also suggested that the immunochemical ELISA detection assay for leukotoxin is a sensitive and convenient method worth considering. In conclusion, it is possible to produce *P. haemolytica* leukotoxin in fermenters in the supernatant from cultures during the logarithmic growth phase, under controlled conditions for vaccine purposes in large quantities with yields that are immunogenic and protective when administered to feedlot calves.

REFERENCES

BALUYUT, C.S., SIMONSON, R.R., BEMRICK, W.J. & MAHESWARAN, S.K. 1981 Interaction of *Pasteurella haemolytica* with bovine neutrophils: Identification and partial characterization of a cytotoxin. *American Journal of Veterinary Research*, 42(11):1920–1926.

CHANG Y-F., RENSHAW H.W. & RICHARDS A.B. 1986. *Pasteurella haemolytica* leukotoxin: Physicochemical characteristics and susceptibility of leukotoxin to enzymatic treatment. *American Journal of Veterinary Research*, 47(4):716–723.

CLINKENBEARD, K.D., MOSIER, D.A. & CONFER, A.W. 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. *Infection and Immunity*, 57(2):420–425.

CONFER, A.W. & DURHAM, J.A. 1992. Sequential development of antigens and toxins of *Pasteurella haemolytica* serotype 1 grown in cell culture medium. *American Journal of Veterinary Research*, 53(5):646–652.

CONFER, A.W. & PANCIERA, R.J. 1994. Testing of two new generation *Pasteurella haemolytica* vaccines against experimen-

tal bovine pneumonic pasteurellosis. *Agri-Practice,* 15(8): 10–15.

GATEWOOD, D.M., FENWICK, B.W. & CHENGAPPA, M.M. 1994. Growth-condition dependent expression of *Pasteurella haemolytica* A1 outer membrane proteins, capsule, and leukotoxin. *Veterinary Microbiology*, 41:221–233.

GENTRY, M.J., CONFER, A.W., WEINBERG, E.D. & HOMER, J.T. 1986. Cytotoxin (leukotoxin) production by *Pasteurella haemolytica*: Requirement for an iron-containing compound. *American Journal of Veterinary Research*, 47(9):1919–1923.

HIGHLANDER, S.K. 1997. Growth of *Pasteurella haemolytica* and production of its leukotoxin in semi-defined media. *American Journal of Veterinary Research*, 58(7):749–754.

MOSIER, D.A., LESSLEY, B.A., CONFER, A.W., ANTONE, S.M. & GENTRY, M.J. 1986. Chromatographic separation and characterization of *Pasteurella haemolytica* cytotoxin. *American Journal of Veterinary Research*, 47(10):2233–2241.

ODENDAAL, M.W., MORRIS, S., DU PREEZ, E. & AITCHISON, H. 1997. The humoral immune response in cattle after immunization with a multivalent IBR/PI_3/ *Pasteurella haemolytica* A1 leukotoxin vaccine. *Onderstepoort Journal of Veterinary Research*, 64:205–212.

ODENDAAL, M.W. 1999. The management of stress-induced respiratory disease caused by *Pasteurella haemolytica* in feedlot cattle. Ph.D thesis. Pretoria: University of Pretoria.

REED, L.J. & MUENCH, H. 1938. A simple method of estimating fifty percent endpoints. *The American Journal of Hygiene*, 27(3):493–497.

SAADATI, M., GIBBS, H.A., PARTON, R. & COOTE, J.G. 1997. Characterization of the leukotoxin produced by different strains of *Pasteurella haemolytica*. *Journal Medical Microbiology,* 46: 276–284.

SHEWEN, P.E. & WILKIE, B.N. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infection and Immunity*, 35(1):91–94.

SHEWEN, P.E. & WILKIE, B.N. 1985. Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. *American Journal of Veterinary Research,* 46(5):1212–1214.

SHEWEN, P.E. & WILKIE, B.N. 1988a. Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. *Canadian Journal of Veterinary Research*, 52:30–36.

SHEWEN, P.E., SHARP, A. & WILKIE, B.N. 1988b. Efficacy testing a *Pasteurella haemolytica* extract vaccine. *Veterinary Medicine*, October: 1078–1083.

SHEWEN, P.E. & WILKIE, B.N. 1988c. Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. *Canadian Journal of Veterinary Research,* 52:30–36.

SREEVATSAN, S., AMES, T.R., WERDIN, R.E., YOO, H.S. & MAHESWARAN, S.K. 1996. Evaluation of three experimental subunit vaccines against pneumonic pasteurellosis in cattle. *Vaccine*, 14(2):147–154.

SUN, Y. & CLINKENBEARD, K.D. 1998. Serum-free culture of *Pasteurella haemolytica* optimized for leukotoxin production. *American Journal of Veterinary Research,* 59(7):851–855.

SUTHERLAND, A.D., DONACHIE, W., JONES, G.E. & QUIRIE, M. 1989. A crude cytotoxin vaccine protects sheep against experimental *Pasteurella haemolytica* serotype A2 infection. *Veterinary Microbiology,* 19:175–181.

WAURZYNIAK, B.J., CLINKENBEARD, K.D., CONFER, A.W. & SRIKUMARAN, S. 1994. Enhancement of *Pasteurella haemolytica* leukotoxic activity by bovine serum albumin. *American Journal of Veterinary Research*, 55(9):1267–1274.

WESSMAN, G.E. 1965. Cultivation of *Pasteurella haemolytica* in a casein hydrolysate medium. *Applied Microbiology*, 13(3): 426–431.

VEGA, M.V., MAHESWARAN, S.K., LEININGER, J.R. & AMES, T.R. 1987. Adaptation of a colorimetric microtitration assay for quantifying *Pasteurella haemolytica* A1 leukotoxin and anti-leukotoxin. *American Journal of Veterinary Research,* 48(11): 1559–1564.