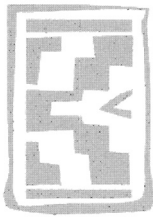

The influence of *Mannheimia haemolytica* A1 seed culture inoculum cell density on the production of leukotoxin in submerged culture supernatant

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ABSTRACT

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Mannheimia haemolytica leukotoxin is produced during the logarithmic growth phase in submerged culture in RPMI 1640 medium with and without the addition of foetal calf serum or albumin. In order to establish a pattern of optimal leukotoxin production in small volumes in submerged cultures and to define some parameters involved, two high leukotoxin producing *Mannheimia haemolytica* strains were grown in RPMI 1640 medium containing either FCS or BSA. The cell growth and leukotoxin production abilities of each strain were determined concomitantly every hour in RPMI 1640 medium containing each of the additives over a time period of 6 h. The growth performance of three dilutions of a standardized seed culture inoculum prepared with each of the cultures and additives were simultaneously compared with each other using the above parameters. The different seed culture inoculum dilutions had a definite effect on the time and quantity of leukotoxin production. Both strains demonstrated peak leukotoxin production after 4 h of active growth.

The addition of albumin to both isolates gave slightly increased leukotoxin levels, and both showed that the peak leukotoxin was not associated with peak cell concentration. Obvious quantitative differences in the ability of different *M. haemolytica* strains to produce leukotoxin were noted. Strain 12296 produced optimal leukotoxin concentration from the medium (1/25) dilution of the seed culture inoculum after 4 h, whereas strain 1/10 produced the same concentration with the low (1/5) dilution seed culture inoculum, possibly reflecting the superior production ability of the first strain. However, each strain of *M. haemolytica* appeared to have its own specific logarithmic cell growth and leukotoxin production pattern. The peak cell density of *M. haemolytica* grown in submerged RPMI 1640 culture medium cannot be used as an indication of optimal leukotoxin levels.

Keywords: Culture, cytotoxin, growth curve, inoculum, leukotoxin, *Mannheimia haemolytica* A1, submerged culture, toxin production

INTRODUCTION

It was due to the efforts of Benson, Thomson & Valli (1978) that the cytotoxic effects of *Mannheimia haemolytica* on bovine alveolar macrophages came to prominence for the first time. The leukotoxin was subsequently produced in culture supernatant by actively growing *Mannheimia haemolytica* type 1 in RPMI 1640 medium without foetal calf serum (FCS) (Baluyut, Simonson, Bemrick & Maheswaran 1981) and with the addition of 7% FCS (Shewen & Wilkie 1982; Shewen & Wilkie 1985). Metabolically active cells produce the leukotoxin, which is secreted extracellularly into the culture medium during the final stages of the lag phase and most of the logarithmic growth phase of *M. haemolytica,* with a decline in production during the stationary phase (Shewen & Wilkie 1985). This aspect is used in an attempt to obtain high or optimal leukotoxin yields. This is in contrast

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to similar toxins produced by *Pseudomonas aeruginosa* (Scharman 1976), *Staphylococcus aureus* (Soboll, Ito & Schaeg 1973) and *Actinobacillus actinomycetemcomitans* (Tsai, McArthur, Baehni 1979), which are harvested from stationary phase cultures and only after bacterial autolysis has occurred. Markham and Wilkie (1980) demonstrated that the cell free culture supernatant of *M. haemolytica* not only impair phagocytosis at low concentrations but are also clearly cytotoxic towards alveolar macrophages at higher concentrations. This aspect was thoroughly investigated and the cytotoxic influence of leukotoxin subsequently confirmed on bovine neutrophils *in vitro* (Baluyut *et al.* 1981), bovine peripheral blood mononuclear leukocytes (Kaehler, Markham, Muscoplat & Johnson 1980a), and bovine pulmonary lavage cells, peripheral blood lymphocytes, neutrophils and cultured peripheral blood mononuclear cells (Shewen & Wilkie 1982). It was also demonstrated that *M. haemolytica* has a cytocidal effect only on the peripheral blood mononuclear leukocytes of cattle, sheep and goats and not on the mononuclear leukocytes of swine, horses or humans (Kaehler, Markham, Muscoplat & Johnson 1980b). The presence of a cytotoxic substance in the culture supernatant produced by *M. haemolytica* was confirmed and illustrated by *in vitro* transmission electron microscopy studies and cytotoxicity assays (Berggren *et al.* 1981).

The *M. haemolytica* leukotoxin belongs to a group of bacterial toxins that are representative of important virulence factors produced by Gram-negative bacteria, and are collectively referred to as the RTX toxins (repeats in toxin). They are characterized by a series of glycine-rich repeat units at the C-terminal end of each protein. These toxins are secreted from the cell without a periplasmic intermediate by a novel mechanism which involves the recognition of a signal sequence at the C-terminus of the toxin by membrane associated proteins that export the toxin directly to the outside of the cell. The structural gene for each protein encodes an inactive toxin which is modified post-translationally to an active cytotoxic form by another gene product before secretion. The genes for toxin synthesis, activation and secretion are for the most part grouped together on the chromosome and form an operon. These toxins all create pores in the cell membrane of target cells, which eventually lead to cell lysis. This appears to be a Ca^{2+} dependent reaction. An important aspect is that these toxins vary in their target cell specificity (Coote 1992). Organisms from species that produce a similar type of leukotoxin active against leukocytes from different animal species include *Actinobacillus pleuropneumoniae*, *A. actinomycetemcomitans* and *Escherichia coli*. There is extensive homology between the DNA sequence of the genes coding for the alpha-haemolysin of *E. coli* and the genes coding for leukotoxin (Lo, Strathdee & Shewen 1987).

The production of leukotoxin by *M. haemolytica* is detected at an earlier stage when it is grown in bovine serum albumin (BSA) supplemented media and its activity is greater in these culture supernatants than when it is grown in nonsupplemented media (Confer & Durham 1992). The activity of leukotoxin produced in RPMI 1640 culture supernatant containing 0,5 % BSA after 2 h growth by *M. haemolytica* increases considerably in comparison to controls that contain no BSA (Waurzyniak, Clinkenbeard, Confer & Srikumaran 1994). Important considerations in the regulation of leukotoxin production in culture medium are the cellular growth kinetics and the leukotoxin produced per cell. It is relatively independent of growth conditions and more closely associated with growth rate. In comparison with brain heart infusion (BHI) broth and unadulterated RPMI 1640, the production of leukotoxin in RPMI 1640 medium with the addition of 2,5 % FCS provides optimal quantities of leukotoxin (Gatewood, Fenwick & Chengappa 1994). It appears that the growth medium and the type of supplements together with the growth conditions may play an important role in maximising the expression of the antigens that are important in developing resistance to pneumonic pasteurellosis (Mosier, Simons, Chengappa & Confer 1994).

The production of toxins used as antigens in commercial vaccines are normally associated with optimal conditions required to produce optimal quantities under defined conditions. Not only is it important to know the leukotoxin producing ability of the strain and the incubation time that is required for it to be produced, but also the needs of the organism in terms of medium requirements and other additives. The purpose of this investigation was to explore the leukotoxin producing abilities of two *M. haemolytica* isolates, with the addition of either FCS or BSA in small quantities of RPMI 1640 culture medium. The study was restricted to a 6 h period with three different seed culture inoculum densities, in an effort to establish the conditions for optimal leukotoxin production. The leukotoxin was quantified in u/mℓ and compared with the optical densities of the cell culture measured at 650 nm.

MATERIALS AND METHODS

Mannheimia haemolytica strains used

The *Mannheimia haemolytica* biotype A serotype 1 strain designated as 01/10, was received from Dr Richard Davies, Department of Microbiology, University of Glasgow, Scotland. It is described by Saadati, Gibbs, Parton & Coote (1997) as a high leukotoxin-producing isolate. The isolate was characterized and its biochemical profiles verified by Dr M. Henton, Section of Bacteriology, Veterinary Research Institute, Onderstepoort. The second *M. haemolytica* A1 strain 12296 was obtained from Professor Samuel

Maheswaran, University of Minnesota, USA and is also described as a high leukotoxin producing isolate (Vega, Maheswaran, Leiniger & Ames 1987).

Preparation of inoculum and measurement of cell growth

The BHI broth was prepared according to the manufacturer's instructions and sterilized in an autoclave at 121 °C for 30 min (Merck NT Laboratory Supplies, Midrand, Gauteng Province). The RPMI 1640 medium containing L-glutamine (Highveld Biological, Kelvin, Gauteng Province), was prepared with either 3,5 % FCS or with 3 % BSA. Ten litres of the RPMI 1640 medium were prepared by adding the required amount of distilled water to 10,41 g/ℓ of medium powder followed by sterile filtration with AP 25 prefilters and 0,22 µ microfilters [Microsept (Pty) Ltd, Bramley, Gauteng Province]. Either sterile 3 % BSA or 3,5 % sterile FCS (irradiated by gamma rays) was added to the RPMI 1640 medium.

The *M. haemolytica* A1 freeze dried culture was taken from the culture collection, resuspended with BHI, plated on three blood tryptose agar (BTA) plates containing 10 % bovine red blood cells (Onderstepoort Biological Products, Onderstepoort) and incubated overnight at 37 °C. The purity and homogenicity of the culture were confirmed by examination of smears stained by Gram's method and three to five colonies were inoculated into tubes containing 200 mℓ BHI pre-warmed broth and cultured for 12–14 h on a shaker at 37 °C. Wet smears were examined under phase contrast to check for any contamination. The BHI culture was centrifuged at 10 000 rpm in a Beckman J2-22 centrifuge [Beckman Instruments (Pty) Ltd, Halfway House, Gauteng Province] for 10 min and the supernatant removed under vertical laminar flow conditions [Labotec (Pty) Ltd, Halfway House, Gauteng Province]. The remaining pellet was resuspended in 50 mℓ of RPMI 1640 medium without any additives and the optical density (OD) of the suspension was measured spectrophotometrically (Novaspec spectrophotometer, SMM Instruments, Midrand, Gauteng Province) at 650 nm, and was standardized to approximately 1,25 (Table 1). This was used as a standardized seed culture inoculum after dilution for all submerged cultures in this trial. The submerged culture refers to the organisms that are growing in a fluid medium.

Three 50 mℓ Schott glass containers were filled with 25 mℓ RPMI 1640 with either 3,5 % FCS or 3 % BSA and were inoculated simultaneously with one of the three seed culture dilutions. Each of the three dilutions of the seed cultures was prepared separately and designated dilution A, B or C depending on the rate of dilution. Dilution A was prepared by removing 0,1 mℓ from 25 mℓ RPMI 1640 medium before 0,1 mℓ of standardized seed culture was added to it. Dilutions B and C were prepared similarly by removing 1 mℓ or 5 mℓ from 25 mℓ RPMI 1640 medium before replacing them with 1 mℓ or 5 mℓ of the standardized seed culture inoculum, respectively. These three inoculums were prepared simultaneously and placed on a horizontal shaker and incubated for 6 h whilst shaking vigorously.

During the incubation period of each batch of media 3 mℓ of growth medium was removed every hour 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, placed in a 1 mℓ Eppendorf tube which was identified and its contents immediately frozen with dry ice. The tubes were stored at –70 °C until the leukotoxin activity could be determined. The remaining cell pellet was resuspended with 1 mℓ of sterile deionized water and the OD measured at 650 nm. Each experiment with each strain and additive (FCS or BSA) was repeated in triplicate (Table 1).

Measurement of leukotoxin activity

The method of Vega *et al.* (1987) utilising bovine neutrophils was used to quantify the amount of leukotoxin present in culture supernatant. One unit of leukotoxin was defined as the reciprocal of the dilution causing 50 % cytolysis of neutrophils and was calculated according to the method of Reed & Muench (1938).

Isolation of bovine neutrophils

One hundred mℓ of calcium and magnesium free phosphate buffered saline stock solution was pre-

TABLE 1 Average optical density of seed culture inoculum of two *M. haemolytica* strains grown in RPMI 1640 medium containing either FCS or BSA

n	*Mannheimia haemolytica* strain	Medium and additive used for LKT production	Average cell density of seed culture 650 nm
5	1/10	RPMI + 3,5 % FCS	1,22
3	12296	RPMI + 3,5 % FCS	1,27
3	12296	RPMI + 3 % BSA	1,26
3	1/10	RPMI + 3 % BSA	1,11

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

RESULTS

Leukotoxin production and increase in cell density by *M. haemolytica* strain 1/10 in RPMI 1640 medium containing 3,5 % FCS

The highest leukotoxin concentration was reached after 3 h for dilution B and 4 h for dilution C, with values of 232 and 309 u/mℓ respectively (Table 2 and Fig. 1). Peak cell concentrations were reached after 5 h for both dilutions B and C with OD of 1,002 and 1,268 respectively (Table 3 and Fig. 2). The leukotoxin levels with dilution A was low at 49 u/mℓ after 6 h.

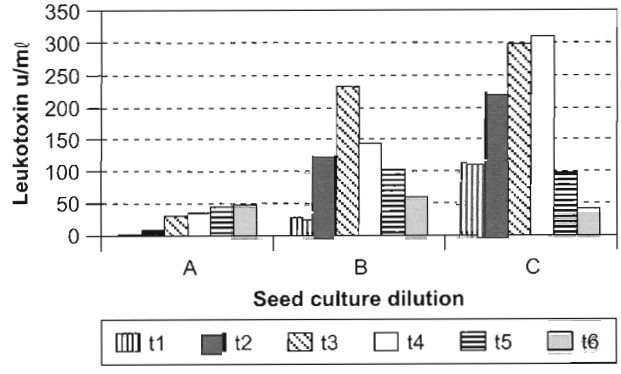

FIG. 1 Graphical presentation of leukotoxin production during active growth of *M. haemolytica* strain 1/10 measured every hour (t1 to t6) in RPMI 1640 medium with 3,5 % FCS inoculated with high (A, 1/250), medium (B, 1/25) and low (C, 1/5) dilution seed culture ($n = 3$)

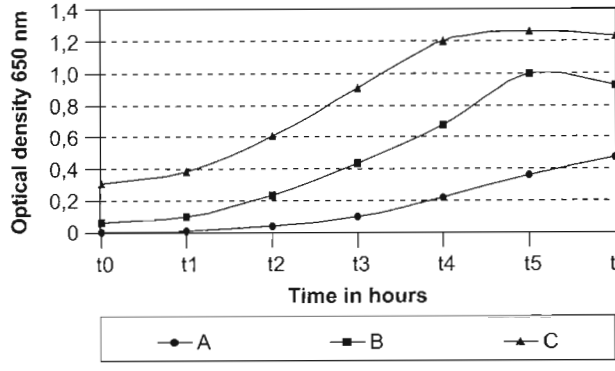

FIG. 2 Graphical presentation of increase in cell density during active growth of *M. haemolytica* strain 1/10 In RPMI 1640 medium with 3,5 % FCS inoculated with low (A 1/250 dilution), medium (B, 1/25 dilution) and high (C, 1/5 dilution) density seed culture ($n = 5$)

TABLE 2 The leukotoxin production of *M. haemolytica* strain 1/10 inoculated by three different inoculum dilutions (A, B, and C) into RPMI 1640 containing 3,5 % FCS measured in units/mℓ ($n = 5$)

Seed culture dilutions	Leukotoxin production in u/mℓ per hour time period					
	t1	t2	t3	t4	t5	t6
A	2	10	30	37	46	49
B	28	125	232	145	104	59
C	112	224	297	309	99	41

TABLE 3 The cell density measured as optical density at 650 nm of *M. haemolytica* strain 1/10 inoculated by three different dilutions into RPMI 1640 containing 3,5 % FCS ($n = 5$) (experiments one to five) (Fig. 2)

Seed culture dilution	Optical density as measured at 650 nm at one hour intervals during growth						
	t0	t1	t2	t3	t4	t5	t6
A	0,0076	0,0136	0,0430	0,1016	0,2272	0,3678	0,4784
B	0,0706	0,1048	0,2420	0,4452	0,6776	1,0020	0,9270
C	0,3130	0,3892	0,6142	0,9168	1,2060	1,2680	1,2340

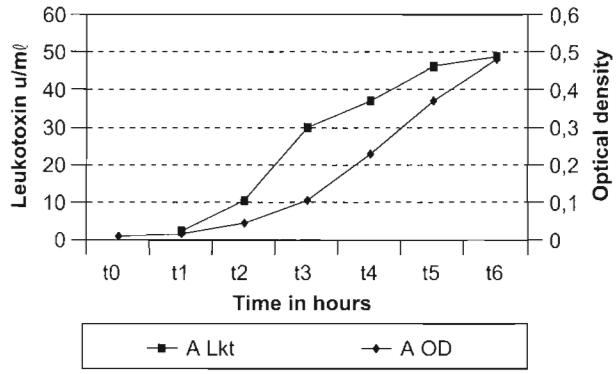

FIG. 3 Comparison between the increase in leukotoxin production and cell density (measured as optical density at 650 nm) during the growth of *M. haemolytica* strain 1/10 in RPMI 1640 containing 3,5 % FCS with a high dilution inoculum A (1/250)

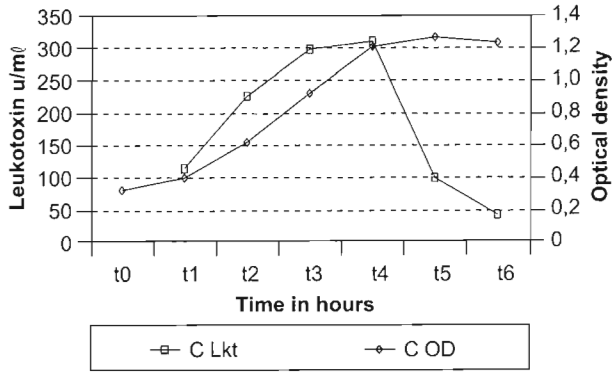

FIG. 5 Comparison between the increase in leukotoxin production and cell density (measured as optical density at 650 nm) during the growth of *P. haemolytica* strain 1/10 in RPMI 1640 with 3,5 % FCS with a low dilution inoculum C (1/5)

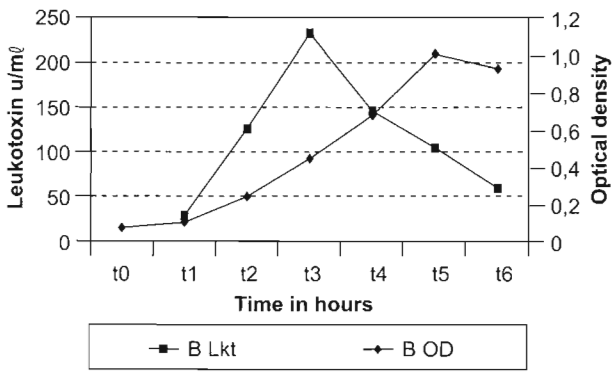

FIG. 4 Comparison between the increase in leukotoxin production and cell density (measured as absorbance at 650 nm) during the growth of *M. haemolytica* strain 1/10 in RPMI 1640 with 3,5 % FCS with a medium dilution inoculum B (1/25)

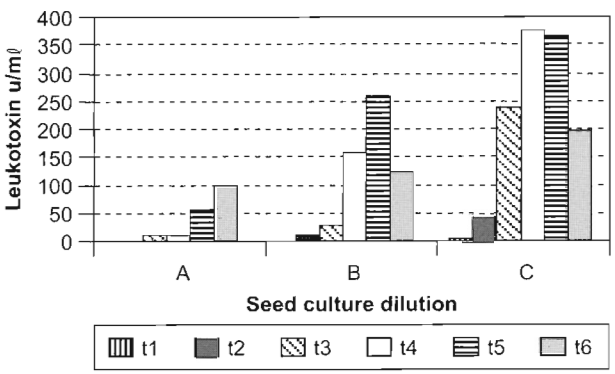

FIG. 6 Graphical presentation of leukotoxin production during active growth of *M. haemolytica* strain 1/10 measured every hour (t1 to t6) in RPMI 1640 medium with 3 % BSA inoculated with high (A, 1/250), medium (B, 1/25) and low (C, 1/5) dilution seed culture ($n = 3$)

The graphical relationship between the leukotoxin production and cell growth, as measured by the optical density at 650 nm for each of the different inoculum dilutions A (1/250), B (1/25) and C (1/5) is shown in Fig. 3, 4 and 5. The leukotoxin production peaked at 3 h and the cell density at 5 h for 1/25 dilution (B). For the 1/5 dilution (C) the leukotoxin peaked at 4 h and the cell density at 5 h.

Leukotoxin production and increase in cell density by *M. haemolytica* strain 1/10 in RPMI 1640 with 3,0 % BSA

The peak leukotoxin concentration produced with the 1/250 seed culture dilution (A) was 99 u/mℓ after 6h, 260 u/mℓ after 5 h for the 1/25 dilution (B) and 377 u/mℓ after 4 h with the 1/5 seed culture (C) dilution. The corresponding cell density measured as OD was 0,62 with seed culture A dilution reached after 6 h, 0,95 for dilution B after 6 h and 1,16 for dilution C after 5 h (Tables 4 and 5; Fig. 6 and 7).

In the high dilution (1/250) seed culture inoculum the *M. haemolytica* 1/10 strain was still in its logarithmic growth phase with a concomitant rise in leukotoxin activity (Fig. 8). With the medium dilution of seed culture the leukotoxin activity reached a peak at 5 h after which it started its decline, but the cell density was still increasing towards 6 h (Fig. 9). The low dilution of seed culture demonstrated peak level leukotoxin activity after 4 h with the cell density reaching its peak logarithmic growth after 5 h before appearing to become static (Fig. 10).

Leukotoxin production and increase in cell density by *M. haemolytica* strain 12296 in RPMI 1640 with 3,5 % FCS

Low levels of leukotoxin activity were detected with the high dilution of seed culture inoculum even after 6 h growth. With the medium and low dilutions of seed culture the peak leukotoxin activity was reached after 4 h at 317 u/mℓ and 3 h at 286 u/mℓ, respectively

TABLE 4 The leukotoxin (u/mℓ) production of *M. haemolytica* strain 1/10 inoculated by three different seedculture densities (A, B and C) into RPMI 1640 containing 3 % BSA ($n = 3$)

Seed culture dilution	Leukotoxin production in u/mℓ per hour time period					
	t1	t2	t3	t4	t5	t6
A	0	1	10	9	55	99
B	1	9	30	158	260	123
C	5	43	240	377	364	196

TABLE 5 The cell density measured as optical density at 650 nm of *M. haemolytica* strain 1/10 inoculated by three different seed culture densities (A, B and C) into RPMI 1640 containing 3 % BSA ($n = 3$)

Seed culture dilution	Optical density measured at 650 nm at one hour intervals during growth						
	t0	t1	t2	t3	t4	t5	t6
A	0,01	0,01	0,03	0,08	0,20	0,44	0,62
B	0,05	0,07	0,16	0,32	0,58	0,95	1,19
C	0,23	0,28	0,43	0,82	1,16	1,23	1,23

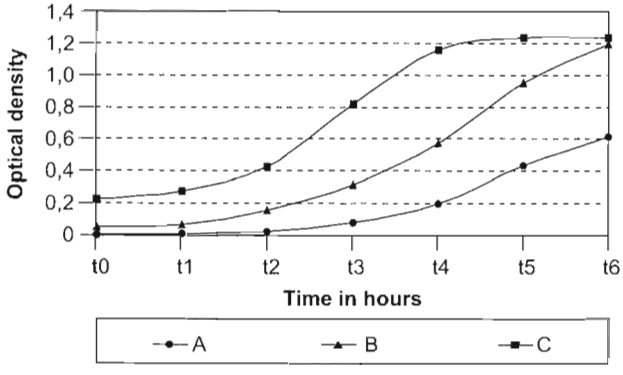

FIG. 7 Graphical presentation of increase in cell density during active growth of *M. haemolytica* strain 1/10 in RPMI 1640 medium with 3 % BSA inoculated with high (A, 1/250 dilution), medium (B, 1/25 dilution) and low (C, 1/5 dilution) dilution seed culture ($n = 3$)

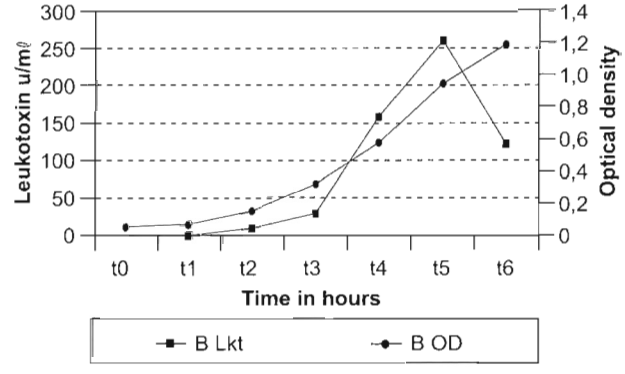

FIG. 9 Comparison between the increase in leukotoxin production and cell density (measured as optical density at 650 nm) during the growth of *M. haemolytica* strain 1/10 in RPMI 1640 with 3 % BSA with a medium dilution seed culture inoculum (B, 1/25)

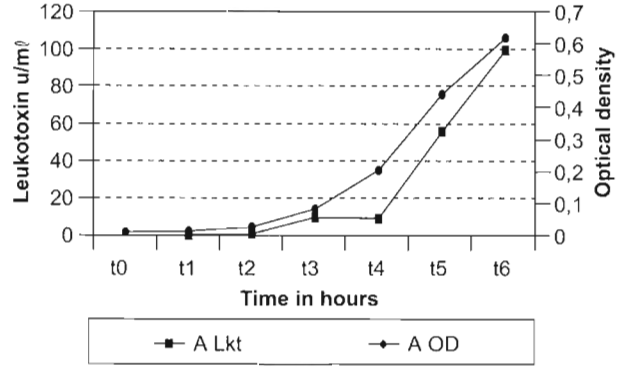

FIG. 8 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 1/10 in RPMI 1640 with 3 % BSA with a high dilution seed culture inoculum (A, 1/250)

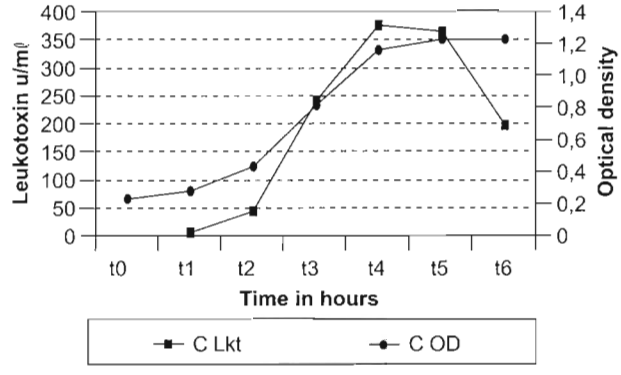

FIG. 10 Comparison between the increase in leukotoxin production and cell density (measured as optical density at 650 nm) during the growth of *M. haemolytica* strain 1/10 in RPMI 1640 with 3 % BSA with a low dilution seed culture inoculum (C, 1/5)

TABLE 6 The leukotoxin production of *M. haemolytica* strain 12296 inoculated with three different seed culture inoculum dilutions into RPMI 1640 containing 3,5 % FCS measured in units/mℓ ($n = 3$)

Seed culture dilution	Leukotoxin production in u/mℓ per hour time period					
	t1	t2	t3	t4	t5	t6
A	3	5	20	46	64	97
B	8	25	147	317	202	84
C	75	173	286	269	67	15

TABLE 7 The cell density measured as optical density at 650 nm of *M. haemolytica* strain 12296 inoculated with three different seed culture inoculum dilutions into RPMI 1640 containing 3,5 % FCS ($n = 3$)

Seed culture dilution	Absorbance as measured at 650 nm at one hour intervals during growth						
	t0	t1	t2	t3	t4	t5	t6
A	0,011	0,018	0,029	0,089	0,172	0,318	0,540
B	0,058	0,104	0,151	0,314	0,708	0,907	1,010
C	0,356	0,385	0,458	0,776	1,063	1,170	1,150

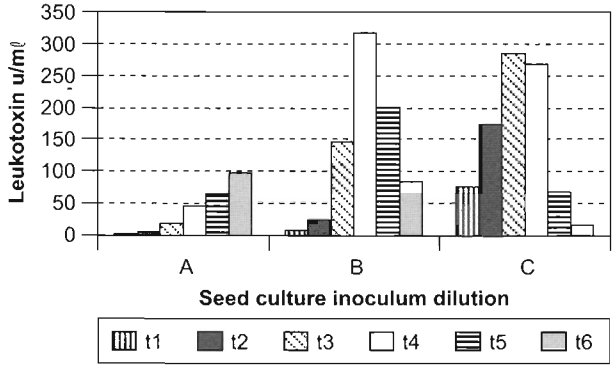

FIG. 11 Graphical presentation of leukotoxin production during active growth of *M. haemolytica* strain 12296 measured every hour (t1 to t6) in RPMI 1640 medium with 3,5 % FCS inoculated with high dilution (A, 1/250), medium dilution (B, 1/25) and low dilution (C, 1/5) seed culture inoculum ($n = 3$)

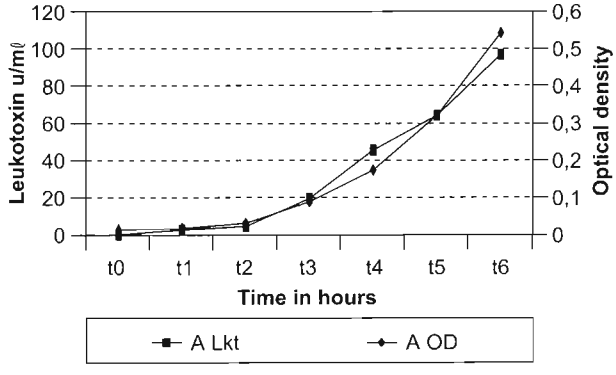

FIG. 13 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 12296 in RPMI 1640 with 3,5 % FCS with a high dilution seed culture inoculum (1/250)

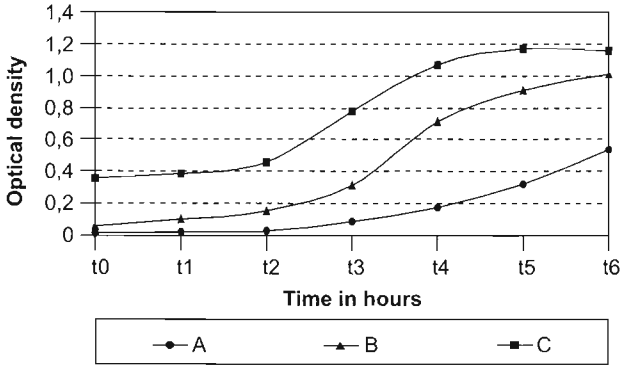

FIG. 12 Graphical presentation of increase in cell density (650 nm) during active growth of *M. haemolytica* strain 12296 in RPMI 1640 medium with 3,5 % FCS inoculated with high (A), medium (B) and low (C) dilution seed culture inoculum ($n = 3$)

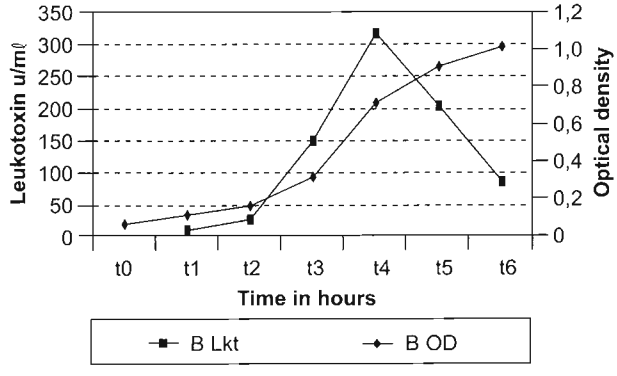

FIG. 14 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 12296 in RPMI 1640 with 3,5 % FCS with a medium dilution seed culture inoculum B (1/25)

TABLE 8 The leukotoxin production of *M. haemolytica* strain 12296 inoculated by three different seed culture dilutions into RPMI 1640 containing 3% BSA measured in units/mℓ (n = 3)

Seed culture dilution	Leukotoxin production in u/mℓ per hour time period					
	t1	t2	t3	t4	t5	t6
A	4	8	21	93	205	114
B	7	67	239	406	136	48
C	50	281	367	244	135	79

TABLE 9 The cell density measured as OD at 650 nm of *M, haemolytica* strain 12296 inoculated with three different seed culture dilutions into RPMI 1640 containing 3% BSA (n = 3)

Seed culture dilution	Optical density as measured at 650 nm at one hour intervals during growth						
	t0	t1	t2	t3	t4	t5	t6
A	0,02	0,04	0,06	0,14	0,29	0,55	0,75
B	0,09	0,17	0,32	0,53	0,85	0,93	1,00
C	0,36	0,51	0,72	0,89	0,94	0,93	0,99

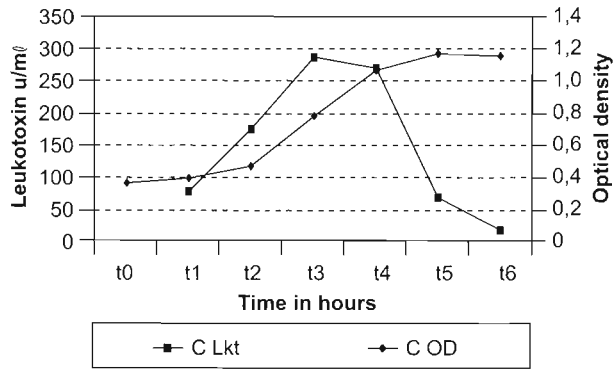

FIG. 15 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 12296 in RPMI 1640 with 3,5% FCS with a low dilution seed culture inoculum C (1/5)

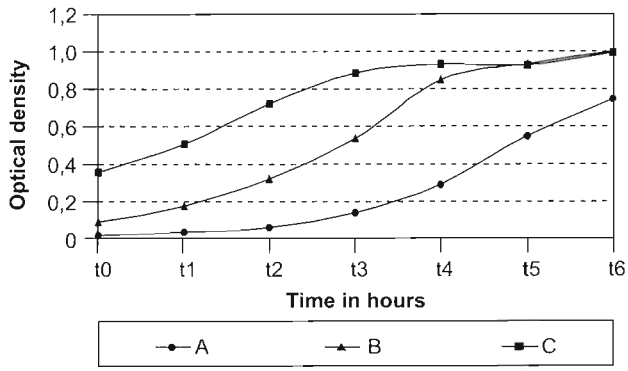

FIG. 17 Graphical presentation of increase in cell density during active growth of *M. haemolytica* strain 12296 in RPMI 1640 medium with 3% BSA inoculated with high (A, 1/250), medium (B, 1/25) and low (C, 1/5) dilution seed culture (n = 3)

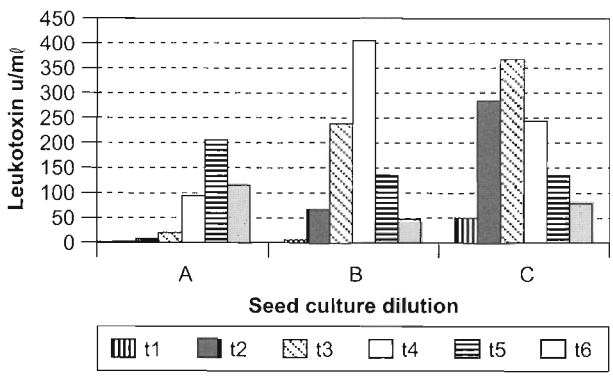

FIG. 16 Graphical presentation of leukotoxin production during active growth of *M. haemolytica* strain 12296 measured every hour (t1 to t6) in RPMI 1640 medium with 3% BSA inoculated with high (A, 1/250), medium (B, 1/25) and low (C, 1/5) dilution seed culture (n = 3)

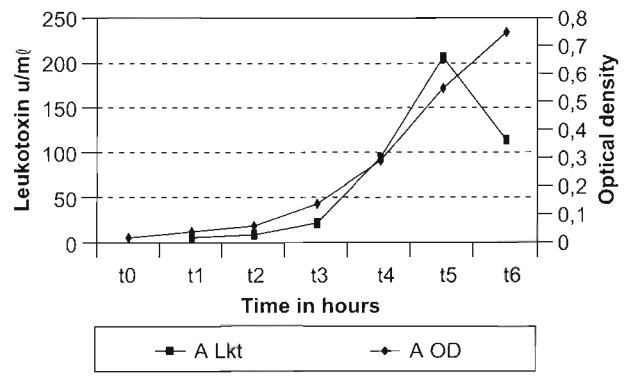

FIG. 18 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 12296 in RPMI 1640 with 3% BSA with a high dilution seed culture inoculum a (1/250)

(Table 6). Peak OD were only reached after 6 and 5 h for B and C dilutions, respectively.

The high dilution seed culture inoculum was still in a rising phase after 6 h (Fig. 13). At peak leukotoxin production after 3 and 4 h for C and B dilutions both cultures were still in the middle of the logarithmic growth phase (Fig. 14 and 15).

Leukotoxin production and increase in cell density by *M. haemolytica* strain 12296 in RPMI 1640 containing 3,0% BSA

The peak leukotoxin production occurred after 5 h at 205 u/mℓ, after 4 h at 406 u/mℓ and 3 h at 367 u/mℓ with the high, medium and low dilution seed culture inoculum, respectively (Table 8 and Fig. 16). It appears that the peak cell density was nearing its peak at 6 h growth for the B (1/25) and C (1/250) seed culture dilutions (Table 9 and Fig. 17).

The peak leukotoxin concentration was produced in the logarithmic growth phase of the cells in each of the three different seed culture dilutions. The cell density of the medium and low inoculum dilutions, however, appeared to have reached their static phases after 6 h (Fig. 18, 19 and 20).

Comparison of the leukotoxin producing abilities of both *M. haemolytica* strains 1/10 and 12296 in RPMI 1640 with 3,5% FCS

The 1/10 strain gave the highest leukotoxin value of 309 u/mℓ after 4 h growth with the 1/5 seed culture inoculum, whereas the 12296 strain gave its highest value of 317 u/mℓ after 4 h with the 1/25 seed culture dilution (Fig. 21).

Comparison of the leukotoxin producing abilities of both M. haemolytica strains 1/10 and 12296 in RPMI 1640 with 3% BSA

The 1/10 strain produced peak leukotoxin levels of 377 u/mℓ after 4 h with 1/5 (C) seed culture inocu-

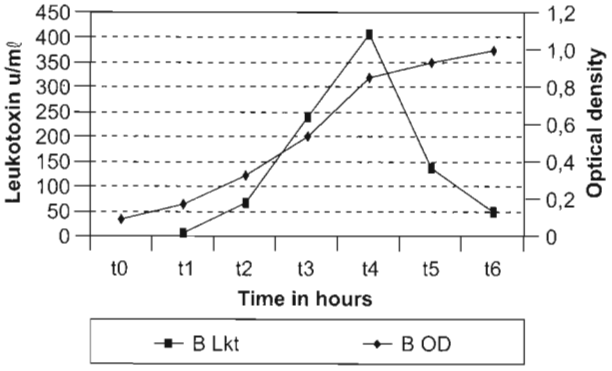

FIG. 19 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 12296 in RPMI 1640 with 3% BSA with a medium dilution seed culture inoculum B (1/25)

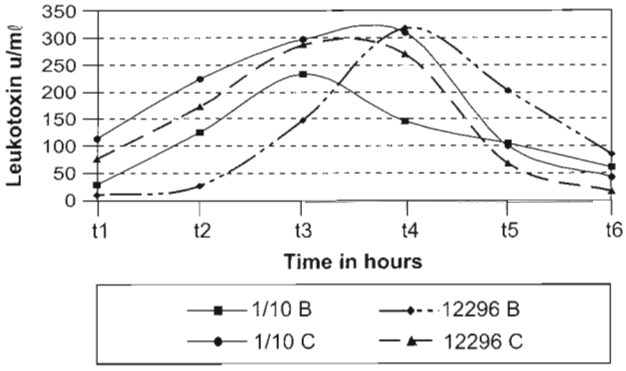

FIG. 21 A comparison between the leukotoxin producing abilities of *M. haemolytica* strains 1/10 and 12296 during active growth over 6 h in RPMI 1640 with 3,5% FCS inoculated with a 1/25 (B) and 1/5 (C) seed culture inoculum

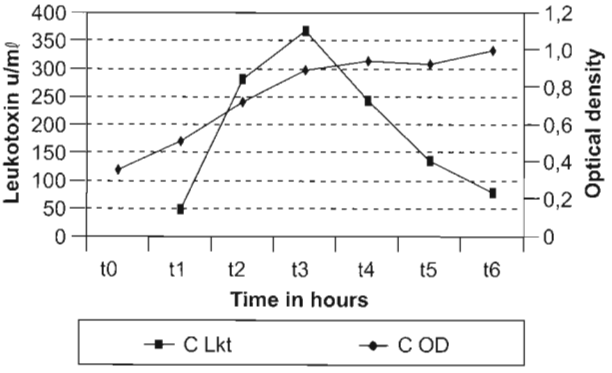

FIG. 20 Comparison between the increase in leukotoxin production and cell density (measured as OD at 650 nm) during the growth of *M. haemolytica* strain 12296 in RPMI 1640 with 3% BSA with a low dilution seed culture inoculum C (1/5)

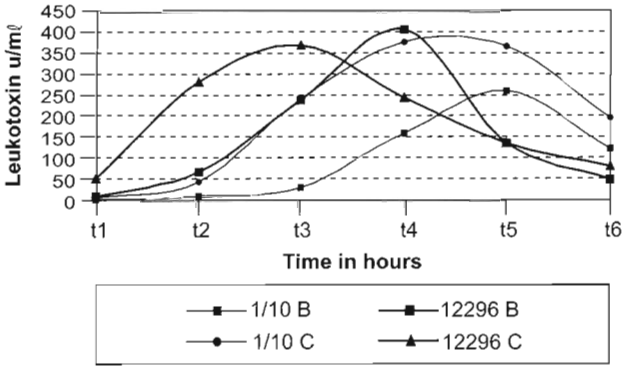

FIG. 22 A comparison between the leukotoxin producing abilities of *M. haemolytica* strains 1/10 and 12296 during active growth over 6 h in RPMI 1640 with 3% BSA inoculated with a 1/25 (B) and 1/5 (C) seed culture inoculum

lum and the 12296 strain produced 406 u/mℓ after 4 h with the 1/25 (B) seed culture inoculum (Fig. 22).

DISCUSSION

This study corroborates previous findings that time dependent variations exist in leukotoxin secretion into culture supernatant by different *M. haemolytica* isolates when grown in RPMI 1640 medium with additives such as FCS or BSA. Though there are other antigens that may also play a role in the pathogenesis of pasteurella pneumonia and which may be produced concomitantly with the leukotoxin in culture supernatant during active growth in RPMI 1640 medium, this study only examined the presence of the leukotoxin during the log phase growth cycle.

The detection of leukotoxin produced by *M. haemolytica* A1 in the supernatant of RPMI 1640 submerged cultures has definite practical advantages when it is intended for use as a vaccine. Under ideal conditions the leukotoxin is harvested at its highest peak production level after a certain time period. This may have an influence on the quality of the vaccine in that the antigen is present in its most pathogenic or biologic active form and it may also have an economic impact on the price of the final product. It is important to create the ideal conditions for toxin production by using the most suitable medium, additives and strain. The final medium in which a toxin or antigen is produced for vaccine purposes is usually the medium that serves as a diluent or carrier for the antigen which is administered to the animal. It has to be safe and free from any possible unacceptable peptides or amino acids especially of animal origin.

Though various media are used to produce the leukotoxin, RMPI 1640 seems the most appropriate and commonly used medium. It is generally regarded as a defined chemically synthetic medium with specified quantities of amino acids and minerals and ideally suited for the purpose of vaccine production. A medium such as BHI is regarded as a complex medium and although highly suitable for growth and toxin production of certain bacterial organisms, it is not ideally suitable for vaccine production due to the presence and unspecified nature of the peptides and amino acids. The use of RPMI 1640 medium to which FCS or BSA is added is a widely accepted method used for the production of leukotoxin (Shewen & Wilkie 1985; Mosier, Simons, Confer, Panciera & Clinkenbeard 1989; Van der Ingh, Visser, Henricks & Binkhorst 1990; Confer & Durham 1992; Gateway *et al.* 1994; Mosier *et al.* 1994) in contrast to other media including BHI and McCoys modified 5A medium (Chang, Renshaw, Martens & Livingstone 1986; Gatewood *et al.* 1994).

The production of leukotoxin in RPMI 1640 for use as a vaccine or antigen carrier has also been successfully evaluated in calves by Shewen & Wilkie (1988a, 1988b), Shewen, Sharpe & Wilkie (1988), Sreevatsan, Ames, Werdin, Yoo & Maheswaran (1996) and Odendaal, Morris, Du Preez & Aitchison (1997). The production of leukotoxin in logarithmic phase cultures in RPMI 1640 containing BSA is also preferred by Confer & Durham (1992) because it provides favourable conditions for growth and leukotoxin production, in spite of the fact that *M. haemolytica* has a doubling time of only 3 h. In most of the leukotoxin production methods described the *M. haemolytica* was not allowed an active growth period but rather a one hour incubation in the RPMI 1640 medium. There is no active growth during this period as measured by the lack of increase in optical density of the bacterial cells in the medium (Odendaal 1999).

The procedures followed in this investigation not only compared FCS and BSA as additives to the RPMI 1640 medium and the leukotoxin producing abilities of two different strains of *M. haemolytica* during active growth cycles but also tested the influence of the density of the seed culture inoculum. The role of BSA in the medium is one of enhancing the leukotoxin activity rather than the growth rate (Waurzyniak *et al.* 1994). Confer & Durham (1992) determined that peak leukotoxin values were reached in RPMI 1640 medium containing 0,5 % BSA after 4 h during the logarithmic growth cycle of the *M. haemolytica*. The culture medium without BSA had a much lower leukotoxin yield, although the growth rate measured as colony forming units only differed slightly. Mosier, Simons, Chengappa & Confer (1994) demonstrated a definite increase of certain protective antigenic complexes in BSA or FCS supplemented RPMI 1640 medium.

In this study there was a higher leukotoxin activity for both *M. haemolytica* strains when BSA was added to the RPMI 1640 culture medium and this was evident for each of the three seed culture inoculum dilutions. The obvious differences were observed between the leukotoxin production yields with each of the different inoculum dilutions is demonstrated in Fig. 1, 6, 11 and 16. The highest dilution gave the poorest yields (Fig. 1, 6, 11 and 16) but the lowest dilution did not necessarily give the highest leukotoxin activity (Fig. 11 and 16). A noteworthy phenomenon was the maximum leukotoxin levels achieved with the 12296 strain using the 1/25 seed culture inoculum after only 4 h of active growth. The leukotoxin levels from the higher 1/5 dilution were much lower (Fig. 11 and 16). The maximum leukotoxin levels were also achieved after 4 h incubation with the 1/10 strain at the low 1/5 (C) seed culture inoculum density, in BSA and FCS (Fig. 1 and 6) and in FCS with B dilution (Fig. 6). From this information, it is evident that the 12296 strain reaches its maximum leukotoxin peak faster when the 1/25 dilution seed

culture inoculum is used than any of the other two dilutions are used. In the case of this particular strain it appears that the highest seed culture inoculum concentration is not always required for optimal leukotoxin production. This characteristic can be exploited for commercial purposes when producing leukotoxin vaccine antigen. It also implies that different strains have different growth capabilities and that they have to be tested and evaluated in small volume submerged cultures as well as in large volume fermenters for leukotoxin antigen production. Peak leukotoxin production for both strains with either additive was obtained after the cell density reached an optical density of > 1,0 at 650 nm in all but one instance, which indicates that a certain measure of cellular activity is required before leukotoxin is secreted into the medium supernatant in large enough quantities.

Another unexplained phenomenon is the sudden decrease of the leukotoxin activity in the culture supernatant after reaching its peak (Fig. 4, 5, 9, 10, 14, 15, 18, 19 and 20). The reason for this is not clear although it can be speculated that the biochemical nutrients required for the synthesis of leukotoxin have been exhausted, or that the simultaneous production of proteolytic enzymes can lead to the decrease or inactivation of its biological activity. This process may be regarded as natural toxoiding. When producing large volumes of leukotoxin this aspect could be crucial and also have a devastating effect on the quality of the product if it is allowed to grow past its peak, resulting in a decreased leukotoxin yield. The increase in cell density beyond the leukotoxin peak level is an indication that leukotoxin is dependent on cell growth and that this is not a reliable indicator for maximum leukotoxin production yield. In conclusion, the density of the cell culture inoculum has an influence on the leukotoxin concentration levels produced, but scope should be given for individual variation amongst high leukotoxin-producing *M. haemolytica* strains.

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