Effects of growth conditions and incubation times on the expression of antigens of *Haemophilus paragallinarum* which are detected by monoclonal antibodies

R.R. BRAGG[1], L. COETZEE[1] and J.A. VERSCHOOR[2]

ABSTRACT

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Haemophilus paragallinarum causes infectious coryza in poultry, and a panel of monoclonal antibodies (Mabs) were established, which detect surface antigens of this bacterium. It was postulated that these Mabs could be used to detect antigenic differences between strains of *H. paragallinarum* used in infectious coryza (IC) vaccines, and isolates made from the field, from poultry vaccinated against IC. It has previously been reported that in South Africa there are three different Mab patterns that have been common to *H. paragallinarum* isolates for the past three decades.

The effects of different growth conditions such as duration of incubation, inoculum size, levels of NAD or NaCl in the medium, and the pH of the medium on these Mab patterns were investigated. It was found that many different factors appear to influence the expression of the antigens detected by the panel of Mabs. It was found that at different stages during the growth cycles, the isolates could be classified into different Mab groups. It was also found that alteration of the inoculum size resulted in Mab-pattern switches. Addition of extra NaCl to the medium, in order to slow the growth rate, was found to result in Mab-pattern switches. pH was found to have signifcant effects on the levels of expression of the antigens detected by the Mabs, although these changes did not result in Mab-pattern switches. The effects of pH were also found to be highly strain dependent. The use of NAD, rather than sterile chicken serum, in the medium did not significantly alter the levels of expression of these antigens.

Alterations of the growth conditions greatly affected the levels of expression of the antigens detected by the Mabs, and were highly strain dependent. It was not possible to predict the effects of a particular growth condition on a particular strain or isolate of *H. paragallinarum*.

Keywords: Effects, expression of antigens, growth conditions, *Haemophilus paragallinarum*, infectious coryza, incubation times, monoclonal antibodies

INTRODUCTION

Infectious coryza, caused by the bacterium *Haemophilus paragallinarum*, is a disease of poultry, with the first serious outbreak in South Africa in 1968 (Buys 1982) on a multi-age farm of approximately 100 000 layers. Soon afterwards, the disease spread to most large production sites and established itself as the most common bacterial infection in layers. This situation is still much the same, and infectious coryza remains a serious disease among layers in South Africa, particularly during the winter months.

Vaccines against infectious coryza have been used in this country since 1975 (Buys 1982), but in the 1980s it became apparent that the vaccines were becoming less effective in controlling the disease. In order to detect any antigenic differences between the strains of *H. paragallinarum* used for production of vaccines, and field isolates made from vaccinated flocks, a panel of monoclonal antibodies (Mabs) against *H. paragallinarum* was produced (Verschoor, Coetzee & Visser 1989). These Mabs were produced against

[1] Department of Poultry Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110 South Africa

[2] Department of Biochemistry, University of Pretoria, Pretoria, 0001 South Africa

two of the strains used in vaccine production in South Africa (0083 and 0222) and two field isolates from vaccinated flocks (M85 and SB86) (Verschoor *et al.* 1989), resulting in a panel of five Mabs which was used to differentiate between the "vaccine" strains and the field isolates.

Bragg, Coetzee & Verschoor (1993a) used these Mabs to investigate a larger sample of field isolates and included the Modesto strain as another vaccine strain in their study. Three different Mab patterns were found for strains 0083, 0222 and Modesto, and it was postulated that these patterns represent the three different serogroups. However, groups of field isolates produced Mab patterns differing from those obtained for the vaccine strains. In the light of these findings, a set of 11 different reference strains of *H. paragallinarum* was studied by Bragg, Gunter, Coetzee & Verschoor (1997) to determine whether any of the reference strains have Mab patterns similar to those obtained for the field isolates. It was found that the Mabs did not produce serogroup-specific Mab patterns when reference strains of *H. paragallinarum* were tested with the Mabs, which implied that the Mabs would not be useful for serotyping South African field isolates of *H. paragallinarum*. However, the possible effects of different growth conditions on the levels of expression of the different antigens detected by the Mabs, needed to be investigated before any decisive conclusion could be reached on the ability of this panel of Mabs to differentiate between field isolates and strains used in vaccine production.

During the production of the Mabs, Verschoor, Boshoff, Coetzee, Van Wyngaardt & Visser (1990) noted high levels of spontaneous fusion between splenocytes derived from mice immunized with whole *H. paragallinarum*, and myeloma cells *in vitro*. Verschoor *et al.* (1990) further established that co-immunization of mice with whole *H. paragallinarum* and an unrelated antigen, followed by co-cultivation of the splenocytes with SP2/0 myeloma cells, resulted in stable hybridoma cell lines producing antibodies against the unrelated antigen. They concluded that *H. paragallinarum* could be used in adjuvants to simplify the production of Mabs. This system may also be used as a model to study spontaneous fusion as a natural immune phenomenon in cancer. In order to study these effects fully, the putative fusogen inducer of *H. paragallinarum* needs to be isolated and characterized. To facilitate these studies, the effects of different growth conditions on the expression of the antigens detected by the panel of Mabs produced by Verschoor *et al.* (1989), should be investigated in order to optimize the yield of selected antigens. There appear to be some indications that the antigen detected by the VF3 Mab, plays some role, or is involved in the phenomenon of spontaneous fusion (Boshoff, Coetzee, Visser & Verschoor 1992).

The effects of different growth conditions and incubation times on the expression of selected antigens of *H. paragallinarum* may also be useful for determining optimal growth conditions and incubation times for expression of antigens involved in protective immunity during the production of commercial infectious coryza vaccines.

MATERIALS AND METHODS

Bacterial strains and isolates

Reference isolates, representing the different serovars described by Kume, Sawata, Nakase & Matsumoto (1983) and renamed by Blackall, Eaves & Rogers (1990), were obtained from Dr P.J. Blackall, Animal Research Institute, Yeerongpilly, Australia. Strains which were obtained and used in these experiments were 0083 (A-1), 0222 (B-1), H-18 (C-1), Modesto (C-2), SA-3 (C-3) and HP-60 (C-4). Samples of strains Spross (B-1) and H-18 (C-1) were kindly supplied by Dr A.A.C. Jacobs of Intervet, Boxmeer, The Netherlands. Strains 0083 and 0222 were obtained from Avimune (formerly Golden Lay Laboratories, Verwoerdburg, South Africa), and both of these strains, as well as the Modesto strain, were reconstituted from own stock.

Isolates SB86 and M85 were supplied by Avimune as freeze-dried cultures. All the other bacterial isolates were made from cases of infectious coryza submitted to this laboratory.

Growth media and purity of inoculum

Modified Casman's medium (Coetzee, Rogers & Veldhuyzen 1983), at a pH of 7,4 ± 0,2, and supplemented with 10% (v/v) sterile chicken serum, was used in all of the experiments unless stated otherwise. All bacteria to be used as inocula were first grown in modified Casman's medium and incubated at 37 °C for 18 h, after which a sample was removed, inoculated onto a blood tryptose agar (obtained from Onderstepoort Biological Products, P/Bag X5, Onderstepoort, 0110 South Africa) (BTA) plate, streaked with a feeder culture of *Staphylococcus aureus*, incubated in a candle jar at 37 °C for 18 h. All of the inocula were processed in this way, unless stated otherwise.

Enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies

The Mabs used in these experiments were produced by Verschoor *et al.* (1989) and consisted of the F1, V1 and VF3 Mabs. Culture fluid from SP2 myelomas was used for background controls. In all experiments, Mab patterns were obtained by performing ELISA on samples collected from the different growth conditions, or after prescribed incubation periods. ELISA

was performed on the isolates according to the methods described previously (Verschoor *et al.* 1989; Bragg, Coetzee & Verschoor 1993b) without modification.

Effects of incubation time

Flasks containing 200 mℓ of Casman's medium were inoculated with 1 mℓ of an 18-h-old culture of either isolates M85, A745/91, A541/92, A1343/90 or strain 0083, and incubated at 37 °C. At one-hourly intervals, for 12 h, 10-mℓ samples were aseptically removed from the flasks. The pH of these samples was determined and the optical density (OD) of the samples was read at 540 nm, using incubated sterile medium was used as a blank. The OD and pH were plotted against time.

Effects of the inoculum size

Tubes containing 10 mℓ of Casman's medium were inoculated with either 20 µℓ, 50 µℓ, 100 µℓ or 200 µℓ of 18-h cultures of either isolates M85, A745/91 or strains 0222 or 0083. In all of the other experiments an inoculum size of 100 µℓ was used.

Effects of using NAD instead of serum in the growth medium

Tubes containing 10 mℓ of Casman's medium which had been supplemented with either 10% (v/v) sterile chicken serum, or NAD at a final level of 200 µg/mℓ (w/v) were inoculated with 100-µℓ samples of either isolates A745/91, SB86 and M85, or strains 0083, 0222, Modesto, HP-60 and SA-3.

Effects of pH

The pH of Casman's medium was adjusted to various values between 7,3 ± 0,05 and 8,8 ± 0,05, and 10 mℓ of each medium was added to tubes. These tubes were inoculated with 100 µℓ of either strains H-18, 0222, SA-3, 0083, Modesto and Spross, or isolates M85 and A745/91.

Effects of NaCl levels

Tubes containing 10 mℓ of Casman's medium, were supplemented with additional samples of sterile NaCl solutions at levels of 0%, 0,5%, 1,0%, 1,5% and 2 %. The tubes were inoculated with 100 µℓ of either strain 0083 and 0222, or isolates A745/91, A340/92, A739/91 and A541/92.

Statistical analysis of data

All of the data generated for the different growth conditions or incubation times were evaluated by the Student *T*-test for significance. The lowest confidence level to be regarded as significant, was taken as 95 %.

RESULTS

The duration of incubation was found to have significant effects on the levels of expression of the antigens detected by the Mabs for strain 0083 (serotype A) and isolate M85 (serotype C). For strain 0083, it was found that at 7 h post inoculation, highly significant changes ($P = 0,005\%$) with V1 and lesser ($P = 0,01\%$) changes in the expression of the F1 antigen were found (Fig. 1). The Mab pattern obtained for strain 0083 up to 7 h of incubation, resembled the WF1 (weakly F1) Mab pattern obtained for the field isolates (Fig. 1). After 7 h of incubation, the Mab pattern typical for strain 0083 (i.e. a strong reaction with the V1 Mab) was found, and it prevailed for the remaining 18 h of incubation time.

Changing of the Mab pattern was also noted with isolate M85 (serotype C) (Fig. 2). For incubation periods of up to 3 h, the Mab pattern resembled the WF1 pattern, changing to SF1 (strongly F1) during 3–7 h of incubation. After 8 h of incubation, the signal obtained for the VF3 Mab was found to be above 2 x background, thus changing into the F1/VF3 group
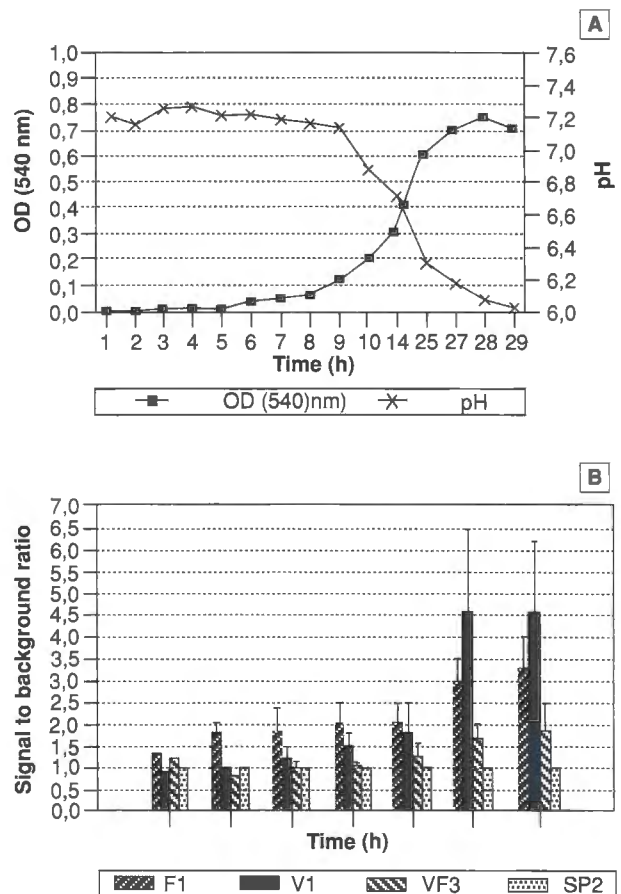

FIG. 1 Mab patterns obtained for strain 0083 after incubation for different times, and growth curves obtained for this strain, showing increased OD (540 nm) and decreasing pH plotted against time (*n* = 4)

FIG. 3 Mab patterns obtained for isolate A745/91 after incubation for different times and growth curves obtained, showing increasing OD (540 nm) and decreasing pH plotted against time ($n = 4$)
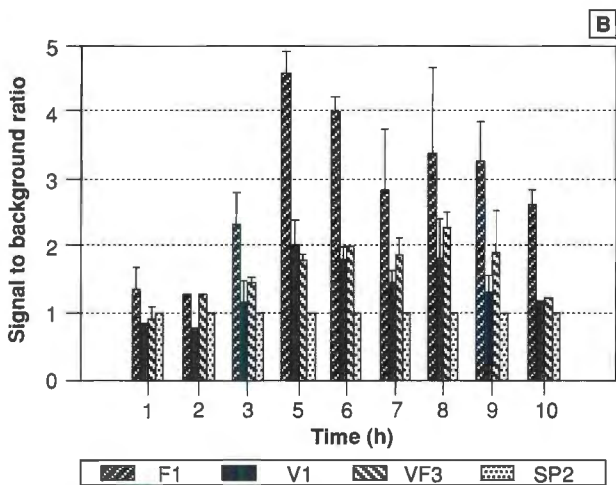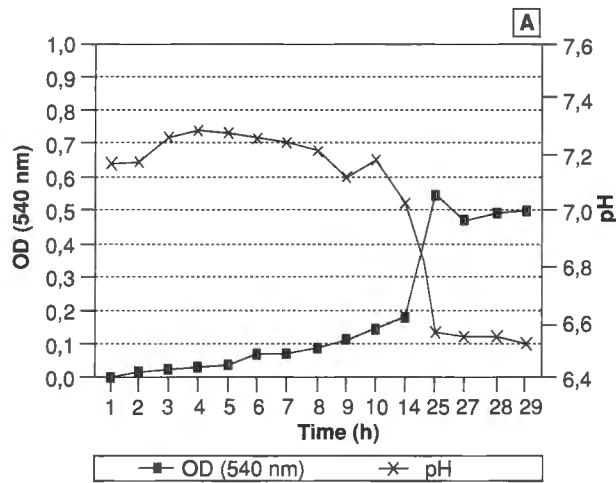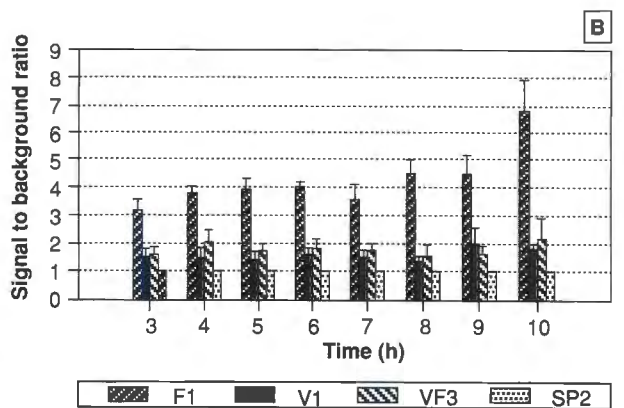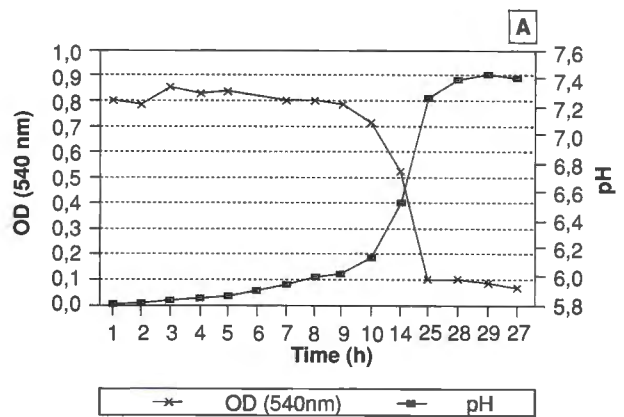

FIG. 2 Mab patterns obtained for isolate M85 after incubation for different times and growth curves obtained for this isolate, showing increasing OD (540 nm) and decreasing pH plotted against time ($n = 4$)

pattern, only to change back to the SF1 group. With isolate A745/91 (serotype A) (Fig. 3), it was found that the SF1 Mab pattern maintained itself from beginning to end without significant switch occurring.

It would therefore appear that the growth stage of the organisms may influence the levels of expression of the antigens detected by the Mabs to such an extent that the Mab-pattern grouping for a particular strain or isolate can be altered, resulting in the classification of the strain or isolate into a different Mab-pattern group. This may occur indirectly when the properties of the medium are altered, owing to the abundance of bacteria late in the growth stage. To test this, bacterial growth of isolates M85 (serotype C) and isolate A745/91 (serotype A) were inoculated with increasing inoculum sizes. When this was done, neither isolate M85 nor A745/91 (Fig. 4) was found to undergo significant switch in the Mab-pattern group.

As experiments were conducted in Casman's medium supplemented with 10% sterile chicken serum, an attempt was made to eliminate the variability in the growth properties due to possible batch variation of chicken sera in the medium. NAD, at levels of 200 µg/mℓ, was investigated as a replacement for serum. No significant differences in the Mab patterns and no switching from one Mab-pattern group to another, was observed compared to when serum was used in the medium when eight different strains or isolates of *H. paragallinarum* (including isolates M85 and A745/91, and strain 0083) were investigated. This suggests that undefined properties in the serum are not a primary factor affecting the expression of these antigens (data not shown).

The results obtained with the growth curves (Fig. 1–3), suggest that antigenic changes between Mab patterns appear to correlate with changes in the pH. Therefore, pH may be the crucial factor determining the levels of antigen expression, and indeed the Mab group into which the isolate or strain is classified during the growth cycle. This was tested by growing
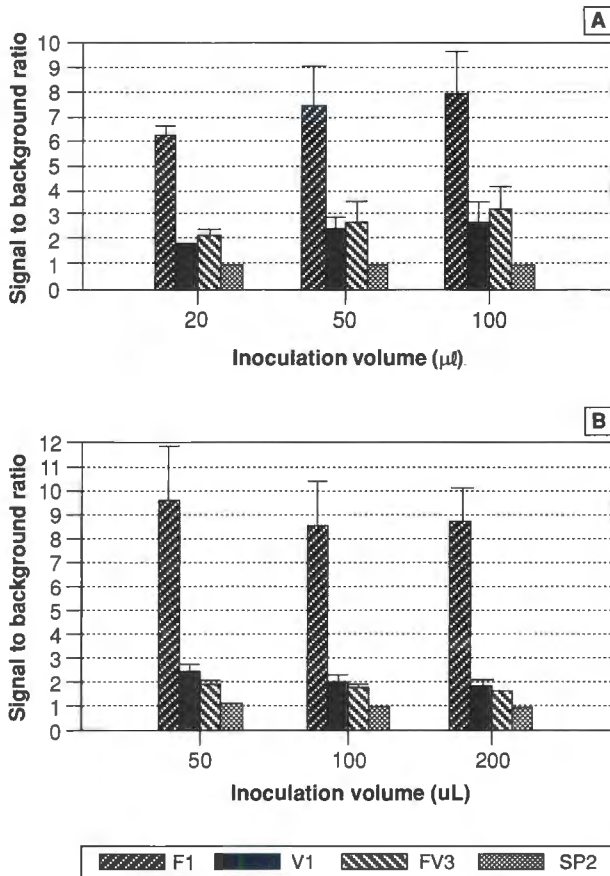
FIG. 4 Mab patterns obtained for isolates M85 (A) and A745/91 (B) when different inoculum volumes were used to inoculate Casman's medium supplemented with 10% sterile chicken serum ($n = 4$)
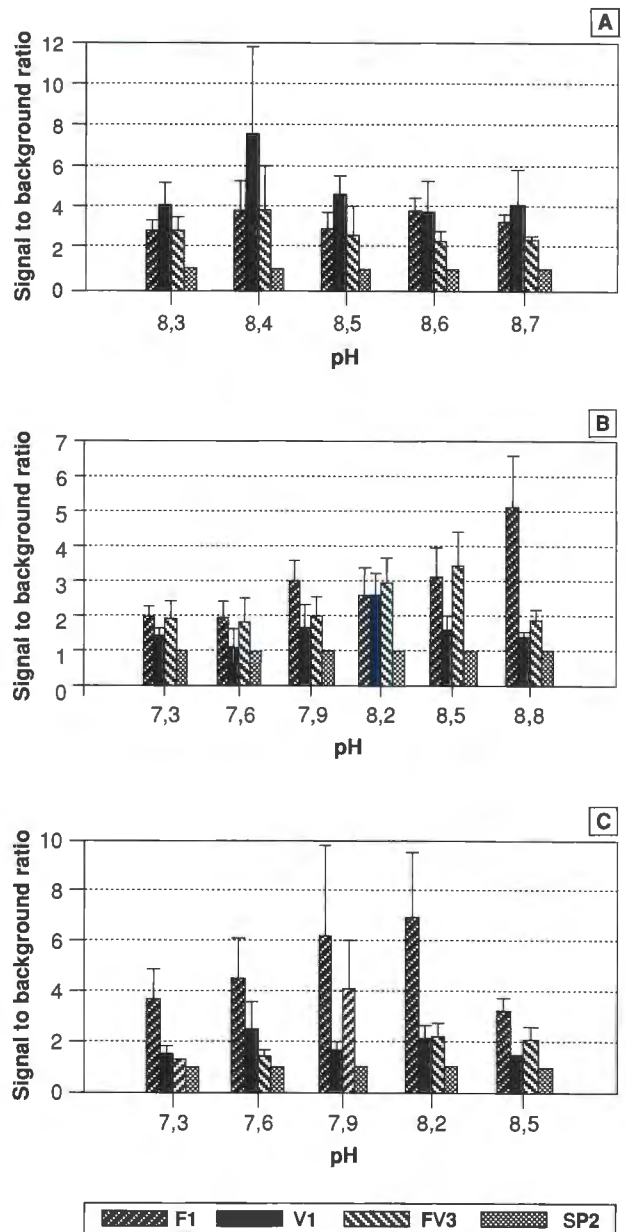

FIG. 5 Mab patterns obtained when reference strains of *H. paragallinarum* [0083 (Graph A), H-18 (Graph B) and Spross (Graph C)] were grown in medium with different pH values ($n = 4$)

different strains and isolates of bacteria in medium with different initial pH values. It was found that significant changes in the Mab patterns, although not resulting in Mab-pattern switches, were found for strain 0083 (Fig. 5). Almost identical results were obtained for strain SA-3 and Modesto (both serogroup C) (data not shown). Significant changes, resulting in Mab-group switching, were noted for strain H-18 (C-1), Spross (B-1) (Fig. 5) and for isolates M85 (C-3) and A745/91 (A-1) (Fig. 6). The Mab-group switch for isolate M85 was from a F1/VF3 grouping at lower pH values, to WF1 at higher pH values. The Mab-group switches for isolate A745/91 occurred firstly from the SF1 group to the F1/VF3 group and then back to the SF1 group, reminiscent of the growth-curve-related effects, typical of M85 (Fig. 2). The Mab-group switch for the Spross strain was from F1/VF3 to SF1, and this switch was reversed for the H-18 strain.

The effects of pH on the levels of expression of the antigens detected by the Mabs, appear to be highly strain or isolate dependent, and no fixed pattern was

observed which could be used as a model to predict the effects of pH on the expression of antigens for all of the strains and isolates.

Mab-pattern changes induced by extra NaCl levels added to the growth medium, were investigated. Addition of NaCl retarded the growth stage and affected the levels of expression of antigens of strain 0083, and of isolates A745/91 and M85. Only in the case of strain 0083, the changes were of such a nature that Mab-pattern type-group switches occurred (Fig. 7).
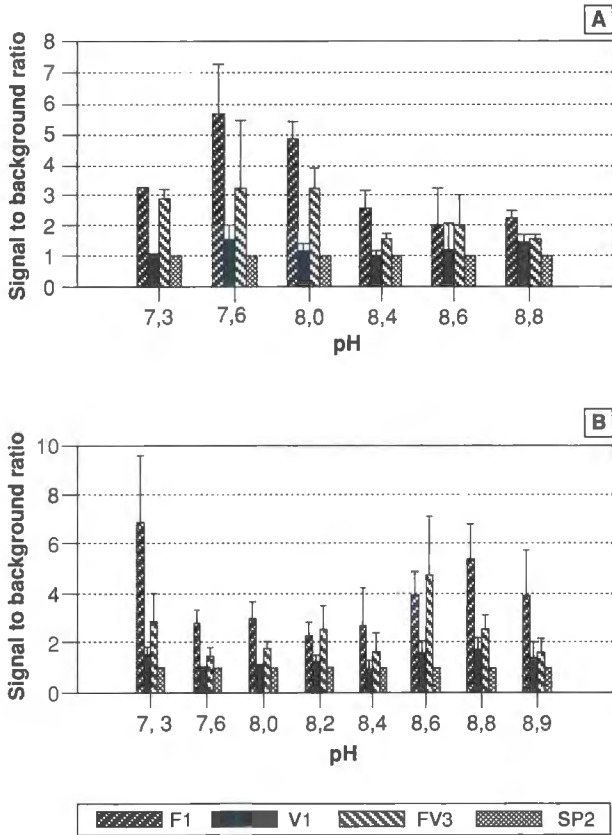
A

B

FIG. 6 Mab patterns obtained for isolates M85 (Graph A) and A745/91 (Graph B) were grown in medium with different pH values ($n = 4$)

FIG. 7 Mab patterns obtained when strain 0083 was grown in medium with added NaCl levels ($n = 4$)

It has been shown in this study that the growth stage of the organisms influences the expression of the antigens. It was found that the addition of NaCl slowed the growth of the bacterium to such an extent, that at 2% added NaCl, only very weak growth, as seen by the turbidity of the culture, was obtained. The Mab pattern of strain 0083 at high levels of added NaCl, corresponded to patterns obtained for this strain after only 3 h of incubation.

DISCUSSION

The growth stage of the bacterium appears to have the most profound effects on the Mab patterns of the different strains and isolates. The changes of the Mab patterns for different strains of *H. paragallinarum* appear to be affected to different degrees by selected growth conditions. As such, a model for the prediction of the effects of different growth conditions for all strains and isolates cannot be established from the data obtained. It can, however, be seen that alteration of the growth conditions can alter the Mab pattern into which a strain or isolate can be classified.
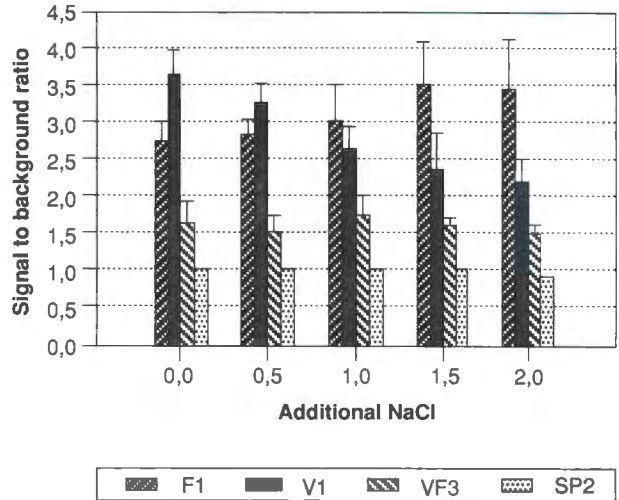
All of the growth conditions investigated had significant effects on the expression of the antigens detected by the Mabs. Some of the effects were highly significant ($P = 0,005$%), while other growth conditions affected the expression of the antigens to a lesser degree ($P = 0,05$%). It was also found that the levels of expression of the antigens detected by the Mabs appear to be highly variable, even under stringently controlled growth conditions. It was found that the expression of the antigen detected by the VF3 Mab, varied with pH of the culture initiation in a way that was strain dependent. For isolate A745/91 (A), it was found that optimal production of the antigen detected by the VF3 Mab occurred at a pH of approximately 8,6, while for isolate M85 (C-3), it was found to be between 7,6 and 8,0. The observation that two serovar C-3 isolates (M85 and SA3) each had a different optimal pH for the expression of this antigen, implies intra-strain variation of expression of the VF3 antigen.

Conditions for the optimal expression of the antigen detected by the F1 Mab also differed for different strains and isolates. High levels of expression of this antigen were detected in media with a pH of approximately 7,3–7,5 for isolates M85 and A745/91. The optimal expression of this antigen in SA-3 appeared to be at a pH of 8,6.

The antigen detected by Mab V1, showed optimal expression at pH 8,4, but could not be tested for its interstrain variability, as only strain 0083 produced significant amounts of the antigen detected by the V1 Mab.

If the antigens recognized by the Mabs should included the antigens involved in stimulating the protective immune response of the chicken, these findings would have significant financial implications for

the manufacture of a coryza vaccine. However, the lipopolysaccharide which is detected by the V1 Mab (Bragg *et al.* 1997), appears to be limited to strain 0083, while the VF3 antigen was also found to be a lipopolysaccharide, although it has a wider distribution of occurrence. Only the F1 Mab recognized a protein antigen, but it has been established that this Mab does not detect the hemagglutinin, as seen by the inability of the F1 and the other Mabs to inhibit haemagglutination of any of the reference strains of *H. paragallinarum* (Bragg *et al.* 1997).

Once it had been established (Bragg *et al.* 1996b) that the panel of Mabs did not detect antigens involved in protective immunity, the relevance of this work decreased. However, it can still be seen from these results that different growth conditions significantly affect the expression of these antigens, and it is therefore possible that these different growth conditions may also affect the antigens involved in stimulating the immune response in a similar manner. In order to investigate and optimize the growth conditions for commercial vaccine production, monoclonal antibodies against the serogroup-specific hemagglutinins are needed.

ACKNOWLEDGEMENTS

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