

**Characterization of the putative Haemagglutinin**  
**in**  
***Haemophilus paragallinarum***

by

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This desertation is dedicated to my grandparents, David and Mara Conradie.

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## LIST OF ABBREVIATIONS

°C	Degrees celcius
µl	Microlitre
pmol	Picomole
2D-gel	Two dimensional gel electrophoresis
A <sub>280</sub>	Absorbance at 280 nanometres
BCA	Bicinchoninic acid
bp	Basepairs
BSA	Bovine serum albumin
Ca <sup>2+</sup>	Calcium (2+)
CaCl <sub>2</sub>	Calcium chloride
Cu <sup>1+</sup>	Copper (1+)
Da	Dalton
dNTP's	Dinucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GA	Gluteraldehyde
HA	Haemagglutination
HCl	Hydrochloric acid
HEPES	N-(2-hydroxyethyl)piperazine-N'(2'-ethanesulphonicacid)
HI	Haemagglutination inhibition
IEF	Isoelectric focusing
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobases
kDa	kilodaltons
M	Molar

mA	Milli-ampere
Mg <sup>2+</sup>	Magnesium (2+)
MgCl <sub>2</sub>	Magnesium chloride
min	Minute
ml	Millimetre
ml.h <sup>-1</sup>	millilitre per hour
ml.min <sup>-1</sup>	millilitre per minute
Mm	Millimetre
mM	Millimolar
Mn <sup>2+</sup>	Manganese (2+)
MnCl <sub>2</sub>	Manganese chloride
MW	Molecular weight
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBC	Red blood cells
rpm	Rotations per minute
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloro acetic acid
Tris	Tris(hydroxymethyl) aminomethane
U	Unit
U/ml	Units per millilitre
V	Volts

v/v	Volume per volume
VB	Veronal buffer
w/v	Weight per volume
xg	Gravitational force
X-Gal	5-Bromo-4 chloro-3-indolyl-A-D-galactopyranoside

## CHAPTER 1

### Literature Survey

#### 1.1 Introduction

*Haemophilus paragallinarum*, the causative agent of infectious coryza (IC), an acute respiratory disease in chickens and fowl, was first isolated in 1931 by De Blicck (1932). The first serious, documented outbreak in South Africa occurred in 1968 (Buys, 1982) on a multi-age layer-farm, soon the bacterium spread to most large production sites and established itself as the most common bacterial infection in layers (Bragg, 1995). The disease has a low mortality rate but leads to a drop in egg production of up to 40 % in layer hens and increased culling in broilers and thus poses significant financial liability to chicken farmers (Arzay, 1987; Bragg, 1995).

One of the reasons for the success of survival for this bacterium is that after recovering from infection, birds become carriers of the bacterium, therefore aiding the spread of *H. paragallinarum* (De Blicck, 1948). Secondly, the bacterial strain belongs to one of nine serovars, which makes combating the spread of the disease through inactivated vaccination ineffective especially due to low cross protection among these serovars. (Rimler *et al.*, 1977; Kume *et al.*, 1980a).

Various potential factors have been identified as potential virulence factors, e.g. the haemagglutinin protein. This protein plays a crucial role in adherence of the bacteria to the host's cells and is considered a possible virulence factor (Sawata *et al.*, 1982; Yamaguchi *et al.*, 1989). Sawata and co-workers (1982) reported at least three different haemagglutinins from *H. paragallinarum* strain 221 with one, HA-L, being serovar specific with the other common types shared by the different serovars in one serogroup.

It would therefore be important to understand the working and interaction of the various virulence factors of *H. paragallinarum*, especially the haemagglutinins, in order to combat this bacterium.

## 1.2 The disease in chickens

*H. paragallinarum* causes an acute respiratory disease in chickens known as infectious coryza, a disease first recognized as a distinct entity in the late 1920's (De Blicck, 1932) and described as roup, cold, contagious or infectious catarrh and uncomplicated coryza (Yamamoto, 1991). Infectious coryza is regarded as a disease limited to the upper respiratory tract (Roberts *et al.*, 1964; Reid and Blackall, 1984) and infection in the lower respiratory tract (Adler and Page, 1962) may be due to synergism between *H. paragallinarum* and other respiratory tract pathogens (Reid and Blackall, 1984). Due to the phenomenon that the disease proved to be infectious only in the nasal passages the name "Infectious Coryza" was adopted (Beach and Schalm, 1936)

The clinical signs (Figure 1.1) associated with this disease include a nasal discharge, conjunctivitis with swelling of the sinuses, face and wattles, diarrhea, decreased feed and water consumption, retarded growth in younger chickens and reduced egg production (Eaves *et al.*, 1989). Lesions associated with the disease reflect an acute catarrhal inflammation of the upper respiratory tract.

Typically a mucoid sinusitis occurs with sloughing, edema and congestion of the sinus mucosa (Reid and Blackall, 1984) as well as an infiltration of the mucosa with mast cells. It has been suggested that these mast cells, along with the heterophiles and macrophages, cause the characteristic lesions of coryza (Ueda *et al.*, 1982).



**Figure 1.1:** A chicken infected with *H. paragallinarum* showing clinical signs of infection, swelling of the face, swollen wattles and a slight nasal discharge. (Photo taken during clinical trials in the Department of Microbiology and Biochemistry,UFS, Bloemfontein, 2000.).

In 1979 Rimler reported that a characteristic feature of the disease is a short incubation period of the bacteria with signs of infection 24-48 hours after intranasal or intrasinus inoculation with either culture or exudates. Susceptible birds exposed by contact to infected cases usually have signs of the disease in 1-3 days. Duration of the disease varied with the inoculum. The natural host for *H. paragallinarum* is the chicken but infectious coryza has been reported in pheasants (Delaplane *et al.*, 1934), companion birds (Dolphin and Olsen, 1978, as cited by Yamamoto, 1991) and one case was reported for guinea fowl (Yamamoto, 1972). Chronically ill or healthy carrier birds serve as the main reservoir of infection, with infectious coryza occurring mostly during fall and winter, although such seasonal patterns may be coincidental to management practices, an example which is the introduction of susceptible pullets onto farms where infectious coryza is present (Yamamoto, 1991). The main mode of transmission of infectious coryza appears to be horizontal either via direct contact, in drinking water or in the air (Matsumoto, 1988; Yamamoto and Clark, 1966).

Eliot and Lewis (1934) reported that 3-5 day-old chicks showed some resistance to infection whilst coryza could routinely be produced through the

use of intranasal inoculation in 7-day-old chicks. Beach and Schalm (1934) found that chickens with ages ranging from 4 weeks to 3 years showed differences in susceptibility towards the disease. Kato and Tsubahara (1967) illustrated typical coryza signs in 90 % of the 4-8 week old and 100 % in chickens of 13 week and older challenged with the bacteria. On farms where multiple age groups are brooded and raised, Clark and Godfrey (1961) found the spread of infection to successive age groups predictable. Infection occurred in a matter of 4-6 weeks after these birds were moved from the brooder house to growing cages near the older groups of infected birds.

### 1.3 *Haemophilus paragallinarum*

Although the bacteria responsible for infectious coryza was only isolated in 1931 by De Blicke (1932), it was already speculated in the 1920's by Beach that a distinct clinical entity was responsible for IC. The reason for the delay in isolation of this organism was due to the masking effect of other pathogens such as fowl pox (Yamamoto, 1991). *Haemophilus* is a genus belonging to the family *Pasteurellaceae* that includes the bacterial genera *Pasteurella* and *Actinobacillus*. When De Blicke first isolated the organism he termed it *Bacillus haemoglobinophilus coryzae gallinarum*, due to the growth requirements believed to apply (De Blicke, 1932). The bacterium was renamed and is now known as *Haemophilus paragallinarum* (Elliot and Lewis, 1934; Delaplane *et al.*, 1934).

Studies done by Nelson (1933b) led to the speculation that three different types of coryza could occur which differed in both onset and duration. These were classified as follows:

- Type I: A short incubation period of 2 days with a short disease cycle of 14 days.
- Type II: A long incubation period of between 10 to 24 days with a short disease cycle of 6 days.

Type III: A short incubation period of 2 days and a long disease cycle of up to 60 days.

From these observations Nelson (1933b) suggested that two agents could be involved in the disease syndromes described. Also notable was that the disease produced in chickens with cultured organisms ran a shorter course than those infected with nasal exudates. In 1938, Nelson was able to isolate bacillus-like organisms in cell culture, which, if injected into chickens, produced the Type II infection. These bacillus-like organisms isolated by Nelson (1938) were found to be *Mycoplasma* (Alder and Yamamoto, 1956) and it was suggested that the Type II infection described by Nelson (1933b) was in fact a *Mycoplasma gallinarum* infection (Edward and Kanarek, 1960). In 1982 Buys suggested that the Type I infection was infectious coryza, with Type II infection caused by *Mycoplasma gallinarum* and Type III infection was a combined infection.

*Haemophilus paragallinarum* is a Gram-negative, polar staining, non-motile bacterium appearing as short rods or coccobacilli in 24-hour-old cultures. Filamentous forms of the bacteria also occur with the older cultures showing pleomorphism (Hinz, 1973; Sawata *et al.*, 1980). The bacilli may occur singly, in pairs, or as short chains (Delaplane *et al.*, 1934).

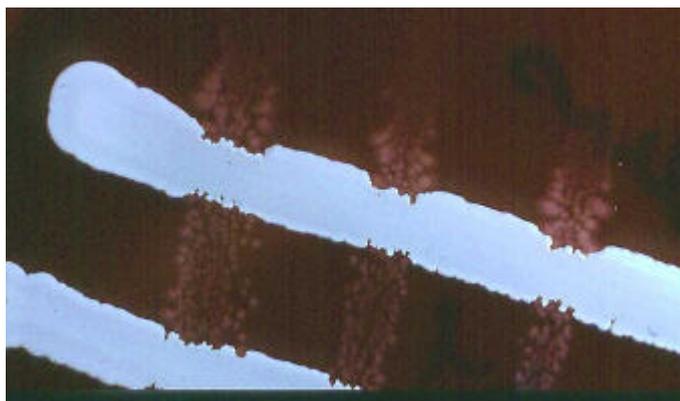
Biochemical properties of the bacteria include the ability to produce acid when grown in fructose, glucose and mannose and an inability to ferment galactose and trehalose (Hinz and Kunjara, 1977; Rimler, 1979). Hydrogen sulfide and indole are not produced, gelatin is not liquefied, and litmus and methylene blue milk are not changed (Blackall, 1989). Nitrates are reduced (Clark and Godfrey, 1961; Page, 1962) and catalase activity is absent (Page, 1962).

The bacterium is a delicate organism that is inactivated rather rapidly outside of the host. Experiments performed by Yamamoto (1978) showed that bacteria stored in the presence of thimerosal at 6°C stayed viable for several days. Using the same conditions but with formalin shortened the viability of the cells to 24 h. Bacteria stored in the exudates at 4°C remain viable for extended periods of time whilst storing of the bacteria in saline at 22°C left the bacteria unable to infect after only 24 h. Reece and Coloe (1985) showed some strains of *H. paragallinarum* to be resistant to sulfonamides. Blackall in 1988 also showed that resistance to streptomycin is common in *H. paragallinarum* and that some strains also occur with resistance to tetracycline.

#### **1.4 Isolation and growth requirements of *H. paragallinarum***

When the first *Haemophilus* species were first isolated it was suggested that the organism required haem (X factor) for growth, it was also noted that the organisms only grew close to *Staphylococcus* species colonies and thus also required NAD (V factor) for growth (Figure 1.2). The requirement for haem in the growth media was reported by a number of other workers (Blackall, 1999) who all suggested that blood or blood products were needed in the medium. This organism was termed *Haemophilus gallinarum* (Elliot and Lewis, 1934) and apparently required both V (NAD) and X (haem) factors.

McGaughey (1932) reported that his organism only required the presence of V factor for growth, but his work was largely overlooked until the 1960's when various workers reported that the isolates made from chickens were not dependant on haem. Page (1962) who worked on isolates from USA and Roberts *et al.* (1964) who worked on isolates from England found that their organism did not require haem for growth. This work was supported by similar results found by other researchers (Hinz, 1973; Rimler *et al.*, 1976).



**Figure 1.2:** Characteristic satellitic growth patterns of *H. paragallinarum* when grown alongside a feeder culture, such as *Staphylococcus aureus*.

In 1979 Rimler examined isolates from 7 different countries, including South Africa, and he could not find any isolates that required haem for growth. Biberstein and White (1969) proposed that isolates made from chickens, which did not require haem for growth should be called *Haemophilus paragallinarum*. Until 1990, all subsequent isolates from chickens with infectious coryza have been found to be dependant on V factor only (Blackall and Reid, 1982) and these isolates have been termed *H. paragallinarum*. The original isolates made by De Blicck (1932), Nelson (1933a) and other early workers, which required both haem and NAD were lost during storage. In an attempt to clarify the haem requirement of *H. gallinarum*, Blackall and Yamamoto (1989a) tested two cultures, which had been isolated in the 1940's and 1950's and labeled as *H. gallinarum* and they found these isolates to be dependant on NAD, but not on haem.

Since 1990, NAD-independent isolates of *H. paragallinarum* were described in South Africa (Mouahid *et al.*, 1992; Bragg *et al.*, 1993b). The NAD independence was shown to be plasmid-encoded and Bragg *et al.* (1993b) demonstrated that transformation of dependant strains with the plasmid would render them NAD independent (Bragg *et al.*, 1993b), and proved that there was a change in antigen expression in the strains now containing the plasmid (Bragg *et al.*, 1995b).

Currently media used for isolation, growth and maintenance of the bacteria include NADH (1.56-25 µg/ml media), the reduced form of NAD (Page, 1962), or NAD (20-100 µg/ml media), the oxidized form (Cundy, 1965). Other requirements include NaCl [1-1.5 % (w/v)] and chicken serum [1 % (v/v)]. These supplements are added to basal media such as brain heart infusion (BHI) and chicken meat infusion (Kume *et al.*, 1980c) with the pH of the different media ranging from 6.9 to 7.4.

The organism can grow under reduced oxygen tension or anaerobically (Rimler *et al.*, 1976) and the normal temperature for growth varies between 34-42°C.

Colonies of *H. paragallinarum* are typically tiny (0.3mm after 24h of growth) with a dewdrop shape (Blackall, 1989). Research has shown the colony morphology to range from mucoid (smooth) iridescent and rough non-iridescent to other intermediate colony forms when inspected under obliquely transmitted light (Rimler, 1979; Sawata *et al.*, 1978).

## **1.5 Serological classification techniques**

The serological classification of *H. paragallinarum* has received a lot of attention in order to find a reliable classification system to include the "non-typable" strains of this bacterium. Conventional tests are used for the classification of *H. paragallinarum* but to date has rendered several problems.

### **1.5.1 Plate agglutination test**

Page (1962) was the first to do work on the serological differentiation between the different strains of *H. paragallinarum* using the plate agglutination test. He detected three different serotypes, which were termed A, B, and C. Two of the original isolates used by Page (1962), 0083 for serotype A and 0222 for serotype B, are still available but the isolates used for characterization of serotype C were lost in the 1960's (Yamamoto, 1991). Rimler *et al.* (1977)

characterized an isolate made by Matsumoto and Yamamoto (1975) as serotype C based on a reaction with rabbit-raised antibodies produced by Page against his serotype C isolates. This isolate (Modesto) is also still available today.

In an independent study in Japan, Kato and Tsubahara (1962), also used an agglutination test and found three agglutinin types, which were called I, II and III. It was later found that II and III were variants of I. This serotype I is represented by strain 221 and this isolate was later found to correspond to strain 0083 which is Page's serotype A (Sawata *et al.*, 1980).

In 1978 Kume and co-workers reported on the isolation of *H. paragallinarum* isolated from chickens from flocks vaccinated with a vaccine made from isolate 221 (Page's serotype A) and these isolates were classified as serotype II (Sawata *et al.*, 1978) with the H-18 as the reference isolate for this group. In later work (Sawata *et al.*, 1980), it was established that Page's serotype A (0083) corresponds biochemically as well as serologically to Sawata's serotype I (221). In 1980 Sawata and co-workers related Modesto (Page C) to H-18 (Sawata's serotype 2). Two exceptions exist to the correlation between the Page and Kume schemes, these being isolates 2493 and 1596. Hinz and Kunjara (1977) placed these isolates into Page's group B. It was however found by Eaves *et al.* (1989) that isolate 2493 is more closely related to Page serogroup A.

The plate agglutination test established simultaneously by Page (1962) and Kato and Tsubahara (1962), although being used through out the world, did have some drawbacks. The major problem with this test was spontaneous agglutination. Irritani *et al.* (1978) however found that the treatment of bacterial cells with trypsin inhibited spontaneous agglutination, and the trypsinized bacteria could still be used to detect *H. paragallinarum* antibodies in chicken serum.

Various field isolates of *H. paragallinarum* have, however, been found to be untypable by the Page scheme (Buys, 1982; Thornton and Blackall, 1984; Blackall and Eaves, 1988). The percentage of untypable isolates varies from 15% (Thornton and Blackall, 1984) to 38% (Blackall and Eaves, 1988). When looking for a scheme for the monitoring of the serological distribution of populations of pathogenic *H. paragallinarum*, such high levels of untypable isolates proves to be problematic, thus eliminating this test as a standard test for classification of isolates.

### **1.5.2 Agar gel diffusion test**

Hinz in 1980 developed a classification scheme for the classification of isolates using the heat-stable antigens detected in a gel diffusion test. He could not confirm a common heat stable antigen between 221 (Kume I) and H18 (Kume II) and established that 0083 and 0222, designated Page A and B respectively, carry distinct determinants. Although successfully applied, this scheme has not been widely used for the serological characterization of *H. paragallinarum*.

### **1.5.3 Haemagglutination (HA) and Haemagglutination inhibition (HI) test**

The HA/HI tests are currently the only tests used for the classification of *H. paragallinarum*. Kato *et al.* (1965) was the first to demonstrate the haemagglutinating ability of *H. paragallinarum* with Sawata *et al.* (1980) demonstrating that the HA activity was only found in untreated cell suspensions of Page A isolates but not in Page C organisms. Yamaguchi *et al.* (1989) demonstrated that hyaluronidase-treatment of the cells from both Page's A and B isolates could haemagglutinate formaldehyde fixed chicken erythrocytes.

Kume *et al.* (1983) established a typing scheme based on the haemagglutinating antigens obtained through potassium thiocyanate extraction and sonication in a HA test performed with glutaraldehyde-fixed chicken erythrocytes (GA-fixed RBC). The different isolates were characterized according to their haemagglutination inhibition (HI) reaction with rabbit raised antisera against the different isolates. This scheme recognized three different serogroups, termed I, II and III, and seven different serovars, HA-1 to HA-7. Serovars HA-1 to HA-3 were found to belong to serogroup I, serovars HA-4 to HA-6 to serogroup II and serovar HA-7 to serogroup III.

Eaves *et al.* (1989) tested 95 isolates, predominantly Australian, but also included 30 isolates from other countries (including South Africa), by the haemagglutinin serotyping scheme described by Kume *et al.* (1983). The same set of reference isolates (Kume *et al.*, 1983) was used and they also found all seven of the haemagglutinating serovars. Another serovar was however discovered consisting of 15 Australian isolates and were termed HA-8. This serovar was found to belong to serogroup I.

Blackall *et al.* (1990b) found another distinct serovar, which was found to fall into the Kume serogroup II, in Australia in 1990 and termed this new group HA-9. The detection of the two new serovars by Eaves *et al.* (1989) and Blackall *et al.* (1990b) highlighted the likelihood that new serovars would continue to emerge, prompting Blackall *et al.* (1990b) to propose the alteration of the nomenclature of the Kume scheme.

It was suggested (Blackall *et al.*, 1990b) that Kume's groups I, II and III be changed to A, C and B respectively to reflect these groups' relatedness to the original groups detected by Page (1962). Serovars within each group could be numbered A-1 to A-4, B-1 and C-1 to C-4 (Table 1.1), creating an open-ended system to allow future new serovars detected to be allocated the next serial number in the serogroup where they belong.

**Table 1.1:** Comparison of the original and newly proposed nomenclature for the Kume serotyping scheme for *H. paragallinarum*. (Adapted from Blackall *et al.*, 1990b)

Reference isolates	Original scheme (Kume)		New Scheme (Blackall)	
	Serogroup	Serovar	Serogroup	Serovar
221	I	HA-1	A	A-1
2403	I	HA-2	A	A-2
E-3C	I	HA-3	A	A-3
HP14	I	HA-8	A	A-4
H-18	II	HA-4	C	C-1
Modesto	II	HA-5	C	C-2
SA-3	II	HA-6	C	C-3
HP60	II	HA-9	C	C-4
2671	III	HA-7	B	B-1

The characterization of the serotype B organisms still appears to be controversial. Page's serotype B strains were found to be untypable when tested by Kume *et al.* (1980a) and Sawata *et al.* (1980) and was considered to be a variant of serotype A or C strains which had lost their type-specific antigens. Sawata *et al.* (1980) regarded the B serotypes untypable because they lacked type-specific heat labile L antigen, however Kume *et al.* (1983) found that two serovar B strains had serovar specific haemagglutinating antigens. Yamaguchi *et al.* (1990b) established that four serotype B isolates of *H. paragallinarum*, including two South African field isolates, produced six different HA antigens, one of which is specific for serovar B. Three of the isolates (0222, 24268 and 24317) showed HA activity against formaldehyde-fixed chicken erythrocytes both before and after treatment with hyaluronidase whereas Spross (the fourth isolate) only showed HA activity after treatment with hyaluronidase.

Currently, the most accepted method for the serological characterization of *H. paragallinarum* appears to be the methods established by Kume *et al.* (1983) and modified by Blackall *et al.* (1990) in which the non-typable isolates are not

treated chemically, but simply washed and stored at 4°C for 3 days before testing and classified using the nomenclature proposed by Blackall *et al.* (1990b).

#### 1.5.4 Specific identification of *H. paragallinarum* via polymerase chain reaction (PCR)

A relatively new PCR based technique for identification of the bacterial strains to confirm their presence in exudates received from chickens showing typical IC signs was designed and described by Chen and co-workers (1996). Using genomic DNA isolated from the Modesto strain, Chen *et al.* (1996) constructed a genomic library and, using southern blots, identified four probes that reacted specifically with 56 *H. paragallinarum* isolates used. None of the four probes reacted with the 24 bacterial isolates used from closely related genera like *Pasteurella* and *Actinobacillus* or field isolates of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*.

**Table 1.2:** The sequences of the primers used by Chen *et al.* (1996) for development of the *H. paragallinarum* specific PCR.

Primer name	Primer sequence
F1	5'-CAA TGT CGAT CCT GGT ACA ATG AG-3'
N1	5'-TGA GGG TAG TCT TGC ACG CGA AT-3'
R1	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'

Using the smallest of these probes he designed 3 primers (Table 1.2). In the combinations of F1/R1 (HPG-1) and N1/R1 (HPG-2) he obtained fragments of about 1.6kb for HPG-1 and 0.5kb for the HPG-2 PCR. Neither one of the PCR's performed on the other bacterial isolates described above gave the desired results. This PCR test is now routinely performed for the identification of *H. paragallinarum* in nasal swabs and for conformation of *H. paragallinarum* grown in the laboratories (Mifflin *et al.*, 1999)

## 1.6 Adhesion of *H. paragallinarum* to cultured chicken cells

The attachment of bacteria to mucosal tissues, as well as their ability to colonize such tissues, is considered an important step in the infectious process. In some bacteria mucosal adhesion has also been associated with their virulence (Jones, 1977). The histopathological change in chickens with experimentally induced coryza has been reported to be an acute catarrhal inflammation of the mucous membranes of the nasal passages, infra-orbital sinuses and trachea (Yamamoto, 1991).

Ueda *et al.* (1982) investigated the relationship between the adhesion of *H. paragallinarum* to chicken embryo fibroblasts (CEF) *in vitro* and its virulence. *H. paragallinarum* V-factor dependant strains 221, FY-3 and GF were used in this study and it was found that strains 221 and FY-3 were pathogenic. Using scanning and transmission electron microscopy, strain 221 exhibited marked adhesion to CEF, the organism adhered to the plasma membrane of CEF, and fuzzy material extended out from them. In some instances the organisms were so closely attached to the plasma membrane that no intervening space could be seen. The organisms were also reported in the cytoplasm of CEF, being enclosed in a membrane bound vesicle (Ueda *et al.*, 1982)

Furthermore, using the scanning electron microscope, it was shown that strain 221 adhered to the cilia of the chicken tracheal epithelium, penetrating into the inter-ciliary spaces and attached by the sides to the ciliary surfaces. Generally the two virulent strains, 221 and FY-3, adhered to the CEF *in vitro*, and with the increased incubation time, the percentage of cells with adherent bacteria increased (Ueda *et al.*, 1982)

Ueda *et al.* (1982) concluded that the adhesion of strain 221 enables them to colonize the surface of the epithelial cells, by resisting the removal function of the bathing secretions and ciliary movement of the respiratory tract. Residence of the organisms between the cilia on the tracheal cells is also thought to protect the cells from the mucocilliary clearance mechanisms.

## 1.7 Virulence factors of *H. paragallinarum*

An important factor influencing bacterial virulence is the efficiency with which the bacteria are capable of adhering to the host cells, leading to colonization and finally infection of the host cells. Mechanisms used by *H. paragallinarum* have been shown to include the use of a capsid, production of toxins and different speculated proteins involved in adherence of the bacteria to the host cell. The amount of colonization and even virulence of the microorganisms may be influenced by the initial adherences of the bacterial to the host cells (Finlay and Falkow, 1997).

### 1.7.1 Capsid of *H. paragallinarum*

Many bacterial species, especially invasive pathogens, express a capsular polysaccharide (CP) that confers significant resistance to the host's immune defenses (van Dam *et al.*, 1990). This capsid has a dual function in that it may be used for adhesion to the cell membrane as well as being antigenic determinants. The antigenic determinants are derived from free heteroglycans found in most bacterial capsid. *Haemophilus influenza*, a member of the *Pasteurellaceae*, is currently classified using their capsular sugars, showing distinct differences against which monoclonal antibodies have been raised.

Rimler *et al.* (1977) speculated that the presence of hyaluronic acid, found in most of his isolates, could play a role as a virulence factor. Kume and Sawata (1984) found that highly encapsulated variants of serotype I organism lost their virulence when treated with hyaluronidase. Other evidence that hyaluronic acid may play a role in virulence was obtained by Sawata and co-workers (1978). They reported that 86 encapsulated strains from both serotypes I and II, now A and C, had similar degrees of pathogenicity for chickens while two non-encapsulated isolates were non-pathogenic.

Sawata *et al.* (1984) showed that the capsule might play a role as a natural defense against the bacteriocidal activity of fresh normal chicken serum. Encapsulated organisms were resistant to the serum whilst non-encapsulated organisms were sensitive towards the serum. It was thus concluded that the capsid might be increasing the potential of virulence.

In other experiments, Sawata *et al.* (1985a, 1985b) inoculated chickens with the encapsulated and non-encapsulated organisms, studying the nasal mucosa using histology (Sawata *et al.*, 1985b), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Sawata *et al.*, 1985a). Chickens inoculated with the encapsulated variants showed marked loss of cilia and microvilli, infiltration of leukocytes and deposition of a mucopurulent substance. Numerous encapsulated organisms were found in association with the cilia and microvilli, and the capsule appears to be the means of attachment of these organisms. On the other hand, very little change of the nasal mucosa was detected in chickens inoculated with the non-encapsulated strains. Factors in the capsule, which facilitate the firm attachment of the bacteria to the nasal mucosa, appear to be important in the virulence of the bacteria.

However, from the work done by Kume *et al.* (1984), in which they correlated the clearance of challenge organisms from the nasal cavity to the HI titre of the serum, it was suggested that no capsular substance was responsible for the development of clinical signs of infectious coryza. Although there is strong evidence to suggest that the presence of hyaluronic acid in the capsule greatly enhances the virulence of the bacterium, the fact that some organisms that do not have hyaluronic acid are still pathogenic, indicates that other antigens must also play some role in virulence.

### 1.7.2 Adhesins of *H. paragallinarum*

The adhesins of *H. paragallinarum*, involved in the plate agglutination and haemagglutination test, was studied quite extensively in the 1980's and early 1990's, with the aim of understanding the mode of infection as well as the possible role as virulence factors (Yamamoto, 1991). The antigenic structure of the adhesions were investigated by Sawata *et al.* (1979) who studied the morphological and serological properties of the then serotype 1 and 2 organisms (serotype A and C), finding a complicated set of antigenic structures. The organisms belonging to serotypes 1 and 2 possessed protein like antigens on their outermost surface. The antigens, classified by the agglutination test, were L1, L2 and L3 and is labile (destroyed after heating at 65°C for 30 min) and trypsin sensitive. Serotype 1 strains contained the L1 and L3 antigens with serotype 2 strains having the L2 and L3 antigens, with two common types of antigens termed HL (heat labile, trypsin resistant agglutinin) and HS (heat stable, trypsin resistant agglutinin) (Sawata *et al.*, 1979), explaining the varying degree of cross reactivity between these two serotypes due to the sharing of the L3, HL and HS antigens.

Kume *et al.* (1980c) showed that nontreated antigen (NTA), cells that were only washed in PBS and stored in PBS containing thiomerosal, prepared from serotype 1 strains agglutinated freshly collected chicken erythrocytes and induced HI specific antibodies. NTA prepared from serotype 2 however lacked these properties.

Sawata *et al.* (1982) later showed that treatment of the cells using potassium thiocyanate (KSCN) and sonication produced antigens from the encapsulated serotype 2 organisms that could agglutinate glutaraldehyde fixed chicken erythrocytes. This haemagglutinin, termed HA-L, is heat labile, trypsin sensitive, hyaluronidase resistant and active against glutaraldehyde fixed RBC, sharing a lot of similarities with the L agglutinins (Sawata *et al.*, 1979). Due to the similarities in the serotype specificities when using the HI test between the HA-L and the L agglutinins, the haemagglutinins were

designated as serotype 1 specific haemagglutinin, HA-L1, and serotype 2 specific haemagglutinin, HA-L2.

In addition to the HA-L haemagglutinin, Sawata and co-workers (1982) also defined two other haemagglutinins using seven different strains belonging to serotype 1. The first is HA-HL, a heat labile, trypsin resistant haemagglutinin and secondly HA-HS, a heat stable, trypsin resistant haemagglutinin (Kume *et al.*, 1983). The HA-L was the variant specific haemagglutinin with HA-HL and HA-HS being the common haemagglutinins among these strains. Kume *et al.* (1983) attempted to classify 17 strains of *H. paragallinarum* belonging to different serotype using the HA/HI test and confirmed the presence of common and serotype specific haemagglutinins (Table 1.3). The type 2 HA's (trypsin resistant) as described by Yamaguchi and Iritani (1980) were present in all the strains classified by the agglutination test. The Yamaguchi and Iritani type 1 HA (trypsin sensitive), HA-L, was found to be both a common and specific haemagglutinin between the strains. The HA-L haemagglutinins, which are defined by their ability to agglutinate gluteraldehyde fixed RBC, is thus more specific than the other haemagglutinins found in *H. paragallinarum* (Kume *et al.*, 1983).

**Table 1.3:** The classification of *H. paragallinarum* using the HA-L serovar specific haemagglutinin (adapted from Kume *et al.*, 1993)

Serotype classified by serotype specific HA-L haemagglutinins	Reference strain <sup>a</sup>	Factor of HA-L haemagglutinin	
		Specific	Common
HA-1	221 (1)	I-1	I-4, I-5
HA-2	2403 (B)	I-2	I-4, I-5
HA-3	E-3C <sup>b</sup> (N)	I-3	I-4
HA-4	H-18 (2)	II-1	II-4, II-5
HA-5	Modesto (C)	II-2	II-4, II-5
HA-6	SA-3 (N)	II-3	II-4
HA-7	2671 (B)	III-1	

<sup>a</sup> Classified by agglutination test

<sup>b</sup> N, non-typable

Yamamoto (1991) suggested that none of the three virulence-associated antigens (described in this section) play a role in protective immunity. Blackall *et al.* (1993) later confirmed this theory, showing that sensitive mutants of *H. paragallinarum*, which retained the type specific haemagglutinin, lost their pathogenicity. However Bragg, (1995) showed that at least one of the mutants was fully pathogenic when inoculated intra-sinously. Yamaguchi *et al.* (1993) suggested that the HA antigen of *H. paragallinarum* plays an important role in both the pathogenicity as well as protective immunity, the group also isolated a mutant, termed S1M, which probably arose spontaneously from the S1 strain (serovar C) in the nasal cavity of chickens. This mutant did not express haemagglutinin and did not produce HI antibodies in chickens. Yamaguchi *et al.* (1993) further established that the S1M strain was not pathogenic to chickens and this led them to suggest that the HA antigen plays an important role in both protective immunity and virulence.

Bragg *et al.* (1995a) found that the expression of the antigens of *H. paragallinarum* as detected by monoclonal antibodies is significantly affected by the organisms' growth requirements. This implies that even the antigens involved in stimulating the immune response may be affected by the different growth conditions.

### **1.7.3 Toxins produced by *H. paragallinarum***

There is evidence that the virulence of *H. paragallinarum* may be associated with toxins (Rimler *et al.*, 1977). Iritani *et al.* (1980a; 1981b) established that crude polysaccharide extracts from serotype A (strain 221) and C (strain S1) contains both a protective as well as a toxic fraction that could be separated by gel filtration. The toxic fraction could cause hydropericardium in chickens but did not induce HI antibodies and was thus not protective. They further established that this toxic substance was common to both serotype A and C. Other proteins may also play a part in the virulence of *H. paragallinarum* but different protein types found by Blackall and Yamamoto (1989b) did not correspond to virulence.

## 1.8 Conclusion

Most of the information available on *H. paragallinarum* from work performed in the 1930's up to the 1990's focused on classification of the bacteria, *H. paragallinarum*. While considerable in-depth work has been done on the factors associated with the virulence of this bacterium, detailed characteristics of the factors and their association with virulence is currently unknown. Combating this disease is currently trial and error based with antibiotic resistance being observed. Further understanding of the factors enhancing infectious modes might lead to development of more effective vaccines and potentially spare the chicken industry from this common 20<sup>th</sup> century scourge.

## CHAPTER 3

### Partial purification and characterization of the Haemagglutinin from *Haemophilus paragallinarum*

#### 3.1 Introduction

Haemagglutinins belong to a group of proteins called adhesins. The adhesins can be classified into two groups consisting of the fimbrial or pilus-like adhesins and the afimbrial or non-pilus like adhesins (Finlay and Falkow, 1997). The fimbrial adhesins includes the type P and IV pili as found in Gram-negative bacteria such as *E. coli* and *Vibrio cholera*. Characteristics of these types of adhesins include the typical fimbrial build, which can be either rigid or flexible with a lectin type-binding site situated on the tip. The tip of the adhesin can have a wide variety of specificities for different but similar receptors (Hultgern *et al.*, 1993). In assay conditions they require the presence of bivalent ions for proper functioning (Jann and Hoschützky, 1991). The afimbrial adhesins include the haemagglutinins from *E. coli*, *Bordetella pertussis* and *Haemophilus influenza* (Sandros and Tuomanen, 1993). These proteins are usually larger in size (up to 220 kDa) and polyvalent (Goldhar., 1995).

Iritani and co-workers (1980b) reported on the HA-L haemagglutinin of *H. paragallinarum* and indicated that it is a protein with an estimated size of 36 kDa, showing similarity to the pili found in *E. coli* at amino acid sequence level. The protein itself was extensively glycosylated with the main sugars being glucose and maltose. The protein was able to agglutinate both fresh as well as gluteraldehyde fixed RBC and was shown to be trypsin sensitive. Unfortunately the research on this protein was not continued and many questions remained unsolved.

A method with a wide variety of uses is affinity chromatography using lectins, such as Concanavalin A, for the isolation of glycoproteins that contain mannose and glucose in their sugar side chains. This method has been

applied with great success for the isolation of receptors for lectins as well as other lectins and sugar binding proteins (Adair and Kornfield, 1974; Findlay, 1974). Furthermore a method for the rapid isolation of integral outer membrane proteins was developed using Triton X-114, a non-ionic detergent, in a two-phase detergent based separation procedure (Thwaites and Kadis, 1991).

Another technique that might be used more often in the future for the investigation of the proteomes of organisms is two-dimensional electrophoresis. This method not only assists in confirming the homogeneity of protein samples, it can also be used for assigning the correct molecular weight and pI combinations to proteins, and with the information and internet database's available putatively identifying the proteins and comparing them to proteins from other organisms (Cash, 1998).

## **3.2 Material and Methods**

### **3.2.1 Chemicals**

Unless otherwise stated, all chemicals were obtained from commercial sources, were of analytical reagent grade or better and were used without further purification.

### **3.2.2 Bacterial strain**

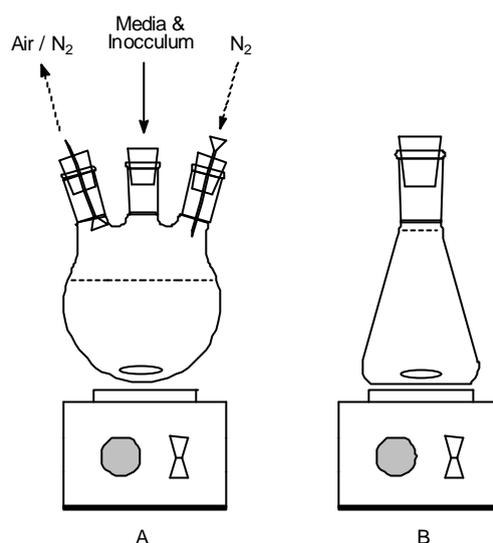
The bacterial strain 46-C3, *Haemophilus paragallinarum* (Hpg) isolated in South Africa and transformed with the plasmid coding for NAD-independence was chosen for the isolation (Taole, 2000). The authenticity of the strain was confirmed using the *Haemophilus* specific PCR test (section 3.2.4), haemagglutination and haemagglutination inhibition tests (section 3.2.5.2) using polyclonal antibodies raised against this bacterial strain in rabbits (Paulse, 1999).

### 3.2.3 Growth of *Haemophilus paragallinarum*

The bacteria were grown and maintained on selective plates (TM/SN), containing Biosate peptone [0.05 % (w/v)]; sodium chloride [0.5 % (w/v)]; starch [0.5 % (w/v)]; glucose [0.25 % (w/v)] and bacteriological agar [0.6 % (w/v)]. The medium was adjusted to pH 7.5 and autoclaved. A broth (TMB), with the same composition as above was used for propagation of the bacteria. Supplements, consisting of heat inactivated chicken serum (10 % (w/v), supplied by Onderstepoort Biological Products), oleic-albumin complex [5 % (w/v)] and thiamine hydrochloride [0.05 % (v/v)] were added to the media after filter sterilization. The solid media (TM/SN) were incubated under increased CO<sub>2</sub> tension inside a candled jar at 37°C (Yamamoto, 1991; Rimler *et al.*, 1974). Liquid cultures were prepared by inoculating bacterium into 10 ml TSB media and grown at 37°C overnight under aerobic conditions (pre-inoculum).

Further growth was achieved either by transferring the pre-inoculum aseptically into 10 ml TMB media and grown at 37°C for 24 h under aerobic conditions or the pre-inoculum was transferred to TMB media (500 ml) and incubated in a three neck flask (Figure 3.1A) with the headspace flushed with nitrogen to provide an anoxic environment, this was placed on a magnetic stirrer to keep the cells in suspension and left at 37°C overnight. These conditions were modified to an Erlenmeyer flask (250 ml) filled with TMB media (270 ml). A rubber stopper (Figure 3.1B) was placed on the opening minimizing oxygen transfer and the culture was grown overnight at 37°C with mild agitation (100 rpm) on a magnetic stirrer.

Cells were harvested at 6 000 xg for 10 min at 4°C. The supernatant was discarded and the pellet washed three times using 0.2 M PBS buffer (pH 7.4). Cells were stored at 4°C to render them haemagglutinable (Blackall *et al.*, 1990a).



**Figure 3.1:** Schematic representation of growth procedures for cultivation of *H. paragallinarum*.

### 3.2.4 *Haemophilus paragallinarum* specific PCR test

Chen and co-workers (1996) described the HPG-2 polymerase chain reaction (PCR), with specific primers for *H. paragallinarum* (Table 3.1)

**Table 3.1:** Sequences of the primers for the *Haemophilus paragallinarum* specific PCR test (Chen *et al.*, 1996).

Primer name	Primer sequence
HP-1F	5'-TGA GGG TAG TCT TGC ACG CGA ATG-3'
HP-1R	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'

The PCR mix contained a single colony of *H. paragallinarum* suspended in sterilized distilled water; MgCl<sub>2</sub> (1.5 mM); dNTP's (10 mM); forward and reverse oligonucleotide primer (100 μmol each) and Taq polymerase (1 U). The PCR conditions were 25 amplification cycles preceded by a "hot start" and performed in a PCR Thermal Cycler (Perkin-Elmer, USA). The hot start entailed a 10 min-denaturing step at 94°C. Each of the following 25 cycles consisted of a 30 s denaturing step at 94°C, a 50 s annealing period at 55°C

and a 45 s elongation period at 72°C. The Taq polymerase was added after the hot start. The PCR products were analyzed on an agarose gel [1% (w/v)] containing ethidium bromide [0.01% (w/v)]. The gel was run with electric field strength of 5.9 V cm<sup>-1</sup> gels for 2 hours and visualized under UV light (Spectroline Transilluminator, USA). A  $\lambda$  phage *HindIII/EcoR1* digested molecular marker was included to estimation of the fragment sizes obtained.

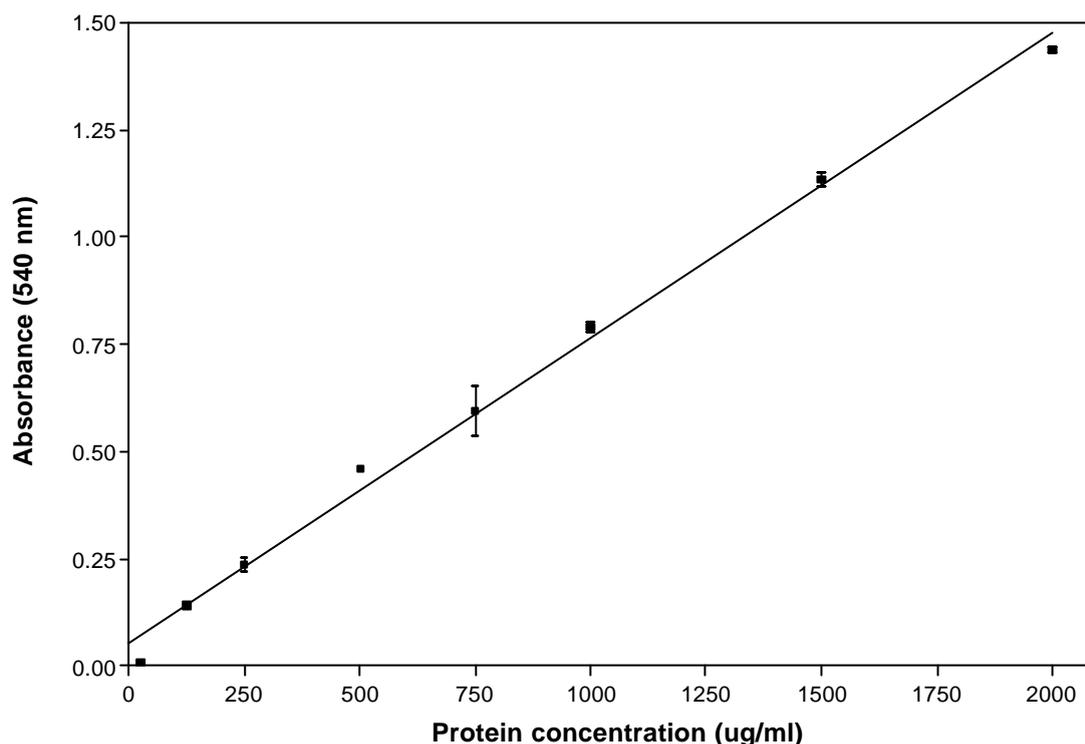
### 3.2.5 Assays

#### 3.2.5.1 Protein assay

Protein concentrations were estimated either by absorbance at 280 nm or by using the micro bicinchoninic acid (BCA) method (Smith *et al.*, 1995), which was supplied as a kit by Pierce (Rockford, IL, USA).

The BCA protein assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration.

The methods used were those supplied with the commercially available kits. A set of protein standards were prepared with bovine serum albumin (BSA), provided by Pierce as part of the BCA protein assay kit, in the range of 25 - 200  $\mu$ g/ml. Standards, as well as protein samples, were prepared in a 96 well flat-bottomed microtitre plate and read at a wavelength of 540 nm using a microtitre plate reader. Standard curves were prepared and used to determine the protein concentration of unknown protein samples (Figure 3.2).



**Figure 3.2.** Standard curve for the BCA protein assay with BSA as protein standard. Standard deviations for triplicate determinations are smaller than the symbols used for the data points.

### 3.2.5.2 Haemagglutination (HA) and Haemagglutination (HI) inhibition test

The haemagglutination test was performed as described by Bragg (1995) using glutaraldehyde fixed chicken red blood cells (GA fixed RBC) prepared according to the method described by Eaves *et al.* (1989) in veronal buffer (VB). Veronal buffer (5 x) was prepared with the following constituents: 5-5-diethylbarbuturic acid (11 mM), barbitone sodium (7 mM), sodium chloride (0.7 M), magnesium chloride (0.2 mM), calcium chloride (0.5 mM) and sodium bicarbonate (12 mM), pH 7.4. The buffer was diluted with Milli-Q water prior to the HA test.

The bacterial suspension or protein extracts (50  $\mu$ l) to be assayed were stored for 3 days at 4°C, washed 3 times in 0.2 M PBS (pH 7.4) and resuspended in 0.2 M PBS (pH 7.4) (Blackall *et al.*, 1990a). These were added to the VB in a

96 well V-bottomed microtitre plate. Two fold serial dilutions were made to which 50  $\mu$ l GA fixed RBC was added and left for 1 h at room temperature. The HA titre was read as the highest dilution of bacteria or protein causing haemagglutination of the GA fixed RBC.

The HI test (Eaves *et al.*, 1989) was performed using 4 HA units of the bacterial suspension (as determined from the HA test) added to 50  $\mu$ l of two fold serial diluted polyclonal antiserum in VB and incubated for 15 min after which 50  $\mu$ l GA fixed RBC was added for a final incubation of 1 hour. The HI unit was read as the highest dilution of antiserum, which inhibited haemagglutination of 4 HA units of the bacteria.

### **3.2.5.3 Dextran assay**

It is well known that HA's functions by binding to sugar entities on the receptor cells of the host. An assay was described in literature by Agrawal and Goldstein (1968) using dextran which is a polysaccharide consisting of D-glucose units linked by  $\alpha(1-6)$  bonds. The assay functions by measuring the binding of the HA to the dextran, at an absorbance of 420 nm. Dextran [1.6 % (w/v)] was incubated at 37°C for 10 min after which either the sample (50  $\mu$ l) or buffer (50  $\mu$ l; blank) was added. This was further incubated for at 37°C for 15 min and the absorbance read at 420 nm using a Spectronic spectrophotometer.

## **3.2.6 Electrophoresis**

### **3.2.6.1 SDS-PAGE**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to monitor the purification process using a 10 % resolving gel and 4 % stacking gel and to determine the relative molecular mass ( $M_r$ ) of the haemagglutinin protein by comparing its electrophoretic mobility with those of standard proteins with known molecular masses.

Protein samples were concentrated by precipitation with trichloroacetic acid (TCA). The protein samples (approximately 10 µg protein) were precipitated by mixing TCA [30 % (w/v)] in a 1:1 (v/v) ratio with the protein sample and left on ice for 10 min, centrifuged at 10 000 xg for 15 min at 4°C. The pellet was subsequently rinsed with ice-cold acetone (-20°C) followed by centrifugation for another 5 min and the supernatant removed. The pellet was dried for 30 min using the Speed-Vac system and resuspended in 25 µl sample buffer.

SDS-PAGE was performed using the “Mighty Small” miniature slab gel electrophoresis unit, SE 200 from Hoefer Scientific Instruments. Electrophoresis was performed on approximately 10 µg protein. The protocol was used as described by Laemmli (1970). The proteins were visualized by silver staining (Switzer *et al.*, 1979).

The protein standards (BIO-RAD) used were myosin (200 000),  $\beta$ -galactosidase (116 250), phosphorylase b (97 400), serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500), lysozyme (14 400) and apoprotinin (6 500), (relative molecular masses in parenthesis).

### 3.2.6.2 Isoelectric focusing (IEF)

Isoelectric focusing was carried out to determine the pI values for the purified protein as well as to confirm purity of the purified protein. The method described by Robertson *et al.* (1987) was used. Electrophoresis was performed using Pharmalyte carrier ampholytes pH 3-10 and loading approximately 20 µg protein sample. The gel was prefocused at 150 V for 30 min and after loading the sample it was focused for 1½ h at 200 V and 1½ h at 400 V. The catholyte was 25 mM sodium hydroxide solution and the anolyte was 20 mM acetic acid. Protein standards with known pI were used as markers to determine the relative pI of the protein in the samples. The proteins were visualized by silver staining (Switzer *et al.*, 1979).

### 3.2.6.3 Two-dimensional gel electrophoresis (2D-gel)

The first dimension (IEF) was prepared as described in section 3.2.6.2. After focusing, the lane containing the protein sample was removed and placed in a transfer solution (50 mM Tris-HCl, pH 6.8; urea (6 M); glycerol [30 % (v/v)]; SDS [1 % (w/v)]; bromophenol blue [0.01 % (w/v)]) for 5-10 min. The strip was then placed directly onto a 10 % SDS resolving gel (3.2.6.1). The gel was run at a constant current (20 mA) and visualized by silver staining (Switzer *et al.*, 1979).

### 3.2.7 Solubilization of the outer membrane proteins

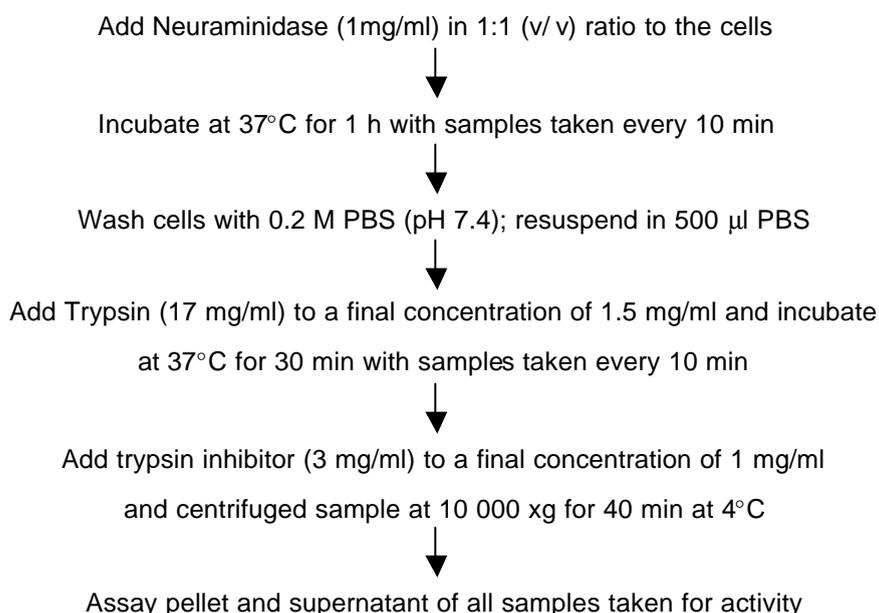
The membrane profile of *H. paragallinarum* was evaluated after treatment of the cells by sonication as previously described by Blackall and Yamamoto (1989b). Cells were grown as described in section 3.2.3, harvested and suspended in HEPES (10 mM, pH 7.4) after which they were subjected to sonication in 3 bursts of 10 s at 50 % power with a 60 % pulse using a Sonifier Cell Disrupter model B-30. The cellular debris was removed by centrifugation at 10 000 xg for 3 min at 4°C. The supernatant was centrifuged again at 10 000 xg for 40 min at 4°C and the pellet resuspended in equal volumes of HEPES (10mM, pH 7.4) and HEPES (10 mM, pH 7.4) containing Triton X-100 [2% (v/v)] to a volume of 400 µl. The suspension was left at room temperature with occasional shaking followed by centrifugation at 10 000rpm for 40 min at 4°C. The supernatant was discarded and the pellet washed with HEPES (10 mM, pH 7.4) and resuspended in HEPES (10 mM, pH 7.4) (Blackall and Yamamoto, 1989b).

The samples were prepared for SDS-PAGE (section 3.2.6.1) and also assayed for activity using the assay described in section 3.2.5.2.

Samples obtained were subjected to column chromatography. Concanavalin A Sepharose 4B resin (105 mm x 10 mm) was equilibrated with 0.1 M Tris-

HCl buffer (pH 7.4) containing 0.5 M NaCl. The non-binding fraction was eluted with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl, followed by elution of the binding fraction using a linear gradient of 0 - 0.5 M methyl- $\alpha$ -D-mannopyranoside with a flow rate of 1 ml.min<sup>-1</sup> and fractions of 1 ml were collected. Elution of the protein was followed using A<sub>280</sub> readings.

### 3.2.8 Enzymatic isolation of the Haemagglutinin



**Figure 3.3:** Flow diagram illustrating the enzymatic digestion procedure as described by Iritani *et al.* (1980b).

Cells were grown and harvested as described in section 3.2.3, incubated in 2 M NaCl at 4°C overnight at 37°C, washed 3 times in 0.2 M PBS buffer (pH 7.4) and suspended in 0.5 ml PBS buffer. The enzymatic digestion was done according to the method (Figure 3.3) described by Iritani *et al.* (1980b). The remaining cells were washed with 0.2 M PBS buffer (pH 7.4) and subjected to more trypsin digestions until no more HA activity was present (Iritani *et al.*, 1980b).

### 3.2.9 Second enzymatic isolation of the Haemagglutinin

From the first isolation it was clear that over-digestion with trypsin caused a loss of HA activity therefore the procedure was adapted to lower concentrations of trypsin. Bacteria were grown and harvested as described in section 3.2.3. The digestions were performed as described in 3.2.8, however, the trypsin and trypsin inhibitor concentrations used were half the concentrations described in 3.2.8.

The supernatants of the digestions were pooled and concentrated using an Amicon stirred cell (Model 8050) fitted with an Amicon YM-10 membrane with a molecular weight cut-off of 10 000 Da. The concentrated sample was applied to a Sephacryl S-400-HR gel filtration column (300 mm x 14 mm) with an application range of 5 000 - 40 000 Da. The column was equilibrated with 0.1 M Tris-HCl buffer (pH 7.4). The flow rate of the column was adjusted to 40 ml.h<sup>-1</sup> and fractions collected (2 ml). Elution of the proteins was monitored spectrophotometrically at 280 nm. Five peaks eluted (A - E) which were separately pooled and the samples (Figure 3.10) with larger molecular weights subjected to affinity chromatography on Concanavalin A Sepharose 4B.

The Concanavalin A column (105 mm x 10 mm) was equilibrated with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl. The non-binding fraction was eluted with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl at 1 ml.min<sup>-1</sup> and fractions (1 ml) were collected. The binding fraction was eluted using a linear gradient of 0 - 0.5 M methyl- $\alpha$ -D-mannopyranoside. The elution of the proteins was monitored using  $A_{280}$  readings. The appropriate protein peaks (Figure 3.11 A) were pooled and concentrated using an Amicon stirred cell as described above and tested for activity as described in section 3.2.5.2. The column was regenerated according to the manufacturers instructions and finally equilibrated with 0.1 M Tris buffer (pH 7.4) containing 0.5 M NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>.

The second and third peaks (Figure 3.11 B) was also purified by the above mentioned affinity chromatography step. Finally the concentration of the different protein peaks was determined as described in section 3.2.5.1 and samples were prepared for SDS-PAGE.

### **3.2.10 Third enzymatic isolation of the Haemagglutinin**

The bacteria were grown, harvested and prepared as described in section 3.2.3. The HA activity was tested as described in section 3.2.5.2.

The isolation of the proteins was performed as described in section 3.2.9. The sequence of the two column steps was reversed and a column with a higher exclusion limit was used. The Concanavalin A column was run as described in the previous section. The protein peaks were monitored at 280 nm. Both the binding and non-binding fractions (Figure 3.13) from the Concanavalin A column step were concentrated with the Amicon stirred cell.

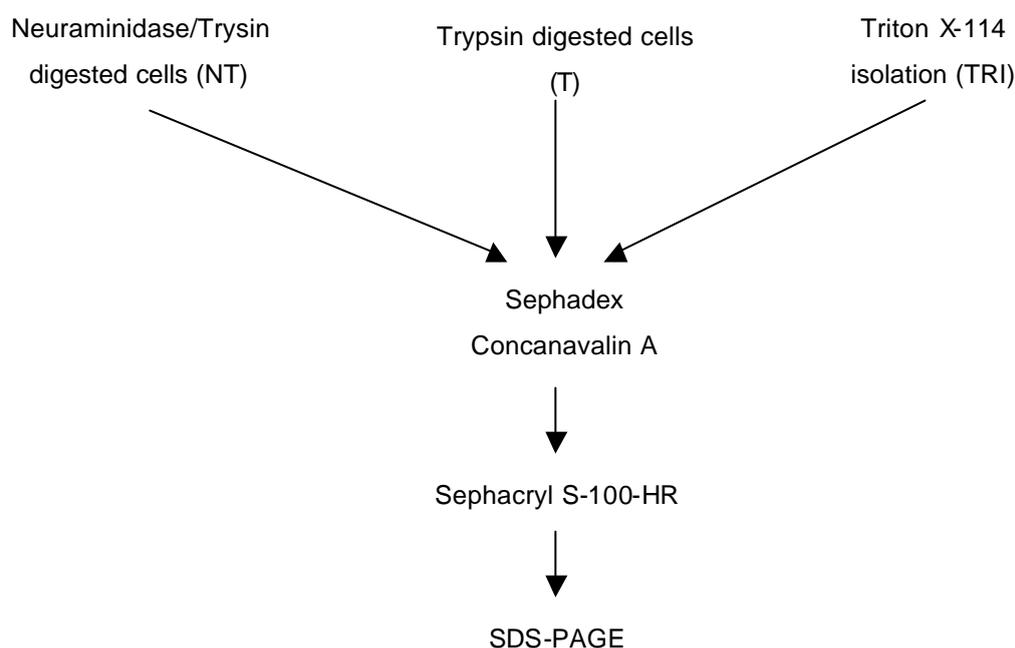
The concentrated samples were loaded onto the Sephacryl S-100-Hr gel filtration column (270 mm x 15 mm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.4). The samples were eluted using a flowrate of 26 ml.h<sup>-1</sup>, monitored at 280 nm and fractions (1 ml) were collected. The protein peaks (Figure 3.15) were again pooled and concentrated using the Amicon stirred cell system (3.2.9), protein concentrations determined (section 3.2.5.1), tested for activity (section 3.2.5.2) and prepared for SDS-PAGE (section 3.2.6.1)

### **3.2.11 Fourth enzymatic isolation of the Haemagglutinin**

All procedures were used as described in section 3.2.10 except that the bacterial suspension was divided into two fractions and the neuraminidase digestion omitted for the one. The flow rate for the Concanavalin A column was adjusted to 20 ml.h<sup>-1</sup>.

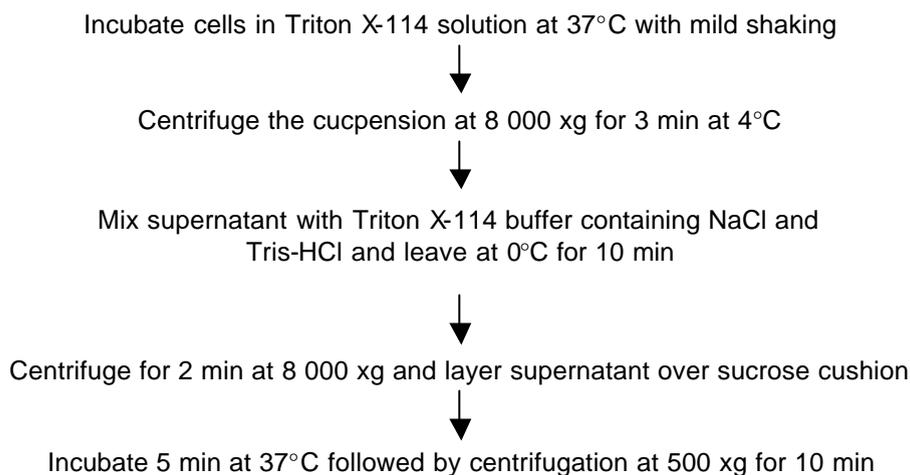
### 3.2.12 Fifth isolation of the Haemagglutinin

Bacterial cells were grown and harvested as described in section 3.2.3 with the cells stored in 0.2 M PBS buffer (pH 7.4). The bacterial suspension was divided into three parts and treated as shown in Figure 3.4. The neuraminidase-trypsin (NT) and trypsin (T) digestions were performed as described in the previous section.



**Figure 3.4:** A flow diagram illustrating the procedure followed for the fifth isolation of the haemagglutinin.

The last third of the cell suspension were subjected to a detergent based two-phase separation procedure using Triton X-114. Bordier (1980) described this method for the separation of integral outer membrane proteins (IOMP) using a nonionic detergent (Triton X-114). Thwaites and Kadis (1991) modified this method for the isolation of integral outer membrane proteins using whole *Actinobacillus pleuropneumoniae* cells (also part of the family Pasteurallaceae).



**Figure 3.5:** A flow diagram of the protocol described by Thwaites and Kadis (1991) for the isolation of integral outer membrane proteins using Triton X-114.

As can be seen in Figure 3.5 the cells were suspended in a Triton X-114 solution [1 % (w/v)] containing DNase (10 U/ml) and RNase (20 U/ml). This suspension was incubated overnight at 37°C with mild shaking (100 rpm). The cellular debris was removed by centrifugation at 8 000 xg at 4°C for 3 min and mixed with Triton X-114 [2 % (w/v)] in 20 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl and left for 10 min at 0°C. This was followed by centrifugation at 8 000 xg for 2 min at 4°C to remove any debris. The supernatant was layered over a sucrose [6 % (w/v)] cushion, incubated at 30°C for 5 min and centrifuged at 500 xg for 10 min. The aqueous phase was removed from the cushion, and the procedure repeated with the same sucrose cushion in order to ensure that all IOMP's had been separated into the detergent phase. The detergent phase (TRI) was collected and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl.

The samples were only subjected to an affinity chromatography (Concanavalin A resin). The column (270 mm x 10 mm) was prepared as described section 3.2.9 with the flowrate adjusted to 30 ml.h<sup>-1</sup> and fractions of 800 µl were collected. Elution of the proteins was monitored using A<sub>280</sub> readings for NT and T (Figure 3.19 A and B) and by means of the method described in section 3.2.5.1 for the TRI sample (Figure 3.19 C). The protein

peaks were pooled, concentrated using the Amicon stirrer cell, tested for activity as described in section 3.2.5.2 and section 3.2.5.3 and samples prepared for SDS-PAGE.

### **3.2.13 Sixth isolation of the Haemagglutinin**

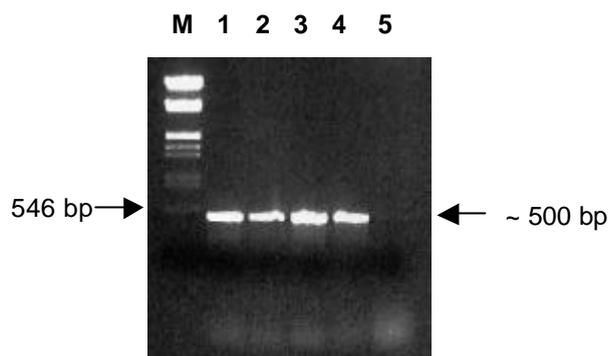
The bacteria were grown, harvested and washed as described in section 3.2.3. The method for the Triton X-114 isolation used in section 3.2.13 was repeated, but the proteins were separated using the Biogel P60 gel filtration column.

The column (300 mm x 10 mm) was equilibrated with 0.1 M Tris-HCl buffer (pH 7.4), the flow rate adjusted to 2.4 ml.h<sup>-1</sup> and fractions (800 µl) collected. Elution of the proteins was monitored using the method described in section 3.2.5.1. The protein peaks (Figure 3.21) were identified and pooled, concentrated using the Amicon stirrer cell and tested for activity as described in section 3.2.5.2 and section 3.2.5.3. Samples were prepared for SDS-PAGE, IEF and 2D-gel electrophoresis.

## **3.3 Results**

### **3.3.1 Confirmation of authenticity of bacterial strain**

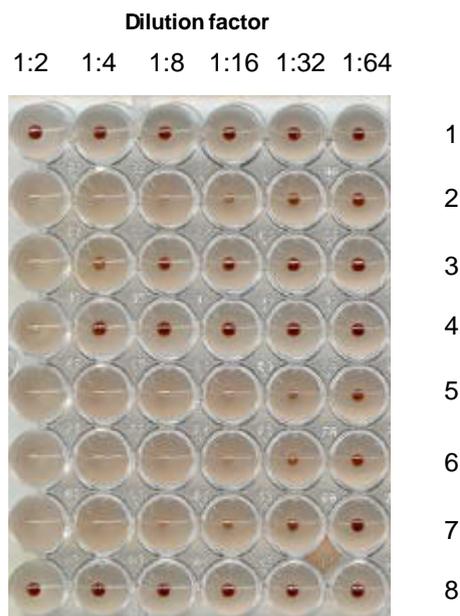
The strain used was confirmed as *H. paragallinarum* using the PCR specific test. DNA fragments correlating to ≈ 500 bp (Figure 3.6) were observed as expected, which correlated with the positive control, a known *H. paragallinarum* strain, when using the HPG 2 PCR. The serovar and strain used was confirmed by the HA and HI tests. This identification procedure was done at regular intervals to confirm that all experiments were being performed on the correct isolate of *H. paragallinarum*.



**Figure 3.6:** Agarose gel electrophoresis of the PCR products obtained for the HPG2-PCR. Lane 1 - 3 represents the PCR products for the *H.paragallinarum* strain used with an estimated size of 500 bp. Lane 4 is the positive control and lane 5 the negative control. The marker shown is  $\lambda$  DNA digested with *HindIII/EcoR1* with the 546 bp fragment indicated.

### 3.3.2 Haemagglutination and haemagglutination tests

All enzymatic digestions were performed on cells displaying a HA of at least 1:32. We had to optimize the assay, modifying the buffer used and adding divalent ions to the buffers. Iritani and co-workers (1980b) did not include any divalent ions and still reported positive assay results. Referring back to section 3.1, the criteria for pilus type adhesins are the need for divalent ions in the solution (Jann and Hoshützky, 1991). It was reported that in other sugar binding proteins, like the lectins, the binding site contained  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  and that the loss of these ions rendered them inactive (reference), it was thus decided to investigate their role in agglutination and optimize the assay conditions. The veronal buffer contained a low amount of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions. The influence of divalent ions at different concentrations was tested by incubating the cells with EDTA (20 mM and 50 mM) for 30 min at 37°C, washing them and testing for activity using veronal buffer without the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . For the estimation of the concentration of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and even  $\text{Mn}^{2+}$  needed, cells were incubated with varying concentrations and different combinations of these ions.



**Figure 3.7:** The HA titre plate with increasing dilutions from left to right. Lanes 1 and 8 are the red blood cell controls; lane 2 the untreated cells; lane 3 cells treated with 20 mM EDTA; lane 4 cells treated with 50 mM EDTA; lanes 5, 6 and 7 cells incubated with 20 mM  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Mg}^{2+}$  respectively.

Figure 3.7 shows the effects of the different conditions on the HA activity of the cells. Lane 2 shows the normal cells showing approximately 3 HA unit's activity. Treatment of the cells with varying concentrations of EDTA almost completely abolished their ability to agglutinate the cells, which might confirm the metal dependency of this protein and assay. On the other hand, the addition of any of the divalent ions restored and even enhanced the HA activity. Enhancement of HA activity was also observed when using combinations of the different ions.

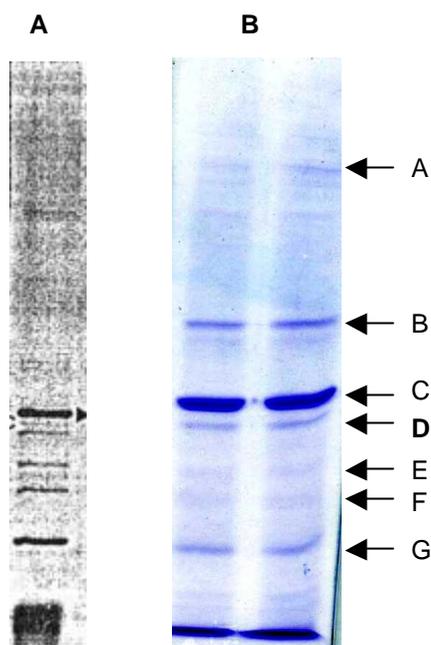
Goldhar (1995), extended the initial work of Jones and Isaacson (1983, as cited by Goldhar, 1995) who worked on adhesins found in *E. coli*. They found that assay conditions for each adhesin had to be optimized in terms of pH of the buffer, temperature of incubation, type of microtitre plate and agitation. Furthermore, haemagglutination is a secondary effect of adhesion and requires a critical density of adhesin molecules on the bacterial surface for the

binding of adhesin-carrying bacteria or cell-free adhesin molecules to many erythrocytes.

This is sometimes difficult with low yields from isolation procedures. Jann and Hoschützky (1991) reported that for the type I, S and P pili of *E. coli* the pH for agglutination would drop from 7.2 for whole cells to a pH of 5 for the purified protein and that haemagglutination only occurred in the presence of divalent ions.

### 3.3.3 Solubilization of the outer membrane proteins

Blackall and Yamamoto (1990) published on the isolation of outer membrane proteins using Triton X-100 and sonication. Their results indicated the presence of 8 clear protein bands when visualized on SDS-PAGE. This procedure was duplicated and the protein extract subjected to affinity chromatography using the Concanavalin A column. This medium might aid in the isolation of the haemagglutinin as suggested by Iritani and co-workers (1980b), because this protein is highly glycosylated with mannose and glucose. Figure 3.8(A) shows the major protein bands as reported by Blackall and Yamamoto (1990) and Figure 3.8(B) the solubilisation pattern obtained with *H. paragallinarum* in this study. The sample, with the active haemagglutinin, was applied to the Concanavalin A, unfortunately very little of the proteins could be retrieved from the column, even after elution with EDTA. The yield was too low for activity measurements and as previously described the activity could not be restored.

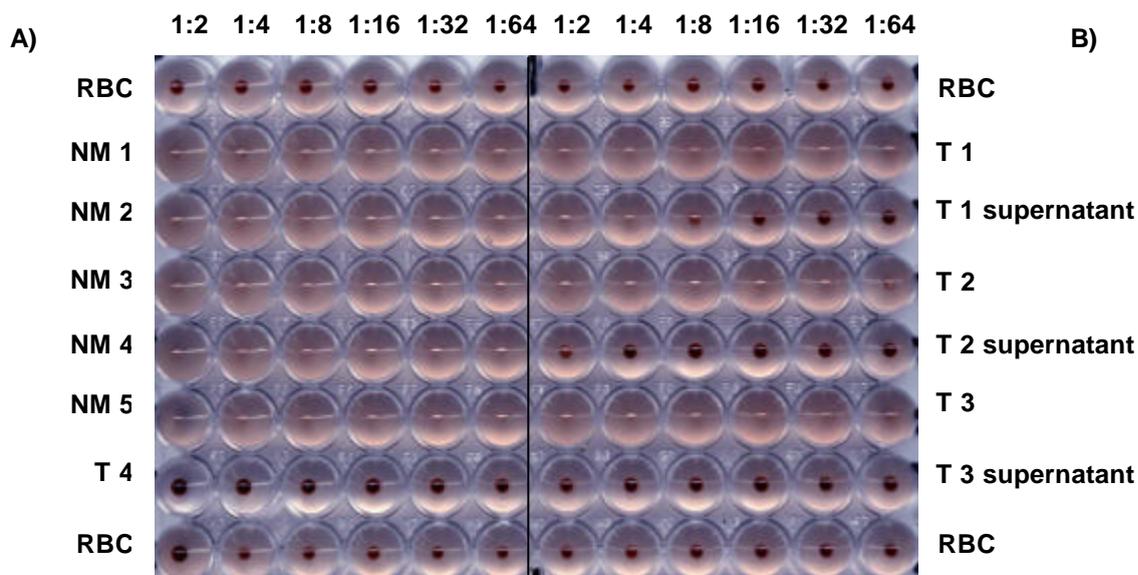


**Figure 3.8:** SDS-PAGE gel of the sonicated sample (1) published by Blackall and Yamamoto (1990) and the sonicated *H. paragallinarum* sample obtained in this study. Seven (A - G) of the possible eight bands can be seen in both pictures.

### 3.3.4 Enzymatic isolation of the Haemagglutinin

The enzymatic isolation of the haemagglutinin was performed as described in section 3.2.8. and monitored over time to assess the effect of trypsinisation.

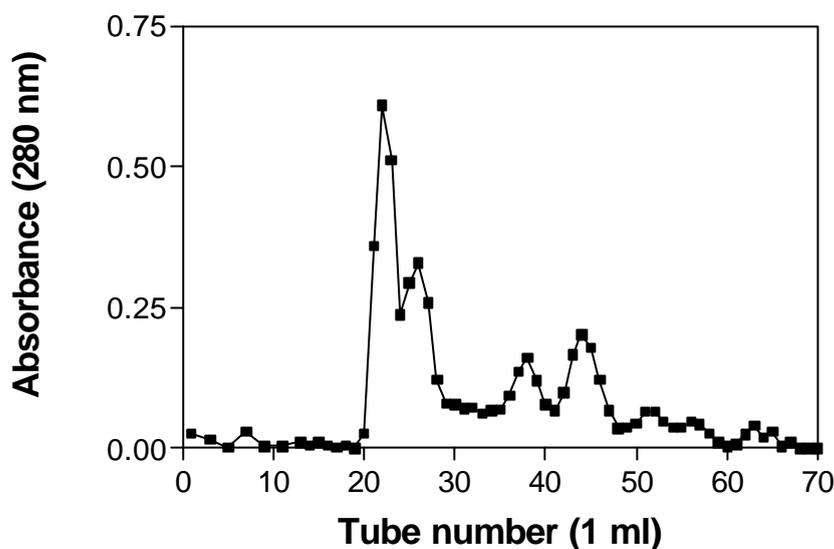
Figure 3.9(A) shows the HA activity of the *H. paragallinarum* cells remained constant during the neuraminidase treatment, suggesting that the cleavage and subsequent removal of N-acetyl neuraminic acid residues does not affect the HA activity in a negative manner. Speculations on the possible enhancement of the activity could not be made; further dilutions would have to be done in order to compare the end point of agglutination. A final concentration of trypsin (1.5 mg/ml) was too high to retrieve active fractions. Figure 3.9(B) shows that the digestion started to degrade the haemagglutinin after 20 min with a loss of 50 % activity, leading to a total loss of activity after 30 min.



**Figure 3.9:** Microtitre plate of the HA assay for the first enzymatic isolation. Side A indicates the samples for the neuraminidase digestions with NM 1 - NM 5 representing the samples taken in 10 min intervals during the digestion. Side B represents the trypsin digestion with T 1, T 2 and T 3 the cells of the samples taken after 10 min, 20 min and 30 min respectively during digestion with the subsequent lane in each case showing the supernatant with the haemagglutinin for each respective time. T 4 is the supernatant after the centrifugation step. RBC indicates the red blood cell controls.

### 3.3.5 Second enzymatic isolation of the Haemagglutinin

The first isolation made it clear that an adaptation of the concentration of trypsin and trypsin inhibitor should be done in order to improve the isolation and yield of the haemagglutinin. The concentrations were halved and digestions allowed to proceed for 20 min. The retrieved sample did not display any HA activity probably because the concentration of the proteins was too low. The sample was applied to the gel filtration column similar to the protocol described by Iritani and co-workers (1980b), they were able to separate the haemagglutinin from the trypsin and trypsin inhibitor using a gel filtration column. Figure 3.10 shows the elution profile obtained for the Sephacryl S-400-HR column.

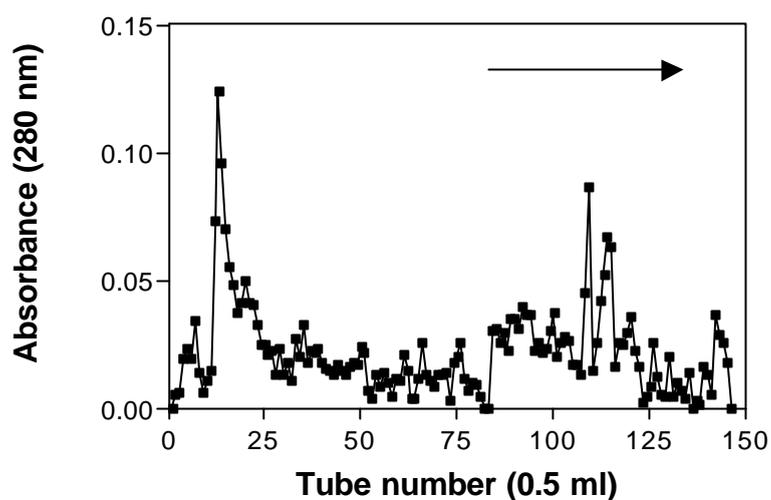


**Figure 3.10:** Sephacryl S-400-HR elution profile of the sample for the first enzymatic digestion.

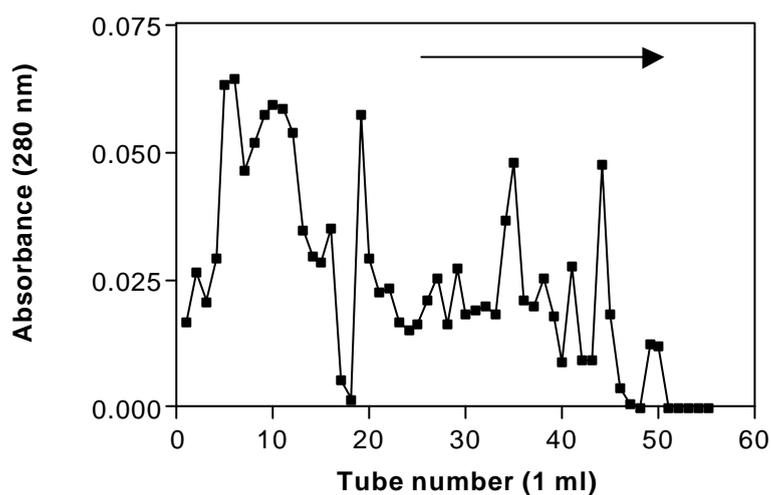
All the protein peaks were pooled and assayed for activity. The pooled fractions were A (20 – 23), B (24 - 25), C (26 - 30), D (35 - 40) and E (42 - 48). On SDS-PAGE, fractions A, B and C displayed more than one protein band with B and C having the same profile (data not shown). A and the combined fractions B and C were subjected to further separation on the Concanavalin A column (Figure 3.11).

Fraction A separated into 5 fractions on Concanavalin A. These fractions were pooled and labeled: NB1 (10 - 17) and NB2 (18 - 23) for the non-binding fractions and B1 (24 - 27), B2 (26 - 31) and B3 (tube 56). For peaks B and C, the peaks of the non-binding fractions were discarded and only the binding fraction evaluated and pooled as BB1 (15 - 18), BB2 (22 - 24) and BB3 (25 - 28).

A)



B)

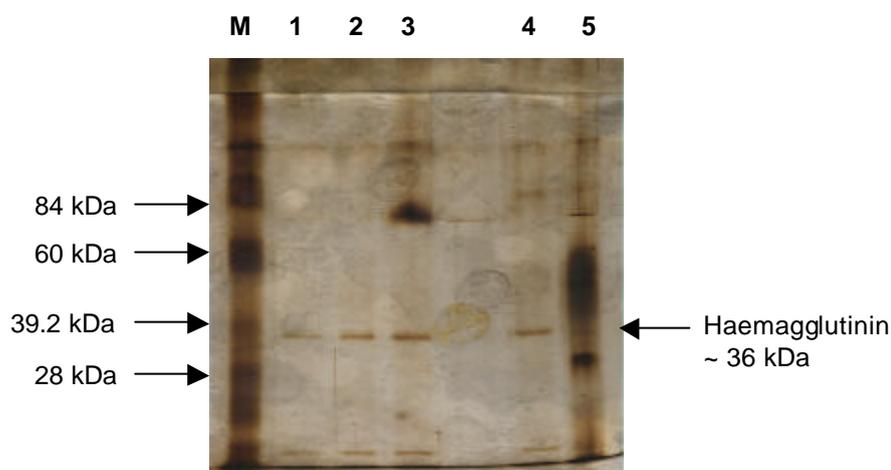


**Figure 3.11:** Concanavalin A Sepharose 4B elution profile for A) fraction A and B) the combined fractions B and C collected from the Sephacryl S-400-HR gel filtration column. In both profiles the arrow indicates the linear gradient of methyl- $\alpha$ -D-mannopyranosyl.

The SDS-PAGE results yielded a protein of the desired size (Iritani *et al.*, 1980b) in almost all the binding fractions, but unfortunately this band was also present in the non-binding fractions. The fractions initially selected from the gel filtration column might all have been part of a single protein fraction (A, B and C). The protein in the non-binding fraction might be due to overloading of the Concanavalin A column. The separation on the Concanavalin A column

can be explained due to the type of interaction between the glycosylated proteins and the Concanavalin A itself which meant that the proteins eluted at different stages.

Unfortunately, in almost all the samples (Figure 3.12), there was a second or third protein present, but what was encouraging was that these bands were relatively small size bands that correlate with the trypsin used for the digestions.

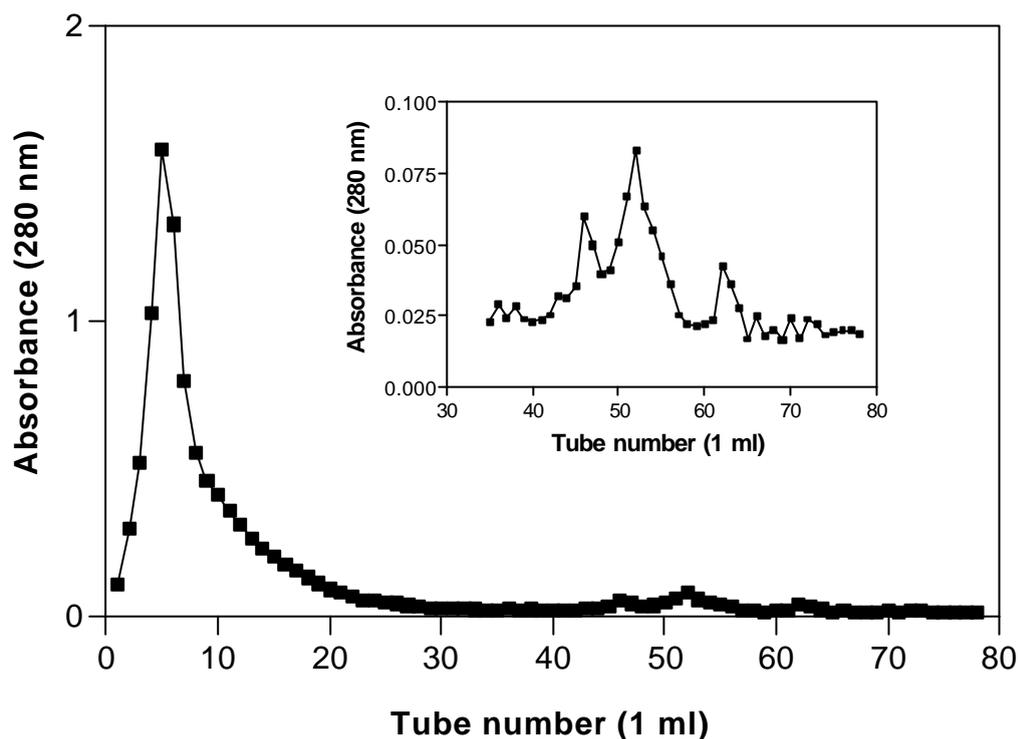


**Figure 3.12:** SDS-PAGE gel patterns of the fractions the Concanavalin A column for the second enzymatic isolation. Lane 1 represents fraction BB4, lane 2 fraction BB3, lane 3 fraction BB2, lane 4 fraction BB1 and lane 5 the sample before it as run on Concanavalin A. M represents the marker (Pierce prestained marker) used.

The sudden loss of HA activity had to be addressed. A possible explanation might be that some of the other haemagglutinins, HA-HL or HA-HS, which are not active against gluteraldehyde fixed red blood cells (Sawata *et al.*, 1982) was isolated. Unfortunately the yield of protein was too low to test all the possibilities or to optimize the assay conditions for the protein.

### 3.3.6 Third enzymatic isolation of the Haemagglutinin

The order of the columns was switched for this isolation and a gel filtration column (Sephacryl S-100-HR) with an exclusion range of 1000 -100000 Da was chosen. The digested sample's supernatant did not display any clear HA activity. The sample was loaded onto the Concanavalin A column and the elution profile obtained can be seen in Figure 3.13.



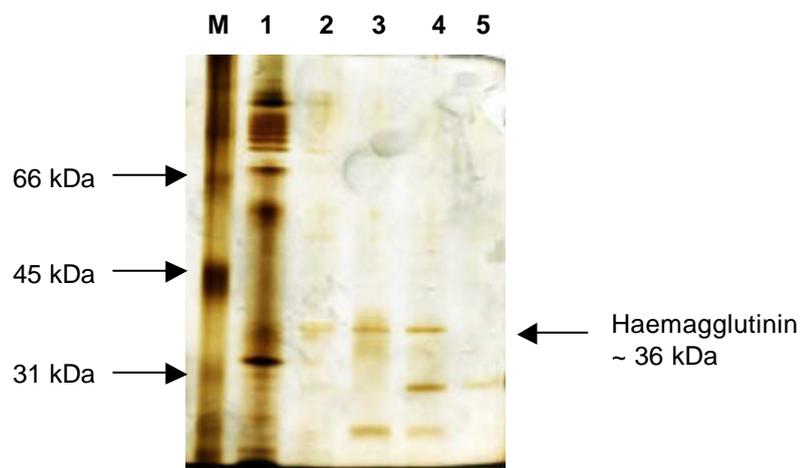
**Figure 3.13:** Concanavalin A Sepharose 4B elution profile for the third enzymatic isolation. Both the non-binding and binding fractions are shown along with an enlargement of the binding fraction. Inserts on graphs show expansion of the binding fractions.

Samples from the Concanavalin A binding fractions were pooled and concentrated for loading onto the gel filtration column (Figure 3.15). Samples were pooled as described in Table 3.2. Samples were analyzed by SDS PAGE (Figure 3.14) and fractions B1B and B1C contained proteins of the expected sizes, unfortunately the fractions also contained the smaller protein band size with an additional third band in B1C. It thus meant that the protein

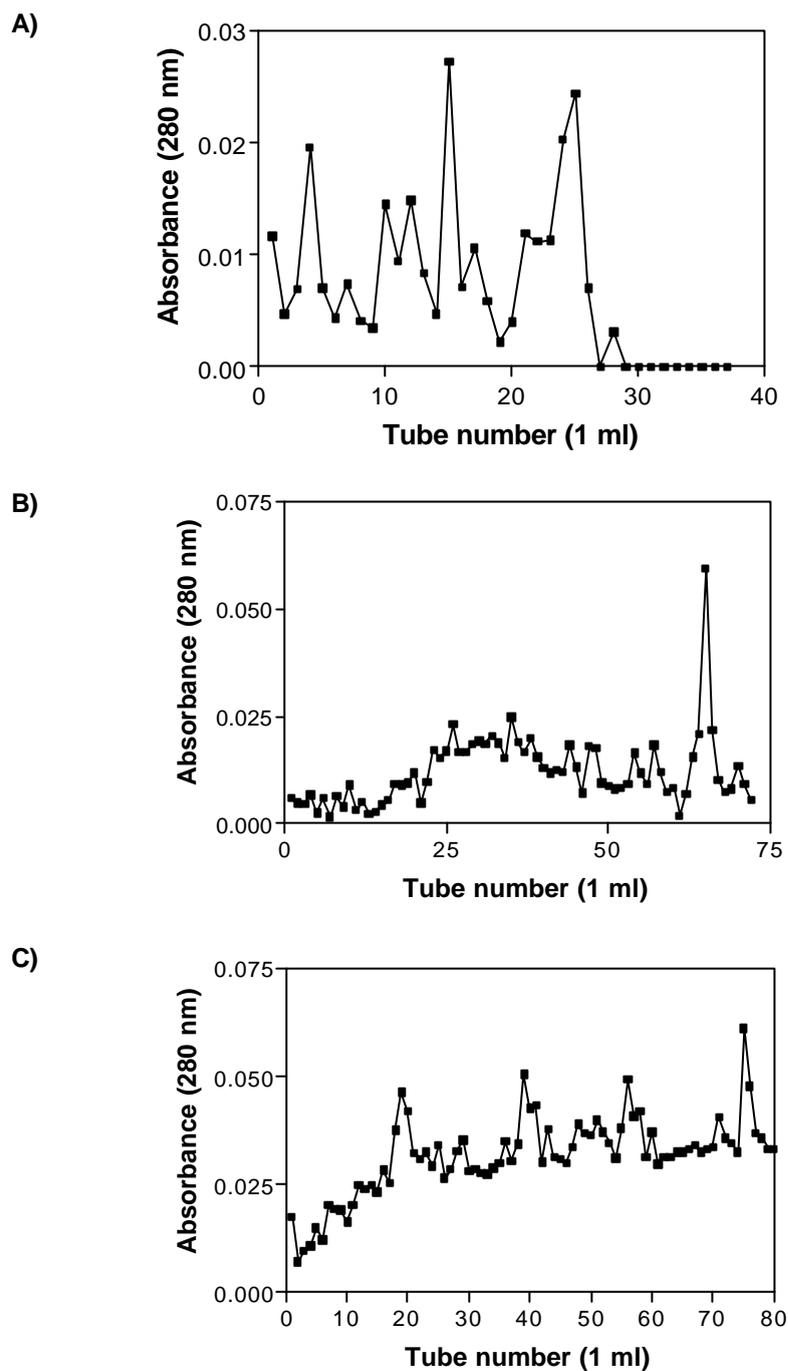
of choice was partially purified, especially when compared to the original sample after digestions (Figure 3.14 lane 1).

**Table 3.2:** Samples obtained during the third enzymatic purification of the haemagglutinin after affinity chromatography, as well as the fractions collected after application to gelfiltration.

Concanavalin A Fraction	Tube number	Gel filtration fractions	Tube number
NB1	1 - 9		
B1	10 - 13	B1A	3 - 5
		B1B	9 - 13
		B1C	14 - 16
		B1D	20 - 26
B2	14 - 22	B2A	61 - 68
B3	26 - 30	B3A	17 - 21
		B3B	37 - 42
		B3C	54 - 59
		B3D	74 - 77



**Figure 3.14:** SDS-PAGE gel patterns of the fractions from the Concanavalin A column for the third enzymatic digestion. Lane 1 represents the sample after digestion with trypsin. Lane 2 is fraction B1A, lane 3 fraction B1B, lane 4 fraction B1C and lane 5 fraction B1D. The marker is indicated by M.



**Figure 3.15:** Sephacryl S-100-HR elution profiles of the samples for the third enzymatic digestion obtained from the binding fractions of the Concanavalin A column, with A) representing fraction B 1, B) fraction B 2 and C) fraction B 3.

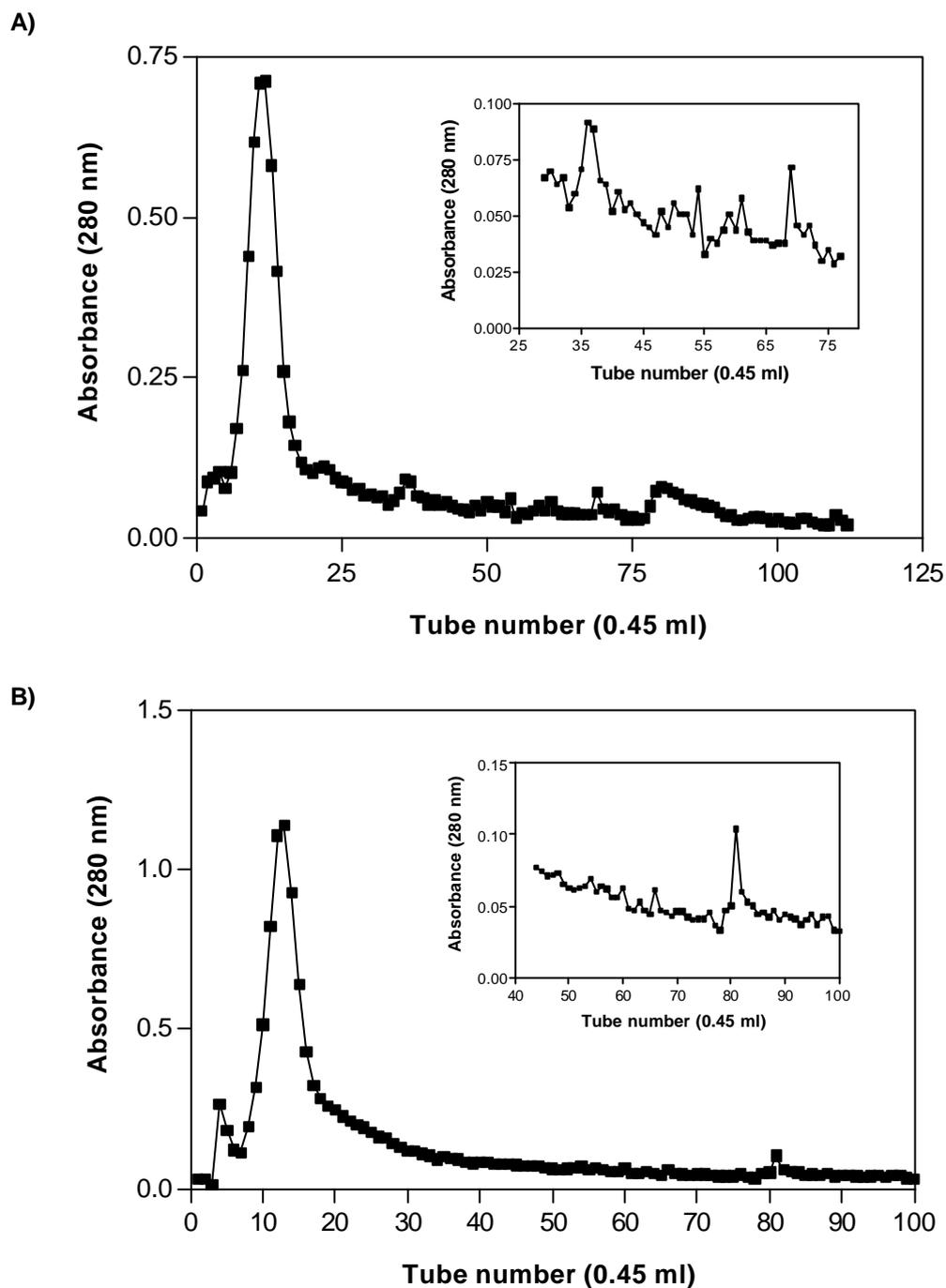
### 3.3.7 Fourth enzymatic isolation of the Haemagglutinin

All efforts to retain and restore the HA activity gave limited success. The possibility that the neuraminidase treatment, which removes N-acetyl neuraminic acid along with the sugar residues associated with this compound could affect the agglutination.

The procedure was followed exactly as described in the previous section with the only difference being the omission of the neuraminidase digestions in half of the sample, thus yielding a neuraminidase/trypsin digested fraction (NT) and a trypsin digested fraction (T).

The columns were eluted at a slower rate and smaller fractions were collected. The elution profiles obtained are given in Figure 3.16 the appropriate peaks were pooled and numbered as described in Table 3.3. These samples were then applied onto the gel filtration columns (Figure 3.17) and peaks were pooled and concentrated for SDS-PAGE analyses.

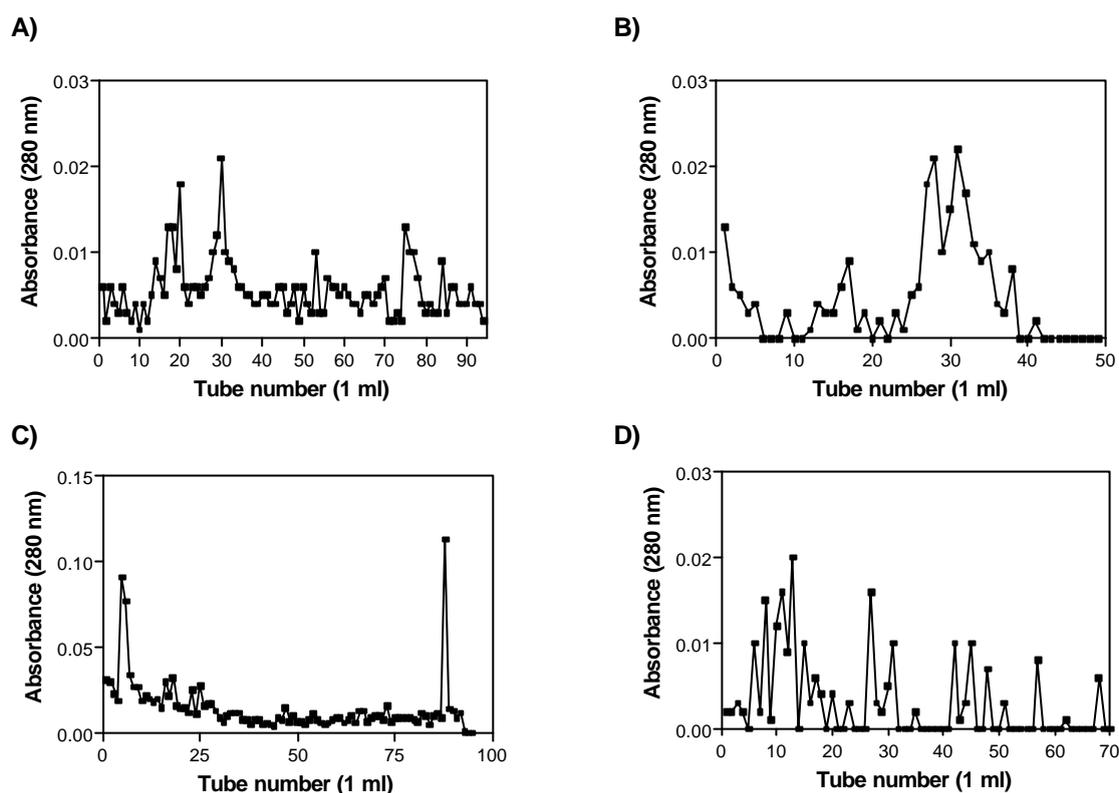
From the data obtained (Figure 3.17) it seems that the gel filtration column did not contribute to the purification of the samples.



**Figure 3.16:** Concanavalin A Sepharose 4B elution profile for the fourth enzymatic isolation for the NT (A) and T (B) samples. Both the non-binding and binding fractions are shown along with an enlargement of the binding fraction. Inserts on graphs show expansion of the binding fractions.

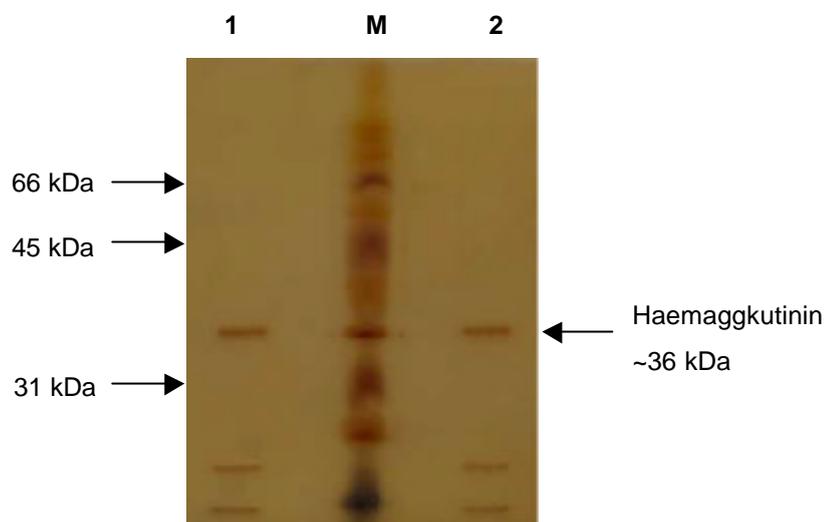
**Table 3.3:** Samples obtained during the fourth enzymatic purification of the haemagglutinin after affinity chromatography, as well as the fractions collected after application to gel filtration.

Treatment	Con A fractions	Tube number	Gelfiltration fraction	Tube number
Neuraminidase/Trypsin	NTNB	8 - 15	NTNB1	13 - 19
			NTNB2	20 - 25
			NTNB3	26 - 35
			NTNB4	36 - 39
	NTBA	33 - 40	NTBA1	12 - 15
			NTBA2	16 - 22
			NTBA3	25 - 36
			NTBA4	52 - 54
			NTBA5	74 - 80
	NTBB	47 - 63	NTBB1	20 - 29
			NTBB2	30 - 33
			NTBB3	34 - 36
			NTBB4	37 - 39
			NTBB5	40 - 44
	NTBC	68 - 74	NTBC1	4 - 10
			NTBC2	15 - 19
			NTBC3	22 - 26
	NTBD	77 - 91	NTBD1	5 - 25
			NTBD2	26 - 40
			NTBD3	41 - 72
Trypsin	TNB	7 - 23	TNB1	18 - 41
			TNB2	42 - 50
	TBA	58 - 66	TBA1	10 - 15
			TBA2	44 - 51
	TBB	77 - 84	TBB1	37 - 45



**Figure 3.17:** Sephacryl S-100-HR elution profiles of the neuraminidase/trypsin digested sample for the fourth enzymatic digestion obtained from the binding fractions of the Concanavalin A column, with A) representing fraction NTBA, B) fraction NTBB, C) fraction NTBC and D) fraction NTBD.

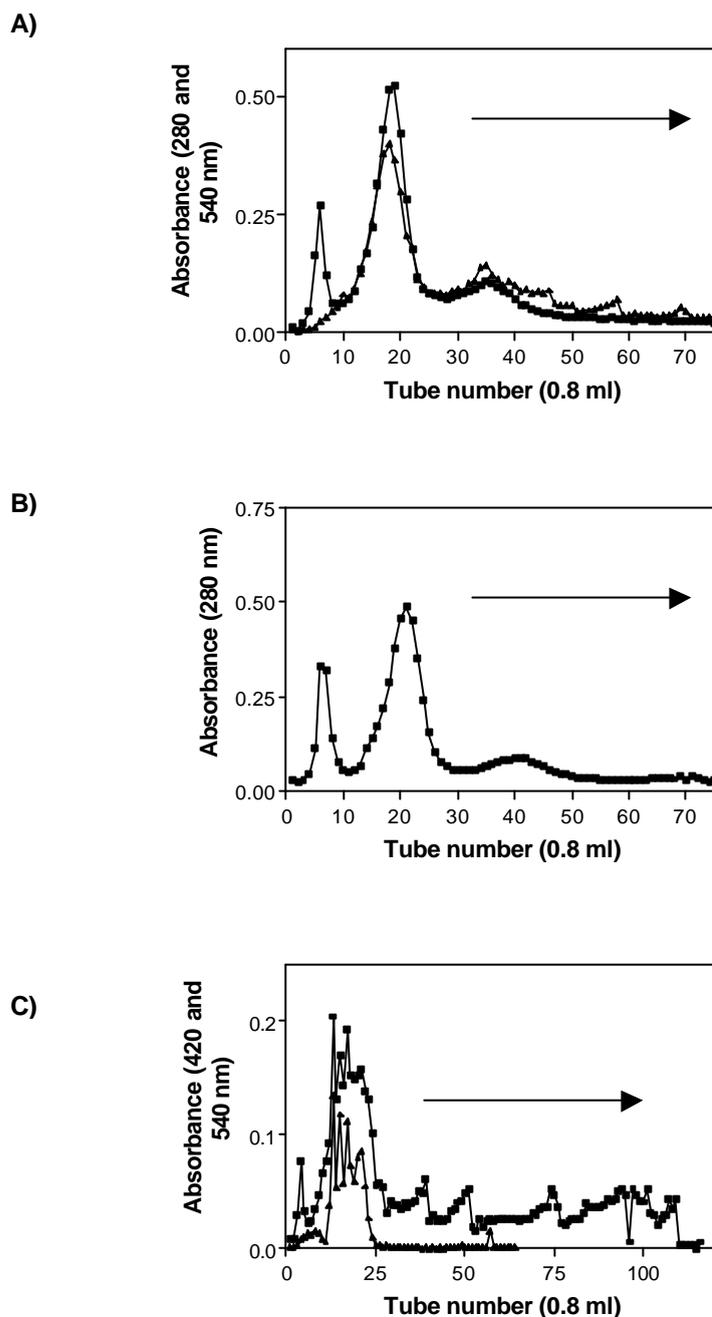
The profile seen in Figure 3.18 lane 1 was a typical protein profile obtained for most of the samples. Some separations yielded an additional band of approximately 100 kDa which might be due to aggregation of the HA subunits (Iritani *et al.* 1980b). The smaller band might also be due to smaller subunits of the HA protein, as is the case with Concanavalin A, where the protein consists of up to four subunits with the  $\text{Ca}^{2+}$  being the binder of the subunits and the  $\text{Mn}^{2+}$  playing an active role in the binding site of the protein (Wang *et al.*, 1971).



**Figure 3.18:** SDS-PAGE gel patterns of the trypsin digested fractions from the Concanavalin A column for the fourth enzymatic digestion. Lane 1 represents fraction TBA and lane 2 fraction TBB. The marker is indicated by M.

### 3.3.8 Fifth isolation of the Haemagglutinin

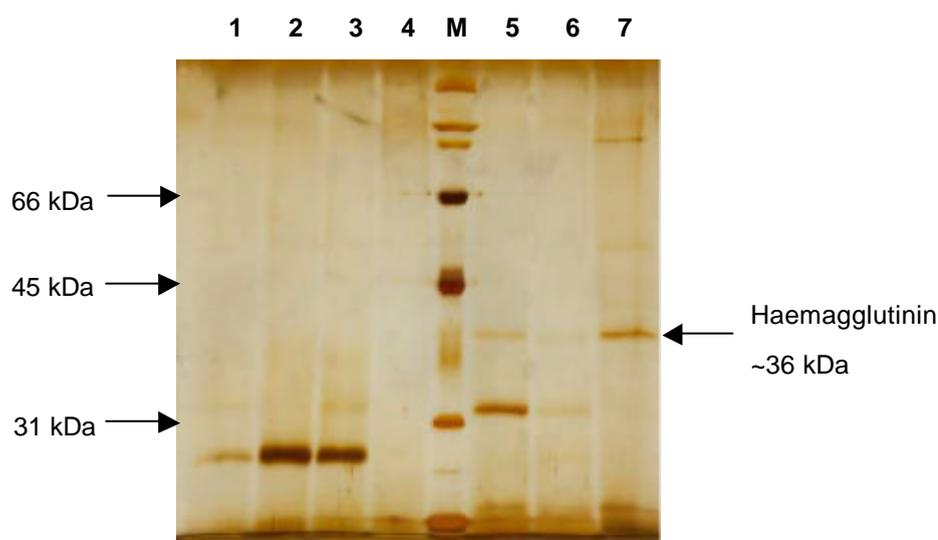
It was clear from the previous purification attempts that the gel filtration step was ineffective and that the Concanavalin A resin had a fairly low binding capacity, prompting the use of a bigger resin volume (four times more) for the affinity chromatography. Digestions described in section 3.2.11 were repeated along with a method described by Thwaites and Kadis (1991) for the isolation of integral outer membrane proteins.



**Figure 3.19:** Concanavalin A Sepharose 4B elution profile for the fifth enzymatic isolation. Both the non-binding and binding fractions are shown for A) the neuraminidase/trypsin digested sample, B) the trypsin digested sample and C) the triton X114 sample. Profile A) contains the  $A_{280}$  ( ) and  $A_{540}$  ( ) values for protein estimation. Profile B) contains  $A_{540}$  ( ) values for protein determination and  $A_{420}$  ( ) for assay values. The arrow indicates the application of the linear gradient of methyl- $\alpha$ -D-mannopyranosyl.

Figure 3.19 shows the Concanavalin A elution profiles for the different samples. With the increased resin volume a higher yield proteins is obtained in the binding fraction for Figure 3.19 (A) and (B). Unfortunately the active fraction (according to the dextran assay) for the triton sample eluted before the application of the gradient.

Protein peaks where pooled and assayed for activity using the HA test. The pooled fractions were NTNB (non-binding fraction; 12 - 25) and NTB (binding fraction; 35 - 42) for the neuraminidase/trypsin sample, TnB (non-binding fraction; 13 - 27) and TB (binding fraction; 35 - 43) for the trypsin sample and TR 1 (non-binding fraction; 6 - 25), TR 2 (binding fraction 1; 35 - 40) and TR 3 (binding fraction 2; 46 - 52) for the triton sample.



**Figure 3.20:** SDS-PAGE gel patterns of the samples from the fifth digestion after separation on the Concanavalin A column. In lane 1 is fraction NTNB, lane 2 is fraction NTB, lane 3 fraction TNB and in lane 4 fraction TB. Lane 5 is fraction TR 1, lane 6 is fraction TR 2 and lane 7 fraction TR 3. The molecular weight markers are indicated by M.

The SDS-PAGE profile (Figure 3.20) revealed a much smaller protein in the NT and T samples, probably due to the trypsin. The TR 1 sample showing dextran as well as haemagglutination activity, showed the similar pattern observed for the fourth isolation, with the protein of about the correct size for the haemagglutinin as reported by Iritani and co-workers (1980b) as well as a

fairly large protein (~100 kDa) and a smaller band (~20 kDa). As no trypsin was used it could be speculated that this might correlate to a smaller outer membrane protein or a subunit of such a protein. Although the activity could be retained, this isolation method still proved to be ineffective as the haemagglutinin itself could not be purified.

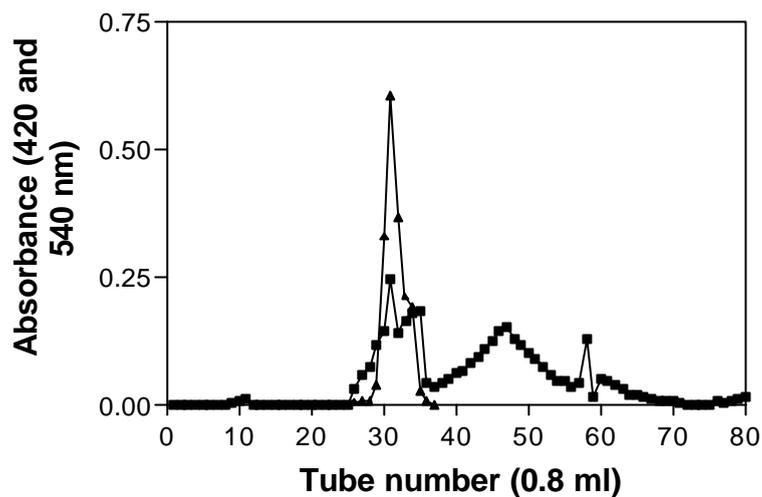
### 3.3.9 Sixth isolation of the Haemagglutinin

It could be concluded from the previous isolation procedure that the Triton X-114 method proved to be effective in isolating a haemagglutinin fraction.

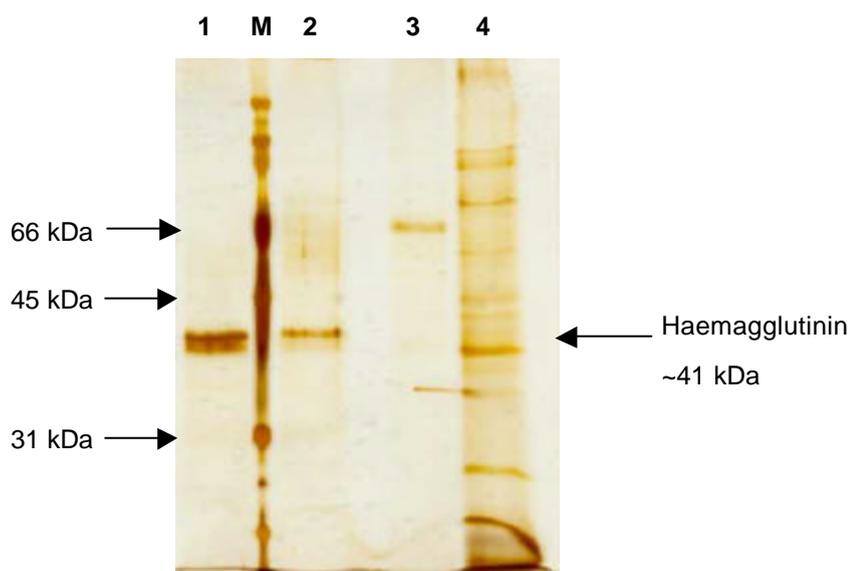
Figure 3.21 shows the elution profile obtained with the gel filtration column, with 3 prominent protein peaks being observed. The first protein peak corresponded to the activities obtained using both the HA and dextran assays. It was thus decided to pool the protein peaks into fractions TR 1 (28 - 32), TR 2 (33 - 36), TR 3 (40 - 52) and TR 4 (57 - 59).

Figure 3.22 represents the SDS-page gel for this isolation and 3 protein bands with almost exactly the same molecular weight are present in lane 1. These proteins correlate well in size to the protein reported by Iritani and co-workers (1980b) since it must be taken into consideration that they removed only the exterior part of the protein and shown here is the entire protein.

The results obtained from the IEF and 2D-gels confirmed the presence of three proteins with molecular masses and pI values as shown in Table 3.4.



**Figure 3.21:** Biogel P 60 elution profile of the Triton X114 sample showing the protein assay (  $\Delta$  ) and dextran assay (  $\square$  ) values.



**Figure 3.22:** SDS-PAGE gel patterns of the fractions obtained after separation on the Biogel P 60. Lane 1 represents fraction TR 1, lane 2 fraction TR 2, lane 3 fraction TR 4 and lane 4 fraction TR 3. The marker is indicated with an M. The arrow indicates the proposed haemagglutinin.

**Table 3.4:** Molecular weight and pI combinations for the three protein bands obtained from the fraction TR 1. Also indicated is the protein to which is similar using the Mw and pI combinations.

Protein fragment	Molecular weight (Da)	pI	Similar to
A	41 000	6.4	Haemagglutinin/Protease from <i>V. cholera</i>
B	40 500	5.2	No significant matches
C	40 000	6.4	Filamentous adhesin from <i>B. pertusis</i> ; switch protein from <i>E. coli</i>

Using these combinations searches were performed on the ExPASy Database (<http://www.expasy.ch>) against all probable Gram-negative bacteria containing both type of adhesins. The most notable similarities (Table 3.4) were found with the Haemagglutinin/Protease from *V. cholera* for fragment A and a filamentous adhesin from *B. pertusis* for fragment C, which is stated in literature (Sandros and Tuomanen, 1993) to be closely related to the type P pili of *E. coli*. This suggests that one of the proteins obtained could be related to that obtained by Iritani and co-workers (1980b), a pilus like protein that it can be classified as a fimbrial adhesin. The similarity with the *V. cholera* protein might suggest that unlike the HA's found in the other *Pasteurellaceae* the afimbrial adhesin is much smaller.

### 3.4 Conclusions

The haemagglutinin from *H. paragallinarum* could be partially purified using a variety of methods, and the determined molecular weight correlated well to that found in the literature (Iritani *et al.*, 1980b). The choice of method would be dictated by the ability to retain activity of the protein. Finally activity was retained with the Triton X-114 isolation method, however as previously described the other isolation methods should not be discarded as the haemagglutinating activity might be “lost” due to agglutination test defects. Furthermore the type of haemagglutinin isolated too plays a role is the type of HA tests used and the results obtained. Other techniques have to be considered to fully purify the haemagglutinin and to unequivocally establish its identity.

The partially pure protein was obtained and characterized with respect to pI and molecular weight, which correlates well to other fimbrial adhesins isolated. This confirms the theory that *H. paragallinarum* has a structurally smaller haemagglutinin in comparison to other HAP organisms. The continued struggle with the HA test possibly are indicative of the assumption that this haemagglutinin are a fimbrial adhesin, which will still agglutinate and play an important role in infection. This emphasizes the ever growing need to investigate these proteins and their action as to refine and improve the vaccines for enhanced cross protection, especially since current vaccines are only mildly effective.

## CHAPTER 4

### Cloning of the Haemagglutinin gene from *Haemophilus paragallinarum*

#### 4.1 Introduction

Previous research on the adhesins of *H. paragallinarum*, in particular the haemagglutinins, did not focus on the genes involved in the expression or assembly of the proteins. Takagi *et al.* (1991) did manage to isolate a gene from the genomic DNA of an *H. paragallinarum* (serotype A) isolate (strain 221) that was responsible for haemagglutination activity when expressed in *E. coli*. The insert was approximately 2.57 kb and the protein produced elicited antibody production in chickens when immunized with the purified protein. The protein itself also bound monoclonal antibodies against the haemagglutinin of this specific strain; this clone was unfortunately lost during storage (Takagi *et al.*, 1991).

The results from Chapter 3 suggested that an adhesin belonging to the afimbrial adhesins could be present. It was therefore important to investigate the presence of a haemagglutinin, possibly belonging to the afimbrial adhesins, since it was shown that both *E. coli* (Duguid *et al.*, 1979) and *H. influenza* (Barenkamp and Geme, 1994) contain a mixture of fimbrial and afimbrial adhesins.

The haemagglutinins belonging to the afimbrial adhesins, are generally relatively large proteins, with the genes encoding them ranging from 12 500 bp (*IspA1*) and 14 800 bp (*IspA2*) for the filamentous haemagglutinins from *H. ducreyi*, considered as one of the largest prokaryotic open reading frames (Ward *et al.*, 1998), to about 7 000 bp for the filamentous haemagglutinin (FHA) from *Bordetella pertussis* (Relman *et al.*, 1989). These genes thus encode proteins of up to 220 kDa for *B. pertussis* and 125 kDa for the filamentous haemagglutinin from *H. influenza* (Barenkamp and Leininger,

1992), larger than the proteins already isolated for *H. paragallinarum* (Iritani *et al.*, 1980b).

## 4.2 Materials and methods

### 4.2.1 Chemicals used

Unless otherwise stated, all chemicals were obtained from commercial sources, were of analytical reagent grade or better and were used without further purification.

### 4.2.2 Bacterial strains used

Bacterial strains used for this section is described in Table 4.1.

**Table 4.1:** Bacterial strains used in molecular characterization with some of their functions and properties.

Bacterial strain	Functions / Properties
<i>Escherichia coli</i> Top 10 <sup>a</sup>	Competent <i>E. coli</i> strain used for transformation experiments with the pGEM 3Z and pGEMT-easy plasmids
<i>Escherichia coli</i> JM109(DE3) <sup>a</sup>	Competent <i>E. coli</i> strain used for transformation experiments with the pET 5a/b/c expression plasmid; contains the T7 RNA polymerase gene for induction of the plasmids used
<i>Pasteurella multocida</i> <sup>b</sup>	Used for genomic DNA isolation and isolation the partial haemagglutinin gene
<i>Haemophilus paragallinarum</i> (serotype A1)	Used for genomic DNA isolation and isolation of the partial haemagglutinin gene

<sup>a</sup> Strains purchased from Promega

<sup>b</sup> Kindly supplied by Dr. M. Henton from the ARC-Onderstepoort Veterinary Institute

### 4.2.3 Plasmids used

All the plasmids used (Table 4.2), were purchased from Promega and contained the  $\beta$ -lactamase gene for ampicillin resistance and the *lacZ* operon allowing for selection.

**Table 4.2:** Plasmids used in molecular characterization with some of their functions and properties.

Plasmid	Function / Properties
PET5 a/b/c expression vector	Expression vectors with different open reading frames allowing expression of cloned genes under transcriptional control of a bacteriophage T7 promoter; expression initiated in bacterial strains containing a chromosomal copy of the T7 RNA polymerase gene.
pGem3Z	Standard cloning vector and <i>in vitro</i> transcription.
pGEMT-easy	Linear plasmid containing T-overhangs for ligation of PCR products.

### 4.2.4 Growth and maintenance of bacteria

*Haemophilus paragallinarum* strains were grown and maintained as described in section 3.2.3 with the addition of 1% (w/v) NAD<sup>+</sup> due to the fact that all the strains used in this chapter were NAD<sup>+</sup>-dependent. Strain identities were verified by using the *Haemophilus* specific PCR (section 3.2.4) and haemagglutination and haemagglutination inhibition test (section 3.2.5.2)

*E. coli* strains were grown and maintained either on LB plates (yeast extract, 0.4 % (w/v); tryptone, 1 % (w/v); NaCl, 1 % (w/v) and bacteriological agar, 0.6 % (w/v)) or LB-broth. After transformation, bacteria were grown on LB-plates supplemented with IPTG (20 mg/ml); X-Gal (20 mg/ml) and ampicillin (30 mg/ml). Cultures were grown at 37°C for 16h.

The *Pasteurella multocida* strain was grown and maintained on blood tryptose agar (BTA) plates supplemented with 5 % (v/v) sheep blood. The bacteria were grown at 37°C and passaged regularly.

#### 4.2.5 Genomic DNA isolation

Total genomic DNA from *Haemophilus paragallinarum* and *Pasteurella multocida* was isolated as described by Towner (1991) using SDS/proteinase K treatment. Cells were grown, harvested and prepared as described in sections 4.2.5 and 3.2.3. The bacterial pellet was resuspended in TE-buffer (1 M Tris-HCl, pH 8; 0.5 M EDTA) in a ratio of 0.5 g cells to 40 ml buffer. After centrifugation at 3000 xg for 5 min at 4°C the pellet was resuspended in Tris-HCl buffer (40 mM; pH 8) containing sucrose (0.7 mM). Lysozyme (20 mg/μl) was added and left for 5 min on ice. EDTA (0.5 M; pH 8) and SDS (10 % (w/v)) were added to the mixture, it was gently mixed and placed on ice. Digestion buffer (50 mM Tris-HCl, pH 9); 1% (w/v) SDS); 0.1 M EDTA; 0.2 M NaCl; 0.5 mg/ml Proteinase K) was added and incubated 16 h at 55°C with mild shaking.

One volume of pH-calibrated biophenol was added and gently mixed for 3 h at 25°C followed by centrifugation at 4000 xg for 10 min. The supernatant was mixed with a phenol-chloroform mixture (50% (v/v) TE-buffer, pH 8; 25 % (v/v) pH buffered phenol; 25 % (v/v) chloroform-isoamylalcohol (24:1)) followed by centrifugation at 3000 xg for 10 min at 25°C and placing on ice for 10 min. To this was added 5 M sodium acetate (pH 5.7) along with ice-cold 99.9 % ethanol. Precipitated DNA was collected and centrifuged for 1 min at 4°C and the pellet was washed with 70 % ethanol. The pellet was air-dried, resuspended in TE-buffer (pH 8) containing RNase (50 μg/ml) and incubated at 30°C until the DNA was dissolved. A sample of the genomic DNA was evaluated on an agarose gel (0.7 %, section) to verify if the DNA was intact.

#### 4.2.6 Polymerase chain reactions amplifications (PCR)

PCR's were performed in a Thermal Cycler (Perkin-Elmer, USA) in a total volume of 50  $\mu$ l. Each reaction consisted of 10x Taq buffer; DNA (10  $\mu$ g); dNTP's (10 mM); forward (PM-F; 5'-GCC GAT ATT GGT GGA GTA GAT ATT AA-3') and reverse (PM-R; 5'-TAT CTT GTT TGG ATA CCA CAT TCG T-3') primers (50 pmol) and Taq polymerase (5 U). The reactions were subjected to a pre-denaturing step at 94°C for 5 min followed by 25-30 cycles denaturing at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 2 min. This procedure was followed for all the experiments except were stated differently.

#### 4.2.7 Agarose gel electrophoresis

DNA was analyzed on a horizontal agarose slab gel [1 (w/v)] with ethidium bromide (0.5  $\mu$ g/ml) in TAE buffer (40 mM Tris acetate; 2mM EDTA, pH 8.3). Electrophoresis was done for 1-2 hours in an electric field strength of 5.9 V.cm<sup>-1</sup> gel and the DNA was visualized with UV light (Spectroline Transilluminator, USA).

The relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with that of the standards run with the samples on each gel. Either 1kB Plus marker (Life Technologies) or *Hind*III/*Eco*R1 digested  $\lambda$  DNA (Promega) were used as standards.

#### 4.2.8 Purification of PCR reactions and PCR products from agarose gels

Purification was achieved using the "NucleoSpin Extract 2 in 1 kit" (Separations) according to the manufacturer's instructions.

## 4.2.9 Restriction enzyme digestions

Restriction enzyme digestions, to verify fragment inserts, were performed according to the supplier's (Roche) recommendations. Usually DNA (approximately 5 µg) was digested with restriction enzyme (1 U) in a reaction containing 10X enzyme buffer at the appropriate optimum temperature for the individual enzymes for 1-2 hours. Products were separated on an agarose gel (section 4.2.7) for evaluation.

## 4.2.10 DNA ligations

For ligation, fragments obtained from genomic DNA digestions (section 4.2.5) were purified from the gel (section 4.2.8) and the desired plasmid and DNA combinations made. Ligations were performed in a total volume of 23 µl containing 10x Ligation buffer and DNA ligase (1 U, New England Biosystems) and incubated at 16°C overnight. PCR products were ligated into pGEMT-easy vector (Promega) at 4°C according to the manufacturers specifications.

## 4.2.11 Bacterial transformations

### 4.2.11.1 Preparation of the bacterial cells for transformation

Competent cells were prepared according to the method described by Tang *et al.* (1994). Briefly, the desired *E. coli* strain was cultured overnight at 37°C in 5 ml LB-broth (section 4.2.4), and 1 ml was transferred to 100 ml LB-broth and allowed to grow to an optical density (OD<sub>600</sub>) of 0.9 - 0.95. The cells were harvested by centrifugation at 4 000 xg for 5 min at 4°C and the pellet resuspended in 10 ml of a solution containing CaCl<sub>2</sub> (80 mM) and MgCl<sub>2</sub> (50 mM). The mixture was left on ice for 10 min and centrifuged at 4 000 xg for 5 min at 4°C. This was repeated twice with the pellet finally being resuspended in CaCl<sub>2</sub> (0.1 M) and glycerol [50 % (v/v)]. The suspensions

were aliquated into 80  $\mu$ l volumes, snap-frozen using liquid nitrogen and stored at  $-70^{\circ}\text{C}$  (Tang *et al.*, 1994).

#### 4.2.11.2 Bacterial transformation

Plasmid (1  $\mu$ g) containing the desired inserts was added to competent *E. coli* cells and left on ice for 30 min then incubated at  $42^{\circ}\text{C}$  for 90 s and placed on ice for 4 min. 800  $\mu$ l LB-media containing 40 % (w/v) glucose was added to the suspension and incubated for 1 hour at  $37^{\circ}\text{C}$ . Cells were harvested by centrifugation at 4 000 xg for 2 min, resuspended in LB-media, plated out onto LB plates containing IPTG, X-Gal and ampicilin (section 4.2.4).

#### 4.2.12 Mini-preps for isolation of plasmid from bacterial cells

Single white colonies were inoculated into 5ml LB-media containing ampicillin (30  $\mu$ g/ml) and incubated at  $37^{\circ}\text{C}$  for 16 h. The cells were harvested by centrifugation for 1 min. The pellets were resuspended in STET buffer (8 % (w/v) sucrose; 5 % (v/v) Triton X-100; 50 mM EDTA; 50mM Tris-HCl, pH8), lysozyme (50 mg/ml) was added and immediately placed in a boiling water bath for 1 min. The samples were placed on ice for 10 min and centrifuged at 10 000 xg for 15 min at  $4^{\circ}\text{C}$ . The pellet was removed using a sterile toothpick, isopropanol was added to the supernatant and incubated for 10 min at  $-20^{\circ}\text{C}$ . The mixture was centrifuged for 10 min at 10 000 xg, the supernatant aspirated and the sample dried in a Speedvac Concentrator and resuspended in TE buffer (1 mM EDTA; 10 mM Tris-HCl, pH 8) containing RNase (50  $\mu$ g/ml).

#### 4.2.13 Southern blot analyses

For Southern hybridization experiments, total genomic DNA (500 ng/ $\mu$ l) was digested with *EcoR*1, *Pst* 1, *Xba* 1, *Hind*III or *Bam*H1 as described in section 4.2.9 and separated on agarose gels (0.7 %; section 4.2.7) for 3 hours. Digested DNA was transferred to a positively charged nylon membrane

(Micron Separations Inc.) using the one-hour downward alkaline capillary transfer described by Chomczynski (1992).

The DIG DNA labeling and detection kit (Boehringer Mannheim) was used for Southern hybridization and detection. Labeling of probes and hybridization detection was performed according to the manufacturers recommendations.

#### **4.2.14 Sequencing of clones obtained**

Sequencing of the fragments was performed using the DYEnamic™ Terminator Cycle Sequencing Premix kit (Amersham Pharmacia Biotech, Inc.) and the ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems). Sequencing reactions were done using the various manufacturers' instructions using the universal T7 and Sp6 primers.

Sequencing was done on an ABI Prism 377 DNA Sequencer (PE Biosystems). The data was analyzed using Sequencing analysis V 3.3. Sequences were reverse complimented and compared using Sequence Navigator V 1.0.1 and assembled using AutoAssembler V 1.4.0 and DNAssist V 1.02. Analyzed sequences were used to search the Genbank Database (<http://www.ncbi.nlm.nih.gov/>).

#### **4.2.15 Testing putative positive clones from a genomic library obtained from Onderstepoort Veterinary Institute**

Clones were obtained from Michelle Goodrum from the Onderstepoort Veterinary Institute (University of Pretoria. A brief summary of work previously done by her entailed that a genomic DNA library was constructed by cloning (section 4.2.10) *EcoR1/BamH1* digested (section 4.2.9) genomic DNA (section 4.2.5) fragments into the pET 5 a/b/c vectors (section 4.2.3). The clones were sent to our laboratory and further experiments was performed as part of this project. Clones displaying agglutination were defined as positive clones, from these clones the 6 best were selected, followed by plasmid

isolations (section 4.2.12) and restriction enzyme analyses (section 4.2.9) with *EcoR1*, *BamH1* or a combination of the two enzymes to estimate the fragment lengths. Before sequencing these clones were cloned (section 4.2.10) into pGEM3Z for a higher plasmid yield, subjected to sequencing (section 4.2.14) and sequences used in BLAST searches performed against the Genbank Database (section 4.2.14).

#### **4.2.16 PCR amplification of the partial haemagglutinin gene from *Pasteurella multocida***

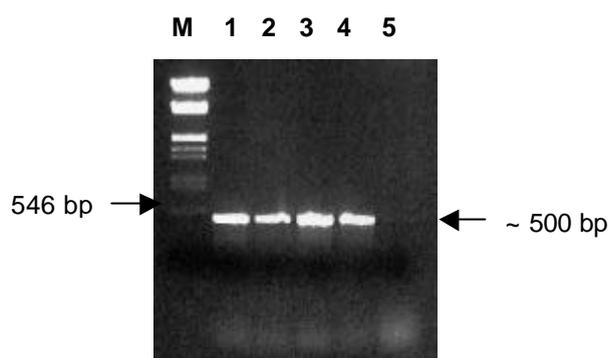
Primers (section 4.2.6) were designed from alignments of nucleotide sequences from the filamentous haemagglutinins from *Pasteurella multocida* and *Haemophilus ducreyi*. PCR's were done as described in section 4.2.6 using genomic DNA isolated from *P. multocida* (section 4.2.5), cloned into pGEMT-easy vectors (section 4.2.10), transformed into competent *E. coli* Top 10 cells (section 4.2.11.2), white colonies grown up and the plasmids isolated (section 4.2.12) followed by the removal of the inserts from the plasmid with *EcoR1* digestions (section 4.2.9) to estimate the size (section 4.2.7). These inserts were sequenced, labeled and used for Southern blots using genomic DNA isolated from *P. multocida* and *H. paragallinarum* serovar A2.

#### **4.2.17 PCR amplification of the partial haemagglutinin gene from *Haemophilus paragallinarum***

PCR's were performed on genomic DNA isolated from *P. multocida*, cloned into pGEMT-easy vectors, transformed into competent *E. coli* Top 10 cells, plasmids were isolated and the inserts removed from the plasmid with *EcoR1* digestions to estimate the size. These insert were sequenced, labeled and used for Southern blots using genomic DNA isolated from *H. paragallinarum* serovar A2.

### 4.3 Results and discussion

The identity of all *H. paragallinarum* strains was confirmed using the *Haemophilus* specific PCR test and the correct fragment length was obtained (Figure 3.6, 4.1). The cells displayed strong HA activity indicating that the haemagglutinin or at least the adhesins were being expressed.



**Figure 4.1:** Agarose gel electrophoresis of the PCR products obtained for the HPG2-PCR. Lane 1 - 3 represents the PCR products for the *H. paragallinarum* strain used with an estimated size of 500 bp. Lane 4 is the positive control and lane 5 the negative control. The marker shown is  $\lambda$  DNA digested with *HindIII/EcoR1* with the 546 bp fragment indicated.

#### 4.3.1 Testing putative positive clones from a genomic library obtained from Onderstepoort Veterinary Institute

The positive clones obtained from Onderstepoort were subjected to haemagglutination tests using the bacteria expressing the cloned genes. Six clones were selected for further testing/characterization. Although the clones did not display a strong HA activity (1:4) it nevertheless differed significantly enough from the controls, indicating that the insert was encoding a protein that contributed to a positive HA test. Plasmids were extracted from the positive clones and the relative lengths estimated (Table 4.3). The inserts from only two of the six clones selected were smaller than the clone (2.57 kbp) described by Takagi and co-workers (1991).

The very low protein expression was problematic for further tests, therefore the fragments were cloned into pGEM3Z plasmids for higher yields, of both protein and DNA, during a shortened growth time.

**Table 4.3:** Classification system and relative sizes for the six positive clones obtained from the Onderstepoort Veterinary Institute

Clone number received	New clone number	Approximate fragment size
5/8	1	2000 bp
3 - 8/2	3	1700 bp
5 - 3/3	5	10 000 bp
6 - 3/7	9	4000 bp
7 - 7/3	21	2900 bp
10 - 9/3	29	2900 bp

Nucleotide sequences obtained from the sequencing of the positive clones, except clone 3, gave no significant or relevant similarities with sequences in the Genbank Database. Clone 3 (Table 4.3) had a relatively high sequence similarity with the flagellar switch protein from *E. coli* (Figure 4.2), something that was not expected, as *H. paragallinarum* is not known as a motile organism (Yamamoto, 1991). The data indicated that the gene obtained codes for a protein with structural similarities to the flagella of *E. coli*. The switch protein is situated in the cytoplasmic membrane, and could function as an anchor for the protein. Another possible sequence similarity indicated that the clone is somewhat similar to a surface polysaccharide antigen, also expressed in *E. coli*. Unfortunately the protein is not well characterized.

These unexpected results prompted a more in-depth search for the haemagglutinin as described by other researchers (Ward *et al.*, 1998). Either the clones obtained from Onderstepoort Veterinary Institute did not contain the genes encoding the specified protein or the protein differed significantly from the conventional described haemagglutinins, therefore a known HAP organism was used as template for the next experimental section.

ecfs	799	CAGCGTCTGTTGCAGGAA	GTGGATTCCGAATCGCTGTT	836
clone 3	1	ATTGTCTGTT-CAGGAATA--	ATTCCGAATCGTTGTT	34
ecfs	837	GATCGCGCTGAAAGGAGCCGAGCAGCCACTGCGCGAGA		874
clone 3	35	GATCG-GCTGAAAGGAGCCGAGCAGCCACTGGGCGAGA		71
ecfs	875	AATTCTTGCGCAATATGTCGCAGCGTGCCGCCGATATT		912
clone 3	72	AATTCTTGCGCAATATGTCGCAGGGTGCCGCCGATATT		109
ecfs	913	CTGCGCGACGATCTCGCCAACCGTGGTCCGGTGCGTCT		950
clone 3	110	CTGGGGGAAGATCTCGCCAACCGTGGTCCGGTGCGTCT		147
ecfs	951	GTCGCAGGTGGAAAACGAACAGAAAGCGATTCTGCTGA		988
clone 3	148	GTCGTAGGTGGAAAAGAACAGAAAGCGATTCTGGTGA		185
ecfs	989	TTGTGCGCCGCCTTGCCGAAACTGGCGAGATGGTAATT		1026
clone 3	186	TTGTGGGCCGGCTTGCCGAAA-TGGGGAGATGGTAATT		222
ecfs	1027	GGCAGCGGCGAGGATACCTATGTCTGAATAATCTGCCG		1064
clone 3	223	GGGAG-GGGGAGGATACCTATGTCTGA-TAATCTGGCG		258
ecfs	1065	TGGAAAACCTGGACGCCGGACGATC		1089
clone 3	259	TGGAAAACCTGGACGCCGGACGATCTCGGGCCACAAAG		296

**Figure 4.2:** Alignment of the sequence obtained from clone 3 with the sequence of the flagellar switch protein of *E. coli* (ecfs).

### 4.3.2 PCR amplification of the partial haemagglutinin gene from *Pasteurella multocida*

The nucleotide sequences from filamentous haemagglutinin from other organisms (section 4.1) indicates that the genes are usually larger than 7 000 bp and that low nucleotide sequence similarities occur between the genes of different organisms. If the positive clone product could be amplified one could do Southern blots analysis for the identification and possibly the cloning of the complete open reading frame. The filamentous haemagglutinins from various organisms in the *Pasteurellaceae* family were aligned to find conserved regions within these genes, the best alignment were used to identify three conserved regions between the sequences of the *P. multocida* and *H. ducreyi* haemagglutinins (Figure 4.3). Primers were designed (section 4.2.6) that would yield an expected product of about a 1000 bp.



```

pmfh 483 -----LVRLGE---RDRONREKRE--KE-----GYFDLPGTLDMKLQELFEKRKQ-----HEAEQKARIEKALLQ-----KS 540
hdfh 1089 IKGKFADLDNKLTVQRIGKIYAGRDLTFNKSNAGGKSEIINRGTINVKNKLSYSDSDVSFENMQSQKVDLYTKIFEAKSDIELTFKINGTHPVYLNFKS 1187

pmfh 541 EQQEK--RVEERKQ-----EKKRQ-----AQDKLAKQVEIAKEMQRVEE 577
hdfh 1188 NNNEKKYRNSENTKNFKSIGDLNEALSASPEAIEAYYSGSSSNYINPVSYLAALGNANSSNPHYLNTALKHILGNGWQDDLKKQENIKVLKQKWED 1286

pmfh 578 IRQ-----REKQLAIQLQE--EKKQQEEKHLS-----EKKQAEQKQAEKVAQERLDIEQQKAYEEMA 636
hdfh 1287 FKKDKGASKMLDLYPNTDKEKAKIFAGIIRNGNDTISDVESEDFKKKYSKFQNGEWAKNDTGTDSYDSTKASEKYKKVENVDHKENIDEHKLNI GKHEI 1385

pmfh 637 KREA EASKNVLLKAIDEE-----R--P-----KVETDPLFRTKLKYINQDDYAGANYFFNKVGLNTKGHQKINVL 699
hdfh 1386 TVPGVSVFENLNKNMMDHQPKLGEIDKSIISELLAQPVYTEKSAARSDSDPRVNQNDKEALDNLVYRTRLSYINQNNYLGAKYFFNQLDTEDDKLGKIKRI 1484

pmfh 700 GDNYFDHQVITRSIEKKVDNHLNQKYNLSDVELVKQLMDNSTTQAQELDLKLGALTKEQQANLTQDIVVYVKTKVKGKDVFPKVFYFASETLVEAQKL 798
hdfh 1485 GDNYFEHQVITRSLIEKVDNHLNLKHLGHDIALVKKLIDSASIQAKDLNLKVGREALTKEQKDNLKEQDIVVYVKTEVNGQEVLPQVYLAKQTIIEVEKQ 1583

pmfh 799 QGLGTGTRVGEAKIKAKDVTNTGTLGRKLNVEASNKIKNQSILSTQETRLVGRKGIENVSRSFANDELGVTAQRSEIKTEGHLHLETDKSDTIDVQ 897
hdfh 1584 RGVGTGQIRAGIIDVKVDDVRNTGTIAGYAVGLEAKNKLKNTGDILSQRSLKLVGKKGLESTGVITYVDETGATKVRKARIKSEGHYILETDKDKNVDLT 1682

pmfh 898 ASDIKAKTSFVKTGDVNLKNTYNTKHAYREKFSPSALQVAELDVAGLKVPLLVGSSPSSYSEHTSEATSEGSIFEVGHLHLAVDRDVNQAGSKIKAKYIT 996
hdfh 1683 ASELKNGTQIKAKDLNLDIYETSYKY--KYEK-----LFGKNGGEIGDR-----VTQTSQAKSVGTDASF DHLHLSLEGDVNQTSNLIKANRT 1765

pmfh 997 TGVVKGNFNTEAGKNIKHVEKEEYSSQLFASAHASGGGTSVRYDYNQDGGNASVGVPTNHTGVGAEAGMSFTHTKDKETLLTHTNSELOVKHGKLVHL 1095
hdfh 1766 TGVVKGDFNTKAGKDLFHRQIDTVISGTVYSASASGGGQSAGISLTI--DQVETIYTNKTATAGANADVTFMFKRTRETETSLTHRNFNALSSELGYVM 1862

pmfh 1096 GYADIGGVDIN-----TKLPEDAQSKAQKEIAASKPEK-----TEQSAQDVAQAQSNANKDKENKAPEIKELSEAEIADLMSEKSKAYFDDFAEQAKKA 1184
hdfh 1863 GKADIGGVDINRDVEVIKTPPEEIAAEQKAAEEAKKAEVKENEASETAAKETEEAENDNVAEKDKTKPKFKKLTDEEIAAAFETKGEDFFAAYKAREEED 1961

pmfh 1185 PENNRFELSAKEIKSSKQKDQYDHESERTTFKVGPEAEAHSAVADMVSHLVKEYRDAQNGTKQDGTVALQHASDVLNIVTGDLAGSSAKLSVERTHETK 1283
hdfh 1962 RKKG-FTLSAEQIESTKARDEKETTYEYELKVGVGAEAEAHSAADAISNKARQIIDTQNGLKQDGTVALQEASDVLNLATGDLAGASAKLKFELSTIEK 2059

pmfh 1284 RTTETGDIVTKIGGNVTLARSQSVNLKNVQSDEQANLTLRAKEDVNVLSGEKTRETTETVSRQKLSHGVAAGCSMMSGACTAGVSTSLGNEYSYTSER 1382
hdfh 2060 KSRGASDGRSILGGRNLNLAARGDITLNNVETTENSHLSLKARDNVNVNSGVTEQKDESNSQLKVTAGASSGCGVMAGGCSAGVSAGVSGSYNESNTE 2158

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Figure 4.3: Continued

pmfh	1383	ET AQNNSFLKARNMKVEAGRDFNVVSSNIDADKLDLHVKGKTNVSKQDTLQKVTGVDYDNL SAGVALSSAT IATPTGNVGFYINETESKRTV NQQAG	1481
hdfh	2159	STSH TNSLLRGKSLRVEAGKDFNLISSNVDDHLHLVDVKGDTNVVSKQDSY SRKERGVNYSV SAGVGVSTAGGARPN GSVGLGVS AENENSKIIVKQQAG	2257
pmfh	1482	IKANKITGQTHDLNLEGGYLVSNDKDNQLKVTGDVTTKALHDQHDKGGTFGLSVG I SERGTTAFNVRGGRAEQKHYNATQKSTLSGVDTSQANVSGQV	1580
hdfh	2258	ISAKRITGEINNLNLTGGYIENKGNPDELNVKGDITTHELKDDEH HKDGGSF GGSVGVSETGVTQVNVNGGRVEQKH YEATQHSSISGINT-KGKTVGNF	2355
pmfh	1581	NTDLTKAKAVTRDDTYASTQFSFEVADIVELGQRAKNKLS-----APNN-----DTDMASGS-----	1632
hdfh	2356	KTDRSQSTEVHRDDTIAATNFNFELGDIAELAKKGKEKWDNRSAKTTSSSQSAHDPRSRSVENGYSELPRFKTADNDAGVDSPRLIKGEAEQAQTLAL	2454
pmfh	1633	-----TLRSR-----STTEADVPTTTRSRVTD-----EADS-	1658
hdfh	2455	TKAGNDVIPEVQSLTQKARQSLVDESPYAEIPALTRPQVKSNI AESIEV PQFR TKVSDGDEGNYAEITFPTNKAAISS TQDVGDTPTPRALRLESESG	2553
pmfh	1659	-----VS VKNPIYESADAVVPTPRSRNVDS-----	1683
hdfh	2554	YESAENLGLIPRGFKSSPKGEYEDISDALEPQTRSRKLDPEPEIYGTINKSPEAIARANAKADEAIQALGYDPRIKPVVPEEAPPALPPRN LQTKAISD	2652
pmfh	1684	-----TD-----LVDN---PLYASATTKANIH DYEEIP---	1708
hdfh	2653	YDDVSYVPDFKVRKTDPEPEIYGTINKSPEAIARANAKADEAIQASGYDPRIKPVVPEDAPPALPPRTQSLIDSTEVS YRSALANVKFDDASPWPQPS	2751
pmfh	1709	AVYSKVGDN-----ADLVRHKTATSDEHLYAEIN-----EPTYSRVGDKNAD-----MRRHNAAGT TDYADVQAHT---	1771
hdfh	2752	ALRSKAFAD E P S S E T P K S R G K R G I S E E S L S S T V Q P R S R K I S E E D S S F E R L P L R I I D N G S D Y A E I L P R N V K Q T N E P A T Q A I R A P K A L D N D V I A E R P S F K	2850
pmfh	1772	-RKADD-----PLPALPN-----Q GK-	1786
hdfh	2851	LRQLDD D V E S V N G I Y S S I K P K A L I E E G T P I T R Q V K T V Q E E T P V T D L V N K R E L V K E D R S L L D K V Q D T F Q P L K V R S K I N D V R S S V E E Y G G E V T F K Y A Q S K G	2949
pmfh	1787	-----ARTVNDGSEHIYTDIS-----	1802
hdfh	2950	EVYNEIVKHAETQNGVCEATCSHWIAKKVND--ENIWTDL YKDGQKGRKGGLNKDAIESIEKLQTEFINAGTATQQFKLTNTWLEEQGVV PKQKYFGKL	3046
pmfh	1803	-----DVG TQT KAIDSTYATVGM PKANAVNLIG-----Q-----NGLG-----	1835
hdfh	3047	SRADEVAGTVSKNDVSALVKAILDIGNESSAVKKISINLEGG SHTV SASIEGQKVVFFDPNFG EITFKDKKSFEKWMKN AFWKKS GYAGKKDTKRFFNV	3145
pmfh	1836	SIYHS-----PD-----SAYKTWQLLDQFANK--G-----G-----DAVFL	1864
hdfh	3146	VNYHKNSKRNKVIDVNQNHIIQQLAGSEGFSPSLPTRPQLANAAGIKSNEMS SLF SWSK LKHLFSRESGKKAQVEGPEIKHLGGVVDKDAFYFPLDKIVT	3244

Figure 4.3: Continued

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pmfh 1865 R--PATEMKCAGAPLKYYTFIVRDYLLRRHTLDKSRLFYN-----AHNKTLF 1908
hdfh 3245 RRDAEGEIRVNMDNIKKAFNPRDKHYNSQEARSLRSLYNQDPSMSGTRFIIENQVIANPFSSADLQSYIQAQQSKLPELGRQARRALPELPTAANKGRC 3343

pmfh 1909 S-----VPIVD----- 1914
hdfh 3344 SRVEEQNIVTRPRVEDVYATVNKGAKHGAEQQPGSFYTKKLVQVSHVPNTEPVYADLHFNRNGRVVRQTEPEVIYEKIRGQQVEVDDPSSLYAKVNRN 3442

pmfh 1915 -----AKVKMLF----- 1921
hdfh 3443 RRLDNVEGFYPPEQLRTRSDKLAEQVSRVPTTEPVYADLRFKSAEDDYAPALPARPELGNAAGFRKAKVKGEESESTWSRLKHLFSRESGKKAQVEGPE 3541

pmfh 1922 -----AEKNIQVNYD-----RSLTAIDLKRLATFNSPEGVVEVPYDFINVPVPPRA-----P 1969
hdfh 3542 IKHLGGVVDKDAFYFPLDKIVTRRDAEGEIRVNMDNIKKAFNPRDKHYNSQEARSLRSLYNQDPSMSGTRFIIENQVIANPFSSADLQSYIQAQQSKLP 3640

pmfh 1970 DAVRQSALAWQEG-KWANDG----- 1988
hdfh 3641 ELGRQARRALPELPTAANKGRGSRVEEQNIVTRPRVEDVYATVNKGAKHGAEQQPGSFYTKKLVQVSHVPNTEPVYADLHFNRNGRVVRQTEPEVIYE 3739

pmfh 1989 -----WVEVEKHT-----LRHRRYANVF-----AVGDVAGVPKGGKTAASVKWQV 2027
hdfh 3740 KIRGQQVEVDDPSSLYAKVNRNRRLDNVEGFYPPEQLRTRSDKLAEQVSRVPTTEPVYADLRFKSAEDDYAPALPARPELGNAAGFRKAKVKGEESEST 3838

pmfh 2028 PVAVAHLLAELEGK-----PCDEIYNG-----YTSCP----- 2054
hdfh 3839 WSRLKHLFSRESGKKAQVEGPEIKHLGGVVDKDAFYFPLDKIVTRRDAEGEIRVNMDNIKKAFNPRDKHYNSQEARSLRSLYNQDPSMSGTRFIIENQV 3937

pmfh 2055 -----LITQLGKG-----ML 2064
hdfh 3938 IANPFSSADLQSYIQAQQSKLPELGRQARRALPELPTAANKGRGSRVEEQNIVTRPRVEDVYATVNKGAKHGAEQQPGSFYTKKLVQVSHVPNTEPVY 4036

pmfh 2065 VEFDYN-----NHLTPSFPGVIAP----- 2083
hdfh 4037 ADLHFNRNGRVVRQTEPEVIYEKIRGQQVEVDDPSSLYAKVNRNRRLDNVEGFYPPEQLRTRSDKLAEQVSRVPTTEPVYADLRFKSAEDDYAPALPAR 4135

pmfh 2084 -----LEELWATWA-----IKTLGLKPT-----YLGMLRG----- 2108
hdfh 4136 PELGNAAGFRKAKVKGEESESTWSRLKHLFSRESGKTKVEEVENEYKSQTNGENSLEIKTLEHIPTRLKNLESPREAQSDLGENALIYGLQRGRQALIS 4234

pmfh 2109 -----LA----- 2110
hdfh 4235 KANAADKEGKNAILLADSYIGKLNLFEGFELTKFAKQVKDGKVTEQDIQNIASFNDETAKLARRSEPKNRINDANVDDNQRRIRELINNEAAVDALKRI 4333

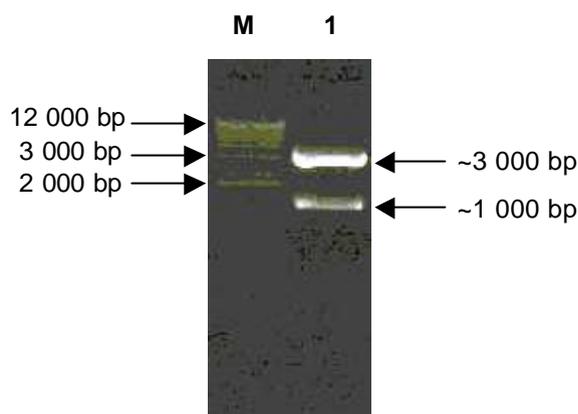
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Figure 4.3: Continued

pmfh	2111		2110
hdfh	4334	ATLSDQEKAMHSTLRANEKFDMDLEESPNTTAENKSIRDYKDTQKALNDARMDFFTEKTKFIAKETLERGGQLYFALDGLVTNSPGFRADTQINMDK	4432
pmfh	2111		2110
hdfh	4433	LKDVFNPNHEHYDSVTSRELRYLYENYKDNPNLKF <sup>T</sup> TLKDHVIANPLK <sup>T</sup> TLK <sup>T</sup> SISEDLKSSPRRARQEGPSLLQVRNLF <sup>T</sup> DKSSSNKRSEKDTAQT <sup>T</sup> SVG	4531
pmfh	2111		2110
hdfh	4532	YRNTNIDIINDKTKGVNHIVENGVEVALTNTDYKPLKLPNVEAAFQ <sup>T</sup> TKLKAENIDPHIEAVK <sup>T</sup> LEIIASSANSIPKEHLLKALIEVTEGKTDEDIN <sup>T</sup> VY	4630
pmfh	2111		2110
hdfh	4631	QKLFNTRQ <sup>N</sup> ISNEVAPT <sup>T</sup> YSLRNLDGKDGKQILRSVAE <sup>T</sup> IYKNLPLSD <sup>T</sup> YQAVRNYVNNRLIEKLSSNRLLLEHLANSKISGNEYA <sup>T</sup> IKYIFD <sup>T</sup> VSRAKQEI	4729
pmfh	2111		2110
hdfh	4730	FEQELNTE <sup>L</sup> APVSLDIMRRKAANILSSEHGSYKDG <sup>T</sup> LSIYDKPVKSAFHSRLKNNGEVLNTIVHELTHHEQDALAKIIDNKGYDAK <sup>L</sup> FDKNNILYITGG	4828
pmfh	2111		2110
hdfh	4829	LGYPKQALERDAFLSGDSVSEAFMKKAKEYHERTKQERKDAKKDEARIAKLYKQWEQEEANKKSASLNGSSQSLDSRSEVEFNRVSHKSVR	4919

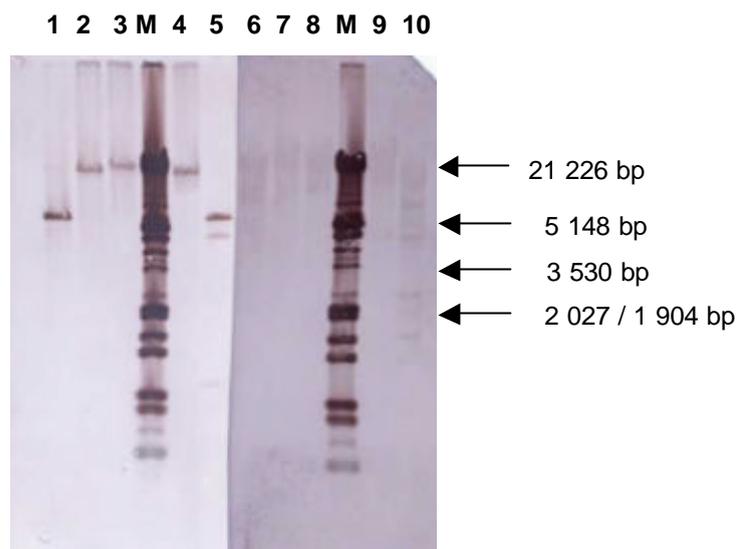
**Figure 4.3:** Continued

The PCR reactions yielded the correct product corresponding to the predicted sizes that was obtained after cloning the product into the pGEMT-easy vector and the plasmid containing the insert digested with *Eco*R1 (Figure 4.4).



**Figure 4.4:** Ethidium bromide stained agarose gel of the PCR product cloned into pGEMT-easy. In lane 1 is the 1 000 bp insert along with the 3 000 bp vector backbone. The marker used (1 kb Plus molecular weight marker) is indicated by the M.

The insert was sequenced and showed a high homology with the haemagglutinin from *P. multocida* (Figure 4.5). The southern blots (Figure 4.6) were done with this probe against restriction enzyme digested genomic DNA from both *P. multocida* and as duplicate against the DNA from *H. paragallinarum*.



**Figure 4.6:** Southern blot hybridization of the confirmed probe with the genomic DNA from *P. multocida* (lanes 1 - 5) *H. paragallinarum* (lanes 6 - 10). M indicates the  $\lambda$  DNA marker digested with *HindIII/EcoR1*; lanes 1 and 6 represents genomic DNA digested with *EcoR1*, lanes 2 and 7 is genomic DNA digested with *Pst1*, lanes 3 and 8 is genomic DNA digested with *Xba1*, lanes 4 and 9 is genomic DNA digested with *BamH1* and lanes 5 and 10 represents genomic DNA digested with *HindIII*.

The southern blots using the genomic DNA from *P. multocida* (Figure 4.6) gave strong hybridizations in all the lanes, as one would expect. The probe did however not hybridize with any of the genomic DNA from *H. paragallinarum*, even after the membrane was stripped and re-incubated at room temperature (normally done at 65°C) and washed with solutions containing low salt concentrations (usually very high concentrations are used), thus creating less stringent hybridization and washing conditions which should allow for hybridization between DNA with lower similarities. The results confirmed the protein results that the *H. paragallinarum* haemagglutinin differed from that of the well-characterized *P. multocida*, the initial results in section 4.3.1 started to reiterate the characteristics of this adhesive protein. It was decided to use these primers on the *H. paragallinarum* genomic DNA as template PCR to amplify the putative haemagglutinin.

A)

***P. multocida* forward primer**

PMFH	1	GCCGATATTGGTGGAGTAGATATTAATACTAAACTACCAGAAGATGCACAAAGCAAAGCACAGAAAGAGATAGCGGCTAGCAAGCCAGAGAAGACCGAG	99
PMF	1	GCCGATATTGGTGGAGTAGCTATTAATACTAAACTACCAGAAGATGCACAAAGCAAAGCACAGAAAGAGATAGCGGCTAGCAAGCCAGAGAAGACCGAG	99
PMFH	100	CAATCTGCACAGGATGTGGCTCAAGCTCAATCAAATGCCAATAAGGATAAGGAAAATAAAGCCCCAGAAATAAAAGAATTATCAGAGGCTGAAATCGCG	198
PMF	100	CAATCTGCACAGGATGTGGCTCAAGCTCAATCAAATGCCAATAAGGATAAGGAAAATAAAGCCCCAGAAATAAAAGAATTATCAGAGGCTGAAATCGCG	198
PMFH	199	GATCTCATGTCAGAAAAATCAAAGCGTATTTTGTATGATTTTGCAGAGCAAGCGAAAAAAGCACCTGAAAACAATCGATTTGAATTGTCTGCCGAAAGAA	297
PMF	199	GATCTCATGTCAGAAAAATCAAAGCGTATTTTGTATGATTTTGCAGAGCAAGCGAAAAAAGCACCTGAAAACAATCGATTTGAATTGTCTGCCGAAAGAA	297
PMFH	298	ATTAAGTCGAGCAAACAAAAAGACCAATATGATCATGAGTCTGAACGGACGACTTTTAAAGTTGGACCTGAAGCGGAGGCTCATTCTG-CGGTTGCCGA	395
PMF	298	ATTAAGTCGAGCAAACAAAAAGACCAATATGATCATGAGTCTGAACGGACGACTTTTAAAGTTGGACCTGAAGCGGAGGCTTATTCTGGCGTTGCCGA	396
PMFH	396	TATGGTGAGCCATTTAGTGAAAGAATATAGAGATGCACAAAATGGGACTAAACAAGACGGTACAGTAGCATTACAACATGCTAGTGATGTCTTAAATAT	494
PMF	397	TATGGGGAG	405

**Figure 4.5:** Alignments of the (A) forward sequence (PMF) and (B) reverse sequence of the 1 000 bp probe from *P. multocida* with the sequence of the filamentous haemagglutinin from *P. multocida* (PMFH). Black lines indicates the primer sequences.

**B)**

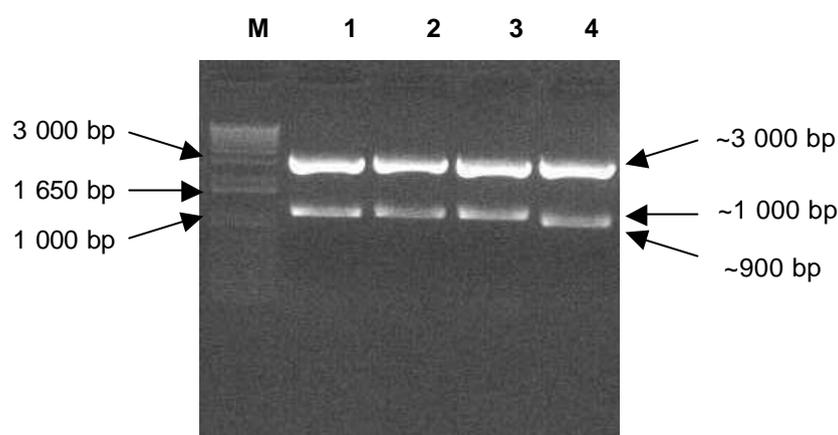
PMR	1		ACATGCTAGTGATGTCTTAAATATT	25
PMFH	397	ATGGTGAGCCATTTAGTGAAAGAATATAGAGATGCACAAAATGGGACTAAACAAGACGGTACAGTAGCATTACA	ACATGCTAGTGATGTCTTAAATATT	495
PMR	26	GTGACGGGGGATTTAAGCGGGGAGTTCAGCTAAATTGTCTGTTGAAAGAACACATTGACCAAAACCGAACGACAGAA	-CGGGGGATATTGTTACTAAG	123
PMFH	496	GTGACGGGGG-ATTTA-GCGGGGAGTTCAGCTAAATTGTCTGTTGAAAGAACACAT-GAGACAAAACCGAACGACAGAAACGGGGGATATTGTTACTAAG		591
PMR	124	ATTGGTGGCAATGTCACACTGTCAGCACGCAGTGGTAGTGTGAACCTTAAAAATGTACAAAGTGATGAACAAGCTAATTTGACCTTAAGAGCAAAGAA		222
PMFH	592	ATTGGTGGCAATGTCACACTGTCAGCACGCAGTGGTAGTGTGAACCTTAAAAATGTACAAAGTGATGAACAAGCTAATTTGACCTTAAGAGCAAAGAA		690
PMR	223	GATGTGAATGTGCTGTCTGGTGAAAAAACACGAGAAACCACAGAAACAGTATCAAGACAGAAACTTTCTCATGGTGTGAACGCAGGTTGCAGTATGATG		321
PMFH	691	GATGTGAATGTGCTGTCTGGTGAAAAAACACGAGAAACCACAGAAACAGTATCAAGACAGAAACTTTCTCATGGTGTGAACGCAGGTTGCAGTATGATG		789
PMR	322	AGTGGCGCCTGTACTGCCGGTGTTAGCACATCACTTGAAGGAAATGAAAGCTATACGTCAGAACGTGAAACGGCTCAAAAATAACAGTTTCTTAAAAGCA		420
PMFH	790	AGTGGCGCCTGTACTGCCGGTGTTAGCACATCACTTGAAGGAAATGAAAGCTATACGTCAGAACGTGAAACGGCTCAAAAATAACAGTTTCTTAAAAGCA		888
PMR	421	CGCAACATGAAAGTTGAAGCAGGTCGCGATTTTAATGTTGTCAGTTCGAATATTGATGCAGATAAGCTCGATCTCCACGTTAAAGGAAAAACGAATGTG		519
PMFH	889	CGCAACATGAAAGTTGAAGCAGGTCGCGATTTTAATGTTGTCAGTTCGAATATTGATGCAGATAAGCTCGATCTCCACGTTAAAGGAAAAACGAATGTG		987
PMR	520	GTATCCAAACAAGATA	535	
PMFH	988	GTATCCAAACAAGATA	1003	

***P. multocida* reverse primer**

**Figure 4.5:** Continued

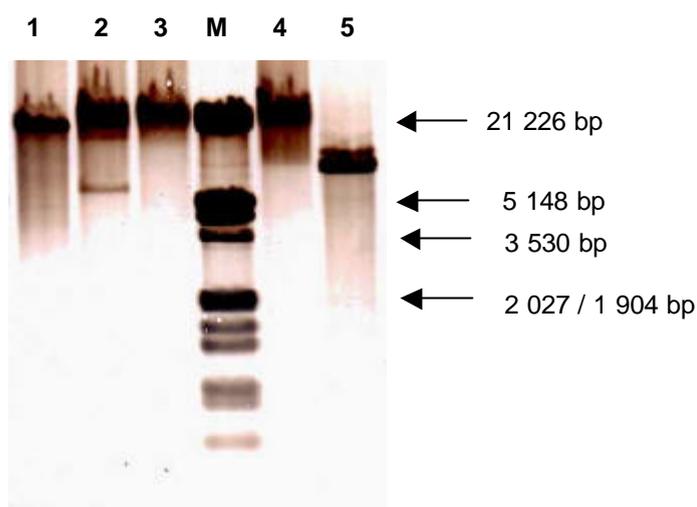
### 4.3.3 PCR amplification of the partial haemagglutinin gene from *H. paragallinarum*

The PCR was not optimally effective using the genomic DNA of *H. paragallinarum* and the procedure was modified to the so-called "touch down" PCR, where 5 cycles were done as described in section 4.2.6 with the annealing temperature changed to 45°C, followed by 20 cycles with an annealing temperature of 50°C.

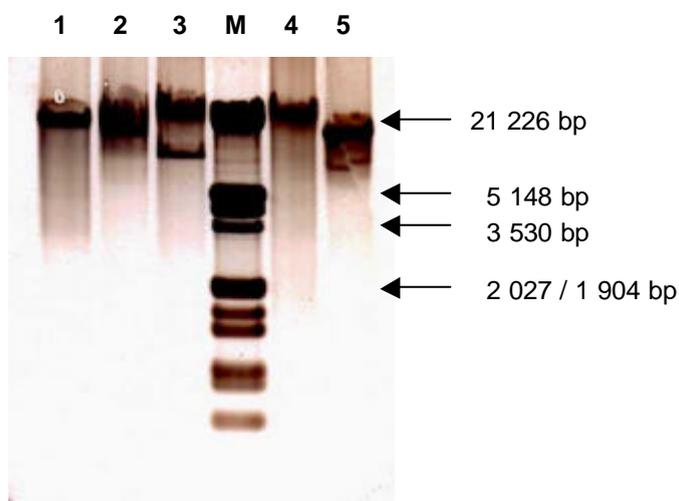


**Figure 4.7:** Ethidium bromide stained agarose gel electrophoresis of the PCR products obtained using the PM-F and PM-R primers on *H. paragallinarum* genomic DNA after cloning into the pGEMT-easy plasmid. Lane 1 - 3 is the 1 000 bp PCR insert along with the 3 000 bp plasmid backbone and lane 4 the 900 bp PCR insert along with the 3 000 bp plasmid backbone. The marker used (1 kb Plus molecular weight marker) is indicated by M.

The products were analyzed on an ethidium bromide stained agarose gel and the size corresponded well with the predicted size of ~ 1 000 bp. After the PCR product was cloned into pGEMT-easy analyzed using restriction enzyme digestions with *EcoR1* indicated two fragments with similar lengths (Figure 4.7) and was termed the 1000 bp and 900 bp insert. These fragments were used as probes in Southern blot analysis to select for the putative haemagglutinin gene(s). The southern blots (Figure 4.8) with the 1 000 bp probes showed hybridization with all 5 digested genomic DNA samples, but the hybridizing sample chosen for further studies was that with approximately 6000 bp with the *Xba1* digested genomic DNA. Another strong signal from



**Figure 4.8:** Southern blot hybridization of the 1 000 bp probe with the genomic DNA from *H. paragallinarum*. M indicates the  $\lambda$  DNA marker digested with *HindIII/EcoR1*; lane 1 represents the genomic DNA digested with *EcoR1*, lane 2 the genomic DNA digested with *Xba1*, lane 3 the genomic DNA digested with *Pst1*, lane 4 the genomic DNA digested with *BamH1* and lane 5 the genomic DNA digested with *HindIII*.



**Figure 4.9:** Southern blot hybridization of the 900 bp probe with the genomic DNA from *H. paragallinarum*. M indicates the  $\lambda$  DNA marker digested with *HindIII/EcoR1*; in lane 1 represents the genomic DNA digested with *EcoR1*, lane 2 the genomic DNA digested with *Xba1*, lane 3 the genomic DNA digested with *Pst1*, lane 4 the genomic DNA digested with *BamH1* and lane 5 the genomic DNA digested with *HindIII*.

The *Hind*III digested DNA indicated a fragment of about 8000 bp. The rest of the hybridizations were with DNA fragments of approximately 18 000 bp.

The second probe (Figure 4.9) also showed hybridization with the DNA in all the lanes, and the hybridizations of interests were the *Pst*I and *Hind*III digested DNA fragments of approximately 9 000 - 10 000 bp.

The probes were sequenced before continuing with any experiments. The sequencing results obtained gave poor alignment (Figure 4.10 A and B) with the DNA gene selections from which the primers was designed and sequence similarities with gene sequences deposited in the Genbank Database correlated with putative proteins from *H. influenza* and *P. multocida*,

Clearly, considerable variation exists in the primary structure of the haemagglutinins even from these closely related species the probes prepared from specific organisms hybridized only with the DNA of that species. This makes comparative studies rather difficult. The question remains if the *H. paragallinarum* "haemagglutinin" detected by Iritani *et al.* (1980b) falls within the class of "classic" haemagglutinins or whether it is some other protein related to pathogenicity of the organism.

**A)**

PMFH	1		
1000bp	1	GCCGATATTGGTGGAGTAGATATTAATACTAACTACCAGAAGATGCACAAAGCAAAGCACAGAAAGAGATAGCGGCTAGCAAGCCAGAGAAGACCG	97
		GCCGATATTGGTGGAGTAGATATTAACGCTAT-----AG-----AGA----AAAGCAATGATATAGAAAAAGTGAAGTGCGGTGAAAAATATAG	80
PMFH	98	AGCAATCTGCACAGGATGTGGCTCAAGCTCAATCAAATGCCAATAAGGATAAGGAAAAATAAAGCCCCAGAAATAAAAGAATTATCAGAGGCTGAAA-	193
1000bp	81	TT-ATTTTTTACCAGCACTTTTTAGGAGTGCAA---AATGC-----AGAATGATTTCTGGGGATCAATATTATTTTCCATTAGTGTAAAC	159
PMFH	194	-TCGCGGATCTCATGTCAGAAAAATCAAAGCGTATTTTTGATGATTTTGCAGA-GCAAGCGAAAAAGCACCTGAAAACAATCGATTTGAATTGTCT	288
1000bp	160	GTTACCCACTTTATTTTTGTTAATTCTTG-GAATATTTTTGCGAAAACGTAACCGTTATTGATGATAGGTTTAGTCAGCAGGCTACGGGAATCGTGT	255
PMFH	289	GCGAAAGAAATTAAGTCGAGCAAACAAAAGACCAATATGATCATGAGTCTGAACGGACGACTTTTAAAGTTGGACCTGAAGCGGAGGCTCATTCTG	385
1000bp	256	TCAAATACCTTACCTGCGTTATTATT-----TTTGAATGTTTTTCAGAAC--CCGA-TTAAGCACTTAAATGAGCAGTTAATGATGAT-CTG	340
PMFH	386	CGGTTGCCGATATGGTGAGCCATTTAGTGAAAGAATATAGAGATGCACAAAATGGGACTAAACAAGACGGTACAGTAGCATTACAACATGCTAGTGA	482
1000bp	341	GGTATGTGTATTCCGCA---CCGTTATTACTTTTCTCTAGCTG--AAATTTTTGCAGAAAAATTTGTGGCAGATAAAAAGAGAGCGTGGTACTTT	431
PMFH	483	TGTCCTAAATATTGTGACGGGGGATTTAGCGGGGAGTTTCTAGCTAAATTTGCTGTTGAAAGAACACATGAGACAAAACGAACGACAGAAACGGGGGAT	579
1000bp	432	TGTC--AAGCGTGTATCGTGAAATT-GCAGT-ATTTTAGGTC--TTGCTTTTTGCCTGAATGCCTATGGTGAGGC-AGCATTAGTTCCCAGCACC	521
PMFH	580	ATTGTTACTAAGATTGGTGGCAATGTCACACTGTCAGCACGCAGTGGT-AGTGTGAACCTTAAAAATGTACAAAGTGATGAACAAGCTAAT-TTGAC	674
1000bp	522	ATTTATGCCGCGACCACAG-CAATAGCATATAATATACTCGGAGTAATTACTTTGAGC---AGATCCTTATCTAATG--GCAAAAGTAAATGTTCAA	612
PMFH	675	CTTAAGAGCAAAGAAGATGTGAATGTGCTGTCTGGTGAAAAAACACGAGAAACCACAGAAACAGTATCAAGACAGAACTTTCTCATGGTGTGAAC	771
1000bp	613	CAAATGATCATAA-ATGGTG	631
PMFH	772	GCAGGTTGCAGTATGATGAGTGGCGCCTGTACTGCCGGTGTTAGCACATCACTTGAAGGAAATGAAAGCTATACGTGAGAACGTGAAACGGCTCAAA	868
1000bp	632		631
PMFH	869	ATAACAGTTTCTTAAAAGCACGCAACATGAAAGTTGAAGCAGGTCGCGATTTTAAATGTTGTGAGTTTGAATATTTGATGCAGATAAGCTCGATCTCCA	965
1000bp	632		631
PMFH	966	CGTTAAAGGAAAAACGAATGTGGTATCCAAACAAGATA	1003
1000bp	632		631

**Figure 4.10:** Alignments of the 1 000 bp (A) and 900 bp (B) sequences obtained with the expected 1 000 bp sequence of the filamentous haemagglutinin of *P. multocida* (PMFH). The *P. multocida* forward primer is indicated by the black line.

**B)**

PMFH	1	GCCGATATTGGTGGAGTAGATATTAATACT--AAACTACCAGAAGATGCACAAAGCAAAGCACAGAAAGAGATAGCGGCTAGCAAGCCAGA--GAAGA	94
900bp	1	GCCGATATTGGTGGAGTAGATATTAACCGGTGGAACGTGGCACAACAGGGGCG----AAATTAGCGAAGCTGTTGGAG-CAAGAAAAGCTGATTGATGA	93
PMFH	95	C-CGAGCAATCTGCACAGGATGTGGCTCAAGCTCAATCAAATGCCAATAAGGATAAGGAAAATAAAGCCCCAGAAATAAAAGAATTATCAGAGGCTGA	191
900bp	94	TGCCACCTATTTGCCGTGGTTGT---TAAAGCCCAAGC-----CCGA---GTAAACAAAATCAAGGCGGGACCTATTCCTTGGAGGTGTGAAGA	179
PMFH	192	AA-TCGCGGATCTCATGTCAG----AAAAATCAAAGCGTATTTTGA-TGATTTTGCAGAGCAAGCGAAAAAGCACCTGAAAAAATCGATTTGAAT	283
900bp	180	CAGTGGCGGATTTGTTGCAACGTTTAAACAGCGGTAAAGAAGTGCAACTGAACGTGC-GTTTTTTAGAGGGAGATACCTTCAAACCTGGCGTA-AAA	275
PMFH	284	TGTCTGCGAAAGAAATTAAGTCGAGCAAACAAAAGACCAATATGATCATGAGTCTGAACGGACGACTTTTAAAGT--TGGACCTGAAGCGGAGGCTC	379
900bp	276	CGCTGGCGAAAGCCCCTTATTTGC---AACAACT-----TTGGCGGATAAAACGGAAGAAGAAATTTATCAATTATTGGATTTG--CCGTGG---	359
PMFH	380	ATTCTGCGGTTGCCGAT-ATGGTGAGCCATTTAGTGAAGAATATAGAGATGCACAAAATGGGACTAAACAAGACGGT----ACAGTAGCATTACAAC	472
900bp	360	ATTTACCGCATAAAAATTGAAGGCTGGCTGTATCTGACACT-TATAGTTACACACCAAAATCGAGCGATCTGG-CGTTGTTGAAACGAGCGGCAGATC	455
PMFH	473	ATGCTAGTGATGTCTTAAATATTGTGACGGGGGATTTAGCGGGGAGTTCAGCTAAATTGTCTGTTGAAAGAACACATGAGACAAAACGAACGACAGAA	570
900bp	456	GTATGAGAGCCTTATTAGAAAA---AACGTGGG---CAGCGCGGAT---GCTAATTTGCCCGTAAAAATGCCT	520
PMFH	571	ACGGGGGATATTGTTACTAAGATTGGTGGCAATGTCACACTGTCAGCACGCAGTGGTAGTGTGAACCTTAAAAATGTACAAAGTGATGAACAAGCTAA	668
900bp	521		520
PMFH	669	TTTGACCTTAAGAGCAAAGAAGATGTGAATGTGCTGTCTGGTGAAAAAACACGAGAAACCACAGAAACAGTATCAAGACAGAACTTTCTCATGGTG	766
900bp	521		520
PMFH	767	TGAACGCAGGTTGCAGTATGATGAGTGGCGCCTGTACTGCCGGTGTTAGCACATCACTTGAAGGAAATGAAAGCTATACGTCAGAACGTGAAACGGCT	864
900bp	521		520
PMFH	865	CAAATAACAGTTTCTTAAAAGCACGCAACATGAAAGTTGAAGCAGGTCGCGATTTTAAATGTTGTGAGTTCGAATATTGATGCAGATAAGCTCGATCT	962
900bp	521		520
PMFH	963	CCACGTTAAAGGAAAAACGAATGTGGTATCCAACAAGATA	1003
900bp	521		520

Figure 4.10: Continued

#### 4.4 Conclusions

The data obtained in the attempted purification and identification of the *H. paragallinarum* haemagglutinin indicates that a protein similar to the haemagglutinin from *H. influenza*, or that from *Mycoplasma gallisepticum*, a protein of about 67 kDa (Markham *et al.*, 1993), or the Haemagglutinin/Protease from *Vibrio cholera* (Booth *et al.*, 1984).

Clearly, the variety of proteins that might be involved with pathogenicity and the phenomenon of haemagglutination observed *in vitro*, makes this a complex issue. The molecular data, although not conclusive, also seems to point in this direction. What has emerged from both approaches however is that the adhesins may be a crucial target for further study. These proteins could be important targets to attenuate the pathogenicity of the infectious organism as far as developing vaccines with better cross protection. One is also tempted to speculate whether this might not partially be the reason for the current confusion in the system of classification of these organisms.

## CHAPTER 5

### Summary

*Haemophilus paragallinarum* is the causative agent of infectious coryza (Blackall, 1999), a disease in chickens responsible for severe economical losses (Arzay, 1987). Current commercial vaccines consists of inactivated bacterial cells (Blackall *et al.*, 1993), but such vaccines protect against the agglutinin serovars present in them, and if chickens were subjected to a serovar not included within the vaccine, it would not provide adequate protection (Blackall and Reid, 1987). Research has been directed towards the haemagglutinin as the main role player during infection (Blackall, 1989), the same protein used for classification of this bacterium. There are a wide array of haemagglutinins present within the nine serovars (Kume *et al.*, 1983), which can account for the low rate of cross-protection between the different serovars used in the vaccines.

The aim of this study was to investigate the haemagglutinins found in *H. paragallinarum*, using both a protein and molecular approach in an attempt to shed more light on the already existing knowledge concerning this protein, in the hope of contributing to a better vaccination procedure.

Approaches to purify the haemagglutinins included the use of sonication in the presence of a detergent (Blackall and Yamamoto, 1989), enzymatic digestions using neuraminidase and trypsin to remove the protein from the exterior of the bacterium (Iritani *et al.*, 1980b) and a two phase detergent based separation procedure using Triton X-114 (Thwaits and Kadis, 1991) for the isolation of the integral outer membrane proteins. This was followed by varying combinations of gel filtration columns and affinity chromatography, either in combination or alone. An inactive partially pure protein, with a relative molecular mass of 36 kDa, could be isolated which correlated with research done by Iritani *et al.* (1983). The use of another gel filtration column,

Biogel P60, along with the Triton X-114 isolation procedure yielded three proteins with approximate molecular masses ranging from 40 kDa to 41 kDa, with one of the proteins displaying haemagglutination (HA) activity. Using the relative molecular mass and pI determined for the three proteins, it was shown that two of the proteins might be haemagglutinins and that it was a fimbrial type adhesin as shown by Iritani and co-workers (1980b).

The first part of the molecular biology approach for the isolation of the haemagglutinin gene consisted of the screening of a genomic library constructed by Michelle Goodrum from the Onderstepoort Veterinary Institute for any clone expressing HA activity, followed by the sequencing of these clones and searching for sequence similarities with other HA agents. Six of the clones expressed some HA activity with only one clone, clone 3, displaying any significant sequence similarities against nucleotide sequences in the Genbank Database, with the sequence of the flagellar switch protein of *E. coli*. The second part was a PCR approach using an amplified part of the *Pasteurella multocida* filamentous haemagglutinin to identify a similar protein in *H. paragallinarum* with the use of Southern blot hybridizations. Using the same primers the DNA was amplified from the genomic DNA of *H. paragallinarum*. Two probes were obtained (900 bp and 1 000bp) and used in Southern blot hybridizations, sequencing and sequence similarity searches against the Genbank Database. Sequencing results from the *P. multocida* confirmed the authenticity of this probe, unfortunately no hybridization was found against the genomic DNA of *H. paragallinarum*. The 900 bp and 1 000 bp probes gave strong signals during hybridization but the sequencing results gave similarities with putative proteins from *P. multocida*.

This project yielded insights into the characteristics of possible HA agents - all to be considered when assessing the best possible vaccine.

## Opsomming

*Haemophilus paragallinarum* veroorsaak aansteeklike neus verkoue in pluimvee (Blackall, 1989), 'n siekte wat groot ekonomiese verliese tot gevolg het (Arzay, 1987). Kommersiële entstowwe bestaan tans uit geï naktiveerde bakteriese selle (Blackall *et al.*, 1993), maar die entstowwe beskerm slegs teen die agglutinasie serogroepe daarin teenwoordig. Sou pluimvee bloodgestel word aan agglutinasie serogroepe wat nie in die entstof teenwoordig is nie, sou voldoende beskerming nie verskaf word nie (Blackall and Reid, 1987). Navorsing het gefokus op die heemagglutinasie agent as die hoof rolspeler gedurende infeksie (Blackall, 1989), dieselfde proteï n wat gebruik word vir klassifikasie van die bakterium. 'n Groot verskeidenheid heemagglutinasie agente is teenwoordig in die nege serogroepe (Kume *et al.*, 1983), wat die swak kruis beskerming tussen die serogroepe wat in die entstowwe gebruik moontlik kan verduidelik.

Die doel van die studie was om die heemagglutinasie agente in *H. paragallinarum* te bestudeer, deur gebruik te maak van beide 'n proteï n en 'n molekulêre benadering in 'n poging om meer lig te werp op die bestaande kennis aangaande die proteï ne. Dit sou hopelik kon bydra om 'n beter entstof te ontwikkel.

Benaderings om die heemagglutinasie agente te suiwer het die gebruik van sonifisering in die teenwoordigheid van 'n suiweringsmiddel ingesluit (Blackall and Yamamoto, 1989), ensiematiese verterings deur gebruik te maak van neuraminidase- en tripsien verterings om die proteï n van die uitwendige oppervlakte van die bakterium te verwyder (Iritani *et al.*, 1980b) sowel as 'n twee fase detergens gebaseerde skeidings prosedure met die gebruik van Triton X-114 vir die isolasie van integrale buite membraan proteï ne (Thwaites and Kadis, 1991). Dit is gevolg deur verskeie kombinasies van jel filtrasie kolomme en affiniteits chromatografie, in kombinasie of alleen. 'n Onaktiewe, gedeeltelike gesuiwerde proteï n met 'n relatiewe molekulêre massa van 36 kDa, kon geï soleer word wat ooreenstem met navorsing gedoen deur Iritani *et al.* (1983). Deur die gebruik van 'n ander jel filtrasie kolom in

kombinasie met die Tiron X-114 isolasie prosedure, kon drie proteïene met molekulêre massas wat wissel tussen 40 kDa to 41 kDa, met een wat heemagglutinasie (HA) aktiwiteit toon geïsoleer word. Met behulp van die relatiewe molekulêre massa en pI van die drie proteïene, kon aangewys word dat ten minste twee van die drie heemagglutinasie agente mag wees en dat hulle tot die fimbriale tipe adhesie proteïene behoort, soos voorheen bewys deur Iritani *et al.* (1980b).

Die eerste deel van die molekulêre biologie benadering vir die isolasie van die gene verantwoordelik vir die heemagglutinasie agente het bestaan uit die sifting van die genoom biblioteek voorsien deur Michelle Goodrum van die Onderstepoort Veeartsenykundige Instituut vir enige kloon wat moontlik die geen wat kodeer vir HA aktiwiteit mag bevat, gevolg deur volgorde bepaling van die klone sowel as soektogte om ooreenkomste met geenvolgordes van ander HA agente te vind. Ses van die klone het aktiwiteit getoon. Net een kloon, kloon 3, het enige volgorde ooreenkomste getoon met volgordes in die Genbank Databasis, en dit met die van 'n flagellêre skakel proteïen van *E. coli*. Die tweede gedeelte het bestaan uit 'n PKR (polimerase kettingreaksie) benadering met 'n geamplifiseerde deel van die filamentagtige heemagglutinasie agent van *Pasteurella multocida* as peiler om 'n soortgelyke proteïen in *H. paragallinarum* te identifiseer met behulp van Southern oordrag hibridisasies. Deur gebruik te maak van dieselfde voorlopers is DNA geamplifiseer vanaf die genoom DNA van *H. paragallinarum*. Twee peilers is geïdentifiseer (900 bp en 1 000 bp) en gebruik vir Southern oordrag hibridisasies. Nukleotied volgorde bepaling is gedoen en daar is in die Genbank data basis vir soortgelyke volgordes gesoek. Ongelukkig kon geen hibridisasie met die genoom DNA van *H. paragallinarum* waargeneem word nie. Die 900 bp en 1 000 bp peilers het sterk seine gegee gedurende hibridisasie maar die volgorde bepalingresultate het slegs getoon ooreenkomste met tentatiewe proteïene van *P. multocida*.

Die projek het dus insig gelewer aangaande die karakteristieke van moontlike HA agente, faktore wat in gedagte gehou moet word as nuwe ensowwe ontwikkel word.

## CHAPTER 6

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