Haemophilus paragallinarum haemagglutinin: role in adhesion, serotyping and pathogenicity

T.G. BARNARD, E. VAN HEERDEN*, R.R. BRAGG and J. ALBERTYN

Department of Microbial, Biochemical and Food Biotechnology, Faculty of Natural and Agricultural Science, University of the Free State, P.O. Box 339, Bloemfontein, 9301 South Africa

ABSTRACT

BARNARD, T.G., VAN HEERDEN, E., BRAGG, R.R. & ALBERTYN, J. 2008. *Haemophilus paragallinarum* haemagglutinin: role in adhesion, serotyping and pathogenicity. *Onderstepoort Journal of Veterinary Research*, 75:11–16

It is suggested that *Haemophilus paragallinarum* requires at least three haemagglutinins for adhesion during infection. This paper reports the partial purification and characterization of the HA-L haemagglutinin from *H. paragallinarum* strain 46-C3, a heat sensitive, trypsin sensitive haemagglutinin that has been shown to be the serovar specific haemagglutinin in this organism. Using the pI and molecular mass obtained, it was shown that this protein shares similarities with other types of adhesins found in Gram-negative bacteria. The haemagglutination assay conditions were optimized at pH 7.5 at 37 °C. It was also shown that activity is enhanced by the addition of Ca^{2+} and Mn^{2+} ions.

Keywords: Haemagglutinin purification and characterization, *Haemophilus paragallinarum*

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 Blieck (1932). The bacterium belongs to the family *Pasteurellaceae* and is a Gram-negative non-motile organism (Yamamoto 1991) requiring NAD^+ (V factor) for growth (Blackall 1999) and causing clinical signs varying from swelling of the face and wattles to diarrhoea (Eaves, Rogers & Blackall 1989). The disease is responsible for severe economical losses in the poultry industry due to a drop in egg production in layer hens and an increase in culling rate in broiler hens (Arzay 1987).

Current commercial vaccines against IC consist of inactivated cells of the causative bacterium mixed with an adjuvant (Blackall, Graydon, Rafiee & Tinworth 1993). Such vaccines protect only against the agglutinin serovars present in them, and if chickens are subjected to a serovar not included in the vaccine, it does not provide adequate protection (Blackall & Reid 1987). Haemagglutinins have been found to be important in pathogenicity and serotyping (Blackall 1989). Yamaguchi, Kobayashi, Masaki & Iritani (1993) confirmed this theory when a mutant strain not expressing any haemagglutinins was isolated, rendering them incapable of producing haemagglutinin antibodies. Sawata, Kume & Nakase (1982) identified three possible haemagglutinins (HA) and termed them HA-HL, a heat labile, trypsin resistant haemagglutinin, HA-HS, a heat stabile, trypsin resistant haemagglutinin (Kume, Sawata, Nakase & Matsumoto 1983) and HA-L, a heat sensitive, trypsin sensitive haemagglutinin. HA-L is the

---

variant specific haemagglutinin while HA-HL and HA-HS the common haemagglutinins among the bacterial strains. Iritani, Katagirl & Arita (1980) isolated a haemagglutinin with relative size of 36 kDa, which was able to agglutinate gluteraldehyde-fixed red blood cells. In 2002, Hobb, Tseng, Downes, Terry, Blackall, Takagi & Jennings reported on a gene encoding a haemagglutinin of approximately the same size.

In this report we describe a haemagglutinin associated with *H. paragallinarum* (strain number 46-C3) which displays some coherent characteristics with the literature cited, but with certain other features which opens a debate on serotyping, immune response and effective vaccination against infectious coryza.

MATERIALS AND METHODS

Bacterial strain used

Haemophilus paragallinarum strain 46-C3, obtained from the Onderstepoort Veterinary Institute, was used in all experiments.

Growth and maintenance of the bacterium

Haemophilus paragallinarum was 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 [1.5 % (w/v)]. The medium was adjusted to pH 7.5 and autoclaved. A broth (TMB), with the same composition as above, excluding the agar, 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 (Bragg, Coetzee & Verschoor 1995). The solid media (TM/SN) were incubated under increased CO_2 tension inside a candled jar at 37 °C (Rimler, Shotts, Brown & Davis 1974; Yamamoto 1991).

Haemophilus paragallinarum specific PCR test

The authenticity of the strain was confirmed using the HPG-2 polymerase chain reaction (PCR), with specific primers for *H. paragallinarum* as described by Chen, Miflin, Zhang & Blackall (1996).

Haemagglutination assay

The haemagglutination test was performed as described by Bragg *et al.* (1995) using gluteraldehyde-fixed chicken red blood cells (GA-fixed RBC) prepared according to the method described by Eaves *et al.* (1989) in veronal buffer (VB). Two-fold serial dilutions of either the protein extracts or bacterial suspensions were made to which GA-fixed RBC were 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.

Dextran assay

The assay used was a modification of the method described by Agrawal & Goldstein (1968). It functions by measuring the binding of the HA to dextran, a polysaccharide consisting of D-glucose units linked by α(1–6) bonds, 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 µℓ) or buffer (50 µℓ blank) was added. This was further incubated for 15 min at 37 °C and the absorbance read at 420 nm.

SDS-PAGE

SDS-PAGE was performed using 10 % stacking gels and 4 % resolving gels as described by Laemmli (1970) in the "Mighty Small" miniature slab gel electrophoresis unit, SE 200 (Hoefer Scientific Instruments). Electrophoresis was performed on approximately 10 µg protein using a constant current of 20 mA.

The method described by Robertson, Dannelly, Malloy & Reeves (1987) was used for isoelectric focusing. Electrophoresis was performed with the use of Pharmalyte carrier ampholytes pH 3–10 and loading approximately 20 µg protein sample. The gel was pre-focused at 150 V for 30 min and after loading the sample it was focused for 90 min at 200 V and 90 min at 400 V. The catholyte was 25 mM sodium hydroxide solution and the anolyte was 20 mM acetic acid.

Two dimensional gel electrophoresis was performed using the IEF as described for the first dimension. The desired lane was removed and placed in a transfer solution (50 mM Tris-HCl, pH 6.8; 6 M urea; 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 and the gel run at a constant current of 20 mA. Proteins were visualized by silver staining (Switzer, Merril & Shifrin 1979).

Purification of integral outer membrane proteins

Outer membrane proteins were purified according

to a method described by Thwaits & Kadis (1991). The detergent phase (TRI) was collected and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl.

Separation of proteins

Proteins were subjected to separation using the Biogel P60 gel filtration resin. 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^{-1} and fractions (800 $\mu\ell$) collected. Elution of the proteins was monitored using the micro bicinchoninic acid (BCA) method (Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, Fujimoto, Goeke, Olsen & Klenk 1985), supplied by Pierce. The protein peaks were identified, pooled and concentrated using the Amicon stirrer cell and tested for activity.

RESULTS

Growth and conformation of *Haemophilus paragallinarum*

The bacterial strain was maintained on TM/SN plates at 37 °C and passaged once a week. PCR was done after each passage to confirm the authenticity of the *H. paragallinarum* strain.

Partial purification of the haemagglutinin

Haemophilus paragallinarum was cultivated and the cells were harvested, washed and incubated in buffer for 2 days at 4 °C before extractions were performed. Activity was monitored during storage using the HA test yielding a titre of 1:256. Following purification of the putative haemagglutinin from *H. paragallinarum*, it was established, using the dextran assay, that the protein was located in the detergent phase, suggesting that it might be an integral membrane protein. The dextran assay was performed as suggested by Agrawal & Goldstein (1968) since Triton X-114 interfered with the HA assay and that a high concentration of antigens is required for a positive HA assay (Goldhar 1995).

After dialysis of the detergent phase, the sample was concentrated and applied onto the Biogel P60 column for separation of the different proteins. The protein peak coinciding with the dextran activity peak (Fig. 1) was pooled, concentrated and tested for activity with the HA assay. The partially purified sample was visualized on SDS-PAGE (Fig. 2) and approximately three protein bands could be distinguished with molecular sizes ranging from 40–41 kDa. These results were confirmed (data not shown) with isoelectric focusing, and 2D gel electrophoresis was used to allocate appropriate pI values to the different molecular mass (Table 2). Shown in Table 1 are some of the relevant results obtained performing searches using the Expasy Database (http://www.expasy.ch) against all probable Gram-negative bacteria containing both types of adhesins. The most notable similarities where found with the Haemagglutinin/protease from *Vibrio cholera* for fragment A and a filamentous adhesin from *Bordetella pertussis* in the case of fragment C—further confirmation of agglutinin properties.
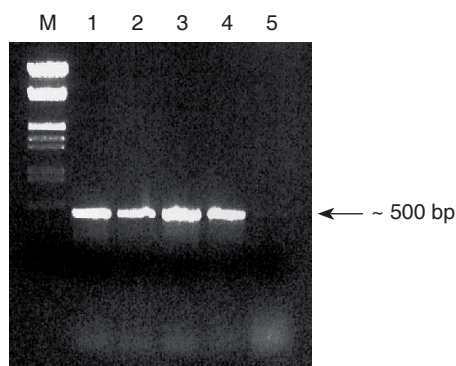

FIG. 1 Agarose gel electrophoresis of the PCR products (lanes 1–3) obtained for the *H. paragallinarum* strain used with an estimated size of 500 basepairs. The positive control is shown in lane 4 with the negative control in lane 5. The marker shown (M) is lambda DNA digested with *Hind* III/*Eco*R1 with the 546 basepair fragment indicated
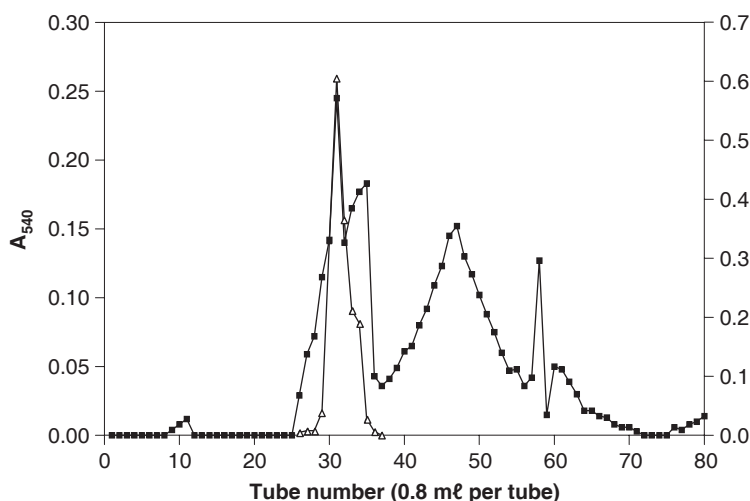

FIG. 2 Biogel P 60 elution profile of the Triton X-114 sample showing the protein assay (■) and dextran assay (△). Fractions 28–32 (TR1), 33–36 (TR2), 40–52 (TR3) and 57–59 (TR4) were pooled and concentrated for HA assay and SDS-PAGE analysis
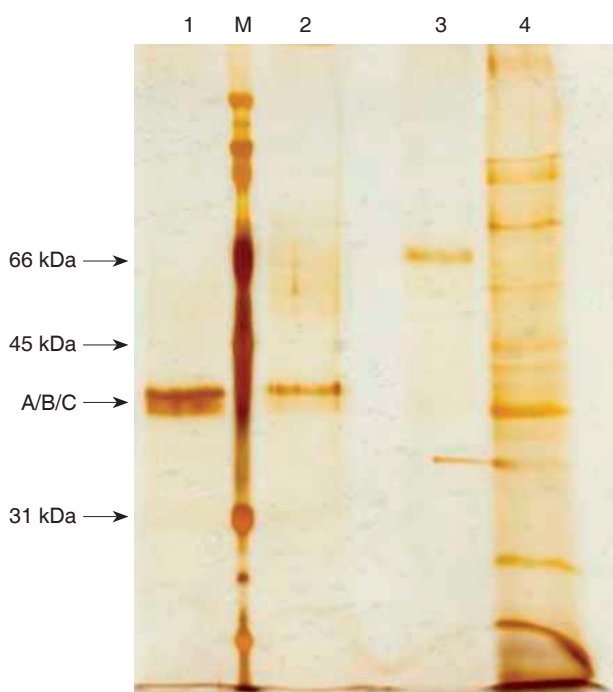
FIG. 3   Three SDS-PAGE gel patterns of the fractions obtained after separation on the Biogel P 60

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 proposed haemagglutinin is shown as A/B/C

Characterization of the partially pure haemagglutinin

The partially pure haemagglutinin obtained was tested for optimum pH and metal dependencies since only 1.56 % of the activity could be retained after the column step. Activity was tested over a wide pH and temperature range and it was established that the optimum conditions for agglutination is pH 7.5 at 37 °C. It was also shown that the protein has a calcium and manganese dependency, since all activity is lost in the presence of 20 mM EDTA but enhanced with either 20 mM calcium or manganese (Table 3). Results reported by Sawata *et al.* (1982) concerning the trypsin sensitivity of the proteins were confirmed (data not shown) since all activity was lost after treatment with trypsin for 10 min. Whole cells treated with trypsin also lost activity over a short period of time confirming the use of this protein during the agglutination process.

DISCUSSION

This paper reports the first isolation and characterization of what is termed the HA-L haemagglutinin from *H. paragallinarum* strain 46-C3, a heat sensitive, trypsin sensitive haemagglutinin that has been

TABLE 1 Activity obtained with the haemagglutination and dextran assay during partial purification of the haemagglutinin. Good activity is indicated with ++++

Procedure	HA titre	Dextran activity
Harvesting of cells	1:64	++
After 3 days storage at 4 °C	1:256	++++
Activity during purification	0	+++
Activity after gel filtration	1:4	+++

TABLE 2 Molecular mass and pI combinations for the three protein bands obtained from fraction TR1. Also indicated is the protein to which it is similar using the molecular mass and pI combination. (A corresponds to the first protein band in Fig. 2, B to the second and C to the third)

Protein fragment	Molecular mass Da)	pI	Similar to
A	41 000	6.4	Haemagglutinin/protease from *V. cholera*
B	40 500	5.2	No significant matches
C	40 000	6.4	Filamentous adhesin from *B. pertusis*; switch protein from *E. coli*

TABLE 3 Display of the activities obtained using the HA assay with the antigen incubated with different metals and chelating agents

Sample	Activity
Red blood cell control (no antigen)	0
Antigen	1:16
Antigen with 20 mM Ca2+	1:32
Antigen with 20 mM Mn2+	1:32
Antigen with 20 mM Mg2+	1:16
Antigen with 20 mM EDTA	1:2

TABLE 4 Adaptation of the tables presented by Blackall *et al.* (1990) and Kume *et al.* (1983) showing the serogroup C organisms to highlight possible factors contributing to the poor protection by vaccines

Reference strain	Classification according to Blackall's serotyping scheme	Factor of HA-L haemagglutinin	
		Specific	Common
H-18	C-1	II-1	II-4, II-5
Modesto	C-2	II-2	II-4, II-5
SA-3	C-3	II-3	II-4
HP60	C-4	Not studied	Not studied

14

shown to be the serovar specific haemagglutinin in *H. paragallinarum* (Sawata *et al*. 1982).

In *H. paragallinarum*, as in many infectious bacterial species, adhesion is one of the most important factors influencing effective infection. According to the literature, the proteins most commonly associated with this role can be divided into two classes of adhesions: afimbrial and fimbrial (Finlay & Falkow 1997). The fimbrial adhesins include the type P and IV pili as found in Gram-negative bacteria such as *Escherichia coli* and *Vibrio cholera*. The afimbrial adhesins include the haemagglutinins from *E. coli*, *Bordetella pertussis* and *Haemophilus influenza* (Sandros & Tuomanen 1993).

The partially pure protein obtained and characterized with respect to pI and molecular mass, correlates well to other fimbrial adhesins isolated (Hobb *et al*. 2002). This confirms the theory that *H. paragallinarum* has a structurally smaller than thought haemagglutinin in comparison to other closely related bacteria. It has been shown by Goldhar (1995) that haemagglutination is a secondary effect of adhesion and requires a critical density of proteins for a positive test, a possible explanation for the lack of activity. Results obtained during this study, and more recent publications, illustrate that a more protein-directed approach should be followed for the better understanding of adhesion and serotyping in *H. paragallinarum.* This is supported by results obtained by Hobb *et al*. (2002) where it was shown that the sequences, both on nucleotide and proposed amino acid levels, do not differ significantly between serovars.

It could be that adhesion, the immune response that follows, and serotyping could be more dependant on the specific glycosilation of the proteins. This is the case in other *Haemophilus* species, such as *H. influenza*, where the serotyping is performed using the different sugar moieties found on the surface proteins.

Another important consideration when working with agglutinins is that their optimum pH differs significantly from when they are still attached to the bacteria to when they are isolated. Also to be considered is the possible different cofactor dependencies found between the different proteins.

Comparison of the results obtained in Table 4 showing only the common and specific HA-L haemagglutinins found in serovar C, emphasizes the ever-growing need to investigate these proteins and their action in order to refine and improve the vaccines for en-

hanced cross protection, especially since current vaccines are only mildly effective.

REFERENCES

AGRAWAL, B.B.L. & GOLDSTEIN, I.J. 1968. Protein-carbohydrate interaction. XV. The role of bivalent cations in concanavalin A—polysaccharide interaction. *Canadian Journal of Biochemistry*, 46:1147–1150.

ARZEY, G.G. 1987. The effects of infectious coryza on the egg production of a layer flock. *Australian Veterinary Journal,* 64: 124–126.

DE BLIECK, L. 1932. A haemoglobinophilic bacterium as the cause of contagious catarrh of the fowl. *Veterinary Journal*, 88:9–13.

BLACKALL, P.J. 1989. The avian haemophili. *Clinical Microbiology Reviews*, 2:270–277.

BLACKALL, P.J. 1999. Infectious coryza: Overview of the disease and new diagnostic options. *Clinical Microbiology Reviews,* 12:627–632.

BLACKALL, P.J. & REID, G.G. 1987. Further efficacy studies on inactivated aluminium-hydroxide-absorbed vaccines against infectious coryza. *Avian Diseases*, 31:527–532.

BLACKALL, P.J., GRAYDON, R.J., RAFIEE, M. & TINWORTH, D. 1993. Towards a live infectious coryza vaccine. X^{th} Congress of the World Veterinary Poultry Association, Sydney, Australia, 16–19 August 1993.

BRAGG, R.R., COETZEE, L. & VERSCHOOR, J.A. 1995. Effects of growth conditions and incubation times on the expression of antigens of *Haemophilus paragallinarum* which are detected by monoclonal antibodies. *Onderstepoort Journal of Veterinary Research*, 64:57–63.

CHEN, X., MIFLIN, J.K., ZHANG, P. & BLACKALL, P.J. 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum. Avian Diseases,* 40:398–407.

EAVES, L.E., ROGERS, D.G. & BLACKALL, P.J. 1989. Comparison of hemagglutinin and agglutinin schemes for the serological classification of *Haemophilus paragallinarum* and proposal of a new hemagglutinin serovar. *Journal of Clinical Microbiology,* 27:1510–1513.

FINLAY, B.B. & FALKOW, S. 1997. Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews*, 61:136–169.

GOLDHAR, J. 1995. Erythrocytes as target cells for testing bacterial adhesins. *Methods in Enzymology*, 253:43–50.

HOBB, R.I., TSENG, H.J., DOWNES, J.E., TERRY, T.D., BLACKALL, P.J., TAKAGI, M. & JENNINGS, M.P. 2002. Molecular analysis of a haemagglutinin of *Haemophilus paragallinarum. Microbiology,* 148:2171–2179.

IRITANI, Y., KATAGIRL, K. & ARITA, H. 1980. Purification and properties of *Haemophilus paragallinarum* hemagglutinin. *American Journal of Veterinary Research,* 41:2114–2118.

KUME, K., SAWATA, A., NAKASE, Y. & MATSUMOTO, M. 1983. Serological classifications of *Haemophilus paragallinarum* with a hemagglutinin system. *Journal of Clinical Microbiology,* 17:958–964.

LAEMMLI, U.K. 1970. Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature*, 227:680–688.

RIMLER, R.B., SHOTTS, E.B., BROWN, J. & DAVIS, R.B. 1976. The effects of atmospheric conditions on the growth of *Hae-*

mophilus gallinarum in a defined medium. *Journal of General Microbiology,* 92:405–409.

ROBERTSON, E.F., DANNELLY, H.K., MALLOY, P.J. & REEVES, H.C. 1987. Rapid isolelectric focusing in a vertical polyacrylamide minigel system. *Analytical Biochemistry*, 167: 290–294.

SANDROS, J. & TUOMANEN, E. 1993. Attachment factors of *Bordetella pertussis*: mimicry of eukaryotic cell recognition molecules. *Trends in Microbiology*, 1:192–196.

SAWATA, A., KUME, K. & NAKASE, Y. 1982. Hemagglutinin of *Haemophilus paragallinarum* serotype 2 organisms: Occurrence and immunologic properties of hemagglutinin. *American Journal of Veterinary Research,* 43:1311–1314.

SMITH, P., KROHN, R., HERMANSON, G., MALLIA, A., GARTNER, F., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150:76–85.

SWITZER, R.C., MERRIL, C.R. & SHIFRIN, S. 1979. A highly effective silver stain for detecting proteins and peptides in polyacrylamide gels. *Analytical Biochemistry,* 98:231–237.

THWAITS, R.N. & KADIS, S. 1991. Immunogenicity of *Actinobacillus pleuropneumoniae* outer membrane proteins and enhancement of phagocytosis by antibodies to the proteins. *Infection and Immunity*, 59:544–549.

YAMAGUCHI, T., KOBAYASHI, M., MASAKI, S. & IRITANI, Y. 1993. Isolation and characterization of a *Haemophilus paragallinarum* mutant that lacks a hemagglutinating antigen. *Avian Diseases,* 37:970–976.

YAMAMOTO, R. 1991. Infectious coryza, in *Diseases of poultry*, 9th ed., edited by M.S. Hofstad, H.J. Barnes, B.W. Calnek, W.M. Reid & E.R. Yoder. Ames: Iowa State University Press.