Hemagglutinins of *Haemophilus paragallinarum* Serotype 1 Organisms

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Various kinds of treated cells were prepared from variants derived from serotype 1 strains of *Haemophilus paragallinarum* by enzymatic or physicochemic treatments. Hemagglutinating properties of the treated cells were investigated against freshly collected and glutaraldehyde-fixed chicken, human being (type O), or rabbit erythrocytes (RBC). At least 3 types of hemagglutinin showing different hemagglutination patterns, designated as hemagglutinins HA-L, HA-HL, and HA-HS, were defined from the results of their biologic properties. Heat-labile, trypsin-sensitive HA-L hemagglutinin agglutinated all RBC used, whereas trypsin-resistant heat-labile, HA-HL or heat-stable, HA-HS antigens agglutinated only fresh chicken or rabbit RBC except those from day-old chicks. Because of the presence of capsule surrounding bacterial cells, nontreated cells prepared from certain variant lacked the ability to agglutinate any RBC used; hyaluronidase-treatment rendered them agglutinate, and the hemagglutinin appeared by this treatment was HA-L hemagglutinin. After trypsin-or heat-treatment of HA-L hemagglutinin, hemagglutinins HA-HL or HA-HS appeared. Electron microscopic studies showed that the outer membrane of the treated cells adhered directly to the surface of RBC. The result suggests that the location of the 3 types of hemagglutinin HA-L, HA-HL, and HA-HS are in the outer membrane of bacterial cells, and indicates the necessity in use of proper hemagglutinin and RBC for experiment.—*Key words: Haemophilus paragallinarum*, Hemagglutinin, Outer membrane.

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Haemophilus paragallinarum causes acute upper respiratory infection in chickens and results in significant economic loss. Prevention of the disease can adequately be accomplished by vaccination with inactivated vaccines. Little is known, however, on antigens and other substances which are responsible for pathogenicity and immunogenicity of the organisms. Antigens participating in an agglutination reaction has been studied by various workers [6, 10, 14, 15] for serologic typing of the organisms.

Hemagglutination of *H. paragallinarum* was first demonstrated by Kato *et al.* [7] using his serotype 1 [14] strain and freshly collected chicken erythrocytes (RBC). The hemagglutinating antigen was correlated with immunogenicity of the serotype 1 organisms [5], and hemagglutination-inhibition (HI) antibodies have been generally used to evaluate the efficacy of the vaccines in Japan.

Iritani and Miyajima [2] showed that chicken RBC's had different susceptibility to *H. paragallinarum* hemagglutinin. Subsequent study [18] indicated that 2 types of heat-labile hemagglutinin, designated as trypsin-sensitive type 1 HA and trypsin-resistant type 2 HA were in serotype 1 [14] strain 221 [6], and the fresh chicken RBC collected from susceptible chicks had receptors against both type 1HA and type 2 HA, and those from partially susceptible ones only against type 1 HA. Therefore, the hemagglutinin described by Iritani and Miyajima [2] would be Yamaguchi and Iritani's type 2 HA [18]. It was apparent that at least 2 types of hemagglutinin were present in the serotype 1 cells of *H. paragallinarum*.

Sawata and Kume have investigated hemagglutinins of genetically stable variants derived from serotype 1 strains [13]. The results indicated that at least 3 types of

hemagglutinin were present on the surface of the serotype 1 organisms; 2 heat-labile hemagglutinins, HA-L and HA-HL, and heat-stable HA-HS. Present study is on the hemagglutinating properties of the 3 types of hemagglutinin. Stable variants derived from serotype 1 strains of *H. paragallinarum* were treated enzymatically or physicochemically and their agglutinability was tested against various RBC's.

MATERIALS AND METHODS

Bacterial strains: Seven variants, designated as hi, li-A, li-B, ni-A, ni-B, R-A, and R-B, were derived from serotype 1 [14] strains 221 [6], 083 [12], or 7411 [14] of *H. paragallinarum* [13]. Differentiating properties of each variant are listed in Table 1 [13]. A procedure for obtaining the variants was described previously [13].

Treatment of variants: Cultures were grown in S broth medium [8, 9] at 37°C for 10 hr. The bacterial cells were collected by centrifugation at 10,000 X *g* for 20 min, and washed once in 0.02 M phosphated buffered solution, pH 7.0, containing 0.85% NaCl (PBS). Nontreated (NT), hyaluronidase (Sigma)-treated (HU), trypsin (P-L Biochemical)-treated (T), 2 M NaCl and trypsin-treated (SAT), or heated cells at 121°C for 2 hr at pH 7.0 (HE 7) from each variant were prepared, according to the methods described previously [15]. All the treated cells were adjusted to a concentration of 1×10^{11} cells/ml using Model 6/20 spectrophotometer (Coleman Instruments), and 0.01% thimerosal was added.

Biologic properties of treated cells prepared from each variant: To heat, trypsin, or formalin sensitivities of the treated cells (NT, HU, T, SAT, or HE 7 cells) showing hemagglutinating activity against fresh chicken RBC, further treatments (heat-treatment at different temperatures, trypsin-treatment, or formalin-treatment) were done according to the methods described previously [16], and

hemagglutinating activity of these cells were examined.

Erythrocytes (RBC): Sources were day-old or 60-day-old line S chicks (Nisseiken Co., Yamanashi, Japan), day-old or 60-day-old H & N chicks (Pfizer Taito Co., Shizuoka, Japan), human being (type O), and NewZealand White rabbits. The glutaraldehyde (GA, Eastman Kodak)-fixed RBC, which were prepared by the method of Bing *et al.* [1], were washed 3 times in PBS and suspended in 1% in PBS containing 0.1% bovine serum albumin (Armour Pharmaceutical) and 0.001% gelatin (Merck). The fresh RBC were washed 3 times and suspended in 0.5% in PBS [16].

Hemagglutination test: The treated cells (1×10^{11} cells/ml) prepared from each variant were tested for their hemagglutinating activity against freshly collected and GA-fixed RBC, according to the methods described in a previous paper [16].

Electron microscope: Preparation of specimens or transmission electron microscopy was followed by the method described by Swaney and Breese [17]. JEM-100 electron microscope (Japan Electric Co., Tokyo, Japan) was used at 80 kilovolt.

RESULTS

Hemagglutination titers of nontreated (NT) cells from variants: NT cells of variants hi, li-B, ni-B, or R-B did not agglutinate RBC (Table 2). On the other hand, NT cells of variants li-A, ni-A, and R-A agglutinated both fresh and GA-fixed RBC of chick and human being (type O) origin. Hemagglutination titers against GA-fixed RBC were higher than those against fresh RBC.

Hemagglutination with treated cells from variants hi and ni-A: NT cells from hi variant (hi-NT) lacked the ability to agglutinate RBC, but by hyaluronidase-treated cells (hi-HU) agglutinated fresh and GA-fixed RBC of 60-day-old line S chicks, human being (type O), or rabbits (Table 3).

Table 1. Morphologic, serologic, and immunologic properties of variants derived from serotype 1 strains of *H. paragallinarum*

Variant	Morphology of colony	Iridescence	Capsule	Variant-specific antigen		Virulence in chicks	Recovery of organisms from inoculated birds	Protective activity against challenge exposure with hi variant of homologous strain
				L	HA-L			
hi	S	ir+	+	— ^{a)}	+ ^{a)}	—	+	+
li-A	S	ir±	±	+	+	+ ^{b)}	+ ^{b)}	+
li-B	S	ir±	±	—	—	—	—	—
ni-A	S	ir—	—	+	+	—	+	+
ni-B	SR	ir—	—	—	—	—	—	—
R-A	R	ir—	—	+	+	—	+	+
R-B	R	ir—	—	—	—	—	—	—

a) Lack of agglutinability by capsules, but the activity appeared soon after treatment with hyaluronidase.

b) When inoculated in chicks, variation from li-A to hi variant *in vivo* was observed.

S=Smooth; R=rough; SR=intermediate between S and R; ir+=high iridescence; ir±=low iridescence; ir—=non-iridescence; L=heat-labile, trypsin-sensitive, hyaluronidase-resistant agglutinin; HA-L=heat-labile, trypsin-sensitive, hyaluronidase-resistant hemagglutinin.

Table 2. Hemagglutination titers of nontreated (NT) cells prepared from variants derived from serotype 1 strains 221, 083, and 7411 of *H. paragallinarum*

Nontreated cells	Day-old line S chicks		60-day-old line S chicks		Human being (type O)	
	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC
hi-NT	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2
li-A-NT	1:8–1:64	1:64–1:256	1:8–1:64	1:64–1:256	1:2–1:32	1:4–1:64
li-B-NT	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2
ni-A-NT	1:64–1:256	1:256–1:512	1:32–1:128	1:512–1:1,024	1:32–1:64	1:128–1:256
ni-B-NT	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2
R-A-NT	<1:2–1:32	1:64–1:512	<1:2–1:16	1:64–1:256	<1:2–1:8	1:64–1:256
R-B-NT	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2

Results were obtained by using 5 lots of NT cells in each strain.

Table 3. Hemagglutination titers of variously treated cells prepared from variants hi and ni-A derived from serotype 1 strains 221, 083, and 7411 of *H. paragallinarum*

Treated cells	60-day-old line S chicks		Human being (type O)		Rabbits	
	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC
hi-NT	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2
hi-HU	1:64–1:128	1:256–1:512	1:32–1:64	1:128–1:256	<1:2–1:4	1:128–1:256
hi-T	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2
hi-SAT	<1:2–1:4	<1:2	<1:2	<1:2	<1:2–1:4	<1:2
hi-HE 7	1:4–1:32	<1:2	<1:2	<1:2	1:64–1:128	<1:2
ni-A-NT	1:32–1:128	1:256–1:512	1:32–1:64	1:128–1:256	<1:2–1:32	1:64–1:128
ni-A-HU	1:64–1:128	1:512–1:1,024	1:32–1:64	1:256–1:512	<1:2–1:32	1:128–1:256
ni-A-T	1:256–1:512	<1:2	<1:2	<1:2	<1:2–1:128	<1:2
ni-A-SAT	1:64–1:128	<1:2	<1:2	<1:2	<1:2–1:16	<1:2
ni-A-HE 7	1:8–1:16	<1:2	<1:2	<1:2	1:128–1:256	<1:2

Results were obtained by using 3 lots of treated cells in each strain.

NT cells from ni-A variant agglutinated fresh and GA-fixed RBC, and hyaluronidase did not affect the reaction. However, treatment with trypsin (T), saline and trypsin (SAT), or heat (HE 7) eliminated antigens responsible for agglutination with GA-fixed chicken RBC, fresh and GA-fixed human RBC. Treatment with T or with SAT did not have obvious effect on hi variant, but, with ni-A variant caused appearance of hemagglutination with fresh RBC of the chicks or rabbits. Heating at 121°C for 2 hr (HE 7) with variants hi or ni-A caused appearance of hemagglutination with fresh RBC of the chicks or rabbits. Similar appearances of hemagglutination with various treated cells from ni-A variant were observed with R-A variant after various treatments.

Biologic properties of treated cells: Trypsin, formalin, and heat sensitivities of the treated cells were investigated (Table 4). Hemagglutinating activities of ni-A-NT and ni-A-HU cells were completely inactivated by heating at 65°C for 30 min. The cells, designated as HA-L hemagglutinin by their biologic properties, was shown to be heat-labile, trypsin-sensitive, formalin-sensitive, hyaluronidase-resistant, and active against GA-fixed RBC. Hemagglutinating activity of ni-

A-SAT cells was inactivated by heating at 100°C for 30 min, but not at 65°C for 30 min. The cells, designated as HA-HL hemagglutinin, was shown to be heat-labile, trypsin-resistant, and formalin-resistant. Hemagglutinating activity of ni-A HE 7 cells was not inactivated by heating at 121°C for 2 hr. The cells, designated as HA-HS hemagglutinin, was shown to be heat-stable, trypsin-resistant, and formalin-resistant. The results apparently showed the presence of 3 types of hemagglutinin classified by their biologic properties in serotype 1 cells.

Sources of chicken RBC: Appearance or disappearance of hemagglutination reactions accompanied by various treatments may be affected by the kinds of receptors present on RBC. To investigate the possibility, treated bacterial cells were reacted with fresh or GA-fixed RBC obtained from day-old or 60-day-old chicks of 2 genetic lines (Table 5).

Hyaluronidase-treated hi variant agglutinated fresh and GA-fixed RBC from the 2 sources. Nontreated or hyaluronidase-treated ni-A variant also showed the reactions. Disappearance of hemagglutinating antigen(s) with the trypsin-treatment or heat-treatment of ni-A variant was confirmed with GA-fixed RBC and fresh RBC from day-old chicks, but

Table 4. Effects of heating on hemagglutination titers of treated cells prepared from ni-A variant derived from serotype 1 strain 221 of *H. paragallinarum*

Treated cells (Hemagglutinin)	Heating at pH 7.0 for 30 min	60-day-old line S chicks	
		Fresh RBC	GA-fixed RBC
ni-A-NT (HA-L)	56°C	1:64–1:128	1:128–1:256
	65°C	<1:2	<1:2
	100°C	<1:2	<1:2
ni-A-HU (HA-L)	56°C	1:64–1:128	1:256–1:512
	65°C	<1:2	<1:2
	100°C	<1:2	<1:2
ni-A-SAT (HA-HL)	56°C	1:64–1:128	<1:2
	65°C	1:64–1:128	<1:2
	100°C	<1:2	<1:2
ni-A-HE 7 (HA-HS)	121°C	1:16–1:32	<1:2

Results were obtained by using 3 lots of treated cells in each hemagglutinin.

Table 5. Influence of RBC collected from 2 genetic lines and RBC from different ages of chicks on hemagglutination of treated cells prepared from variants hi and ni-A derived from serotype 1 strain 221 of *H. paragallinarum*

Treated cells (Hemagglutinin)	Day-old line S chicks		60-day-old line S chicks	
	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC
hi-HU (HA-L)	1:128–1:256	1:512–1:1,024	1:64–1:128	1:256–1:512
ni-A-NT (HA-L)	1:64–1:128	1:512–1:1,024	1:32–1:128	1:256–1:512
ni-A-HU (HA-L)	1:128–1:512	1:512–1:1,024	1:64–1:128	1:512–1:1,024
ni-A-T (HA-HL)	<1:2	<1:2	1:256–1:512	<1:2
ni-A-SAT (HA-HL)	<1:2	<1:2	1:64–1:128	<1:2
ni-A-HE 7 (HA-HS)	<1:2	<1:2	1:8–1:16	<1:2

Results were obtained by using 5 different lots of treated cells in each hemagglutinin. The results obtained by using RBC collected from H & N chicks were almost similar to those observed in Table 5.

Table 6. Hemagglutination patterns of 3 types of hemagglutinin of *H. paragallinarum* serotype 1

Hemagglu- tinin	Appearance of hemagglu- tinating activity in		Day-old chicks		60-day-old chicks		Human (type O)		Rabbits	
	Variant	Treated cells	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC	Fresh RBC	GA-fixed RBC
HA-L	hi	HU	+	+	+	+	+	+	+/-	+
	ni-A	NT, HU	+	+	+	+	+	+	+/-	+
	R-A	NT, HU	+/-	+	+/-	+	+	+	+/-	+
HA-HL	hi	T, SAT	–	–	+/-	–	–	–	+/-	–
	ni-A	T, SAT	–	–	+	–	–	–	+/-	–
	R-A	T, SAT	–	–	+	–	–	–	+/-	–
HA-HS	hi	HE 7	–	–	+	–	–	–	+	–
	ni-A	HE 7	–	–	+/-	–	–	–	+	–
	R-A	HE 7	–	–	+	–	–	–	+	–

was not observed with fresh RBC from 60-day-old chicks. The results might be resulted in lack of a receptor to hemagglutinins HA-HL and HA-HS on fresh RBC of day-old chicks or in destruction of a receptor on RBC of 60-day-old chicks by treatment with glutaraldehyde.

Hemagglutination patterns of 3 types of hemagglutinin: Hemagglutination patterns of the 3 types of hemagglutinin varied greatly (Table 6). Chicken, human being (type O), and rabbits RBC, both fresh and GA-fixed, were agglutinated HA-L hemagglutinin in HU cells of hi variant as well as in NT and HU cells of variants ni-A and R-A. HA-HL hemagglutinin in both T and SAT cells of variants hi, ni-A, and R-A agglutinated only fresh RBC of 60-day-old chicks or rabbits.

HA-HS hemagglutinin in HE 7 cells of variants hi, ni-A, and R-A agglutinated only fresh RBC of 60-day-old chicks or rabbits.

Electron microscopic observation of hemagglutination by the 3 types of hemagglutinin: Encapsulated and pilated bacterial cells were not found among the treated cells. Outer membrane of 221-ni-A variant treated with hyaluronidase (HA-L hemagglutinin, Fig. 1) or trypsin (HA-HL hemagglutinin, Fig. 2), were found to adhere directly to the surface of fresh RBC of 60-day-old line S chick. After heating at 121°C for 2 hr at pH 7.0, outer membrane of 221-ni-A variant also adhered directly to the surface of RBC (HA-HS hemagglutinin, Fig. 3).

Fig. 1. Ultrathin section stained with uranylacetate of the hemagglutination occurred between 221-ni-A-HU cells (HA-L hemagglutinin) of *H. paragallinarum* serotype 1 and fresh RBC of 60-day-old line S chicks. Features included: (b) bacterial cell, (o) outer membrane of bacterial cell, and (r) fresh chicken RBC. Magnification, $\times 40,000$.

Fig. 2. Ultrathin section stained with uranylacetate and ruthenium red of the hemagglutination occurred between 221-ni-A-SAT cells (HA-HL hemagglutinin) of *H. paragallinarum* serotype 1 and fresh RBC of 60-day-old line S chicks. Magnification, $\times 90,000$.

Fig. 3. Ultrathin section stained with uranylacetate and ruthenium red of the hemagglutination occurred between 221-ni-A-HE 7 cells (HA-HS hemagglutinin) of *H. paragallinarum* serotype 1 and fresh RBC of 60-day-old line S chicks. Magnification, $\times 90,000$.

DISCUSSION

Present study was done to show the 3 types of hemagglutinin in serotype 1 [13] and to differentiate them. Serotype 1 of *H. paragallinarum* had at least 3 types of hemagglutinin designated as HA-L, HA-HL, and HA-HS, and their hemagglutination pattern was different, respectively. HA-L hemagglutinin agglutinated both fresh and GA-fixed RBC from chicken, human being (type O), or rabbits. However, hemagglutinins HA-HL and HA-HS agglutinated only fresh RBC of 60-day-old chicks and rabbits. Hemagglutinin HA-L was masked by capsule, whereas hemagglutinins HA-HL and HA-HS also appeared to be masked by HA-L hemagglutinin. Electron microscopic studies showed that the serotype 1 hemagglutinins are located on outer membrane of bacterial cells.

Biologic properties of the 3 types of hemagglutinin were different, respectively. Hemagglutinating ability of serotype 1 HA-L hemagglutinin was inactivated with trypsin,

formalin, and heating at 65°C for 30 min, whereas it was not inactivated with hyaluronidase. The hemagglutinin was shown to be heat-labile, trypsin-sensitive, formalin-sensitive and hyaluronidase-resistant, and agglutinate against GA-fixed RBC. Serotype 1 HA-HL hemagglutinin was shown to be heat-labile, trypsin-resistant and formalin-resistant, whereas serotype 1 HA-HS hemagglutinin was shown to be heat-stable, trypsin-resistant and formalin-resistant. Biologic properties of serotype 1 HA-L hemagglutinin were similar to those of Yamaguchi and Iritani's type 1 HA [18], whereas HA-HL hemagglutinin was to their type 2 HA.

Sawata *et al.* [16] previously reported that serotype specificity of hemagglutination was correlated with HA-L hemagglutinin of serotype 1 (HA-L1 hemagglutinin) and serotype 2 (HA-L2 hemagglutinin). In the present investigation, it was apparent that only trypsin-sensitive HA-L1 hemagglutinated GA-fixed RBC among the 3 types of serotype 1 hemagglutinin. Therefore, serotype specificity

of both serotypes 1 and 2 [16] resides in HA-L hemagglutinin active against GA-fixed RBC. HA-L hemagglutinin, if it is present in all strains, may be used for serologic typing of *H. paragallinarum*. On the other hand, trypsin-resistant hemagglutinins HA-HL and HA-HS did not agglutinate GA-fixed RBC. However, such a difference in hemagglutination pattern may be useful for differentiating HA-L hemagglutinin from hemagglutinins HA-HL or HA-HS.

Iritani *et al.* [3] suggested that hemagglutination of *H. paragallinarum* serotype 1 might be caused by pili. Previously, Sawata and Kume [13] demonstrated that the capsule of serotype 1 cells of *H. paragallinarum* completely inhibited agglutination both in homologous antiserum and freshly collected chicken RBC, and this was easily restored with hyaluronidase. The presence of such inhibitor in the cells of *H. paragallinarum* was preliminarily suggested by Rimler *et al.* [11, 12] and by Iritani *et al.* [4]. Hemagglutination of HA-L2 hemagglutinin was also activated by sonication following treatment with potassium thiocyanate [16]. Electron microscopic studies suggested that the location of hemagglutinins HA-L, HA-HL, and HA-HS is on outer membrane.

Sawata and Kume [13] suggested that the presence of HA-L hemagglutinin in *H. paragallinarum* serotype 1 cells were responsible for adherence or colonization, but not for virulence in chick. Present results showed that variants hi, ni-A, and R-A had at least 3 types of hemagglutinin HA-L, HA-HL, and HA-HS. HA-L hemagglutinin was masked with capsule in hi variant. After treatments with trypsin or heating at 121°C for 2 hr, HA-L hemagglutinin was completely removed, and, simultaneously hemagglutinins HA-HL or HA-HS appeared. The results indicate that both hemagglutinins HA-HL and HA-HS are completely masked with HA-L hemagglutinin. HA-HS hemagglutinin appeared to be at the most inner part of outer

membrane.

Sawata *et al.* [16] also suggested that HA-L hemagglutinin was a protective antigen of *H. paragallinarum*. In fact, HI test has been used for quality control of the *H. paragallinarum* serotype 1 vaccines. Yamaguchi and Iritani [18] found adult chicken RBC having only receptor to type 1 HA (HA-L hemagglutinin). In the present investigation, we could not find such RBC, but fresh RBC collected from day-old chicks, fresh human being (type O) RBC, and all the GA-fixed RBC used had only the same receptor. The result showed that specific antibodies against HA-L hemagglutinin can be detected using GA-fixed chicken RBC to estimate immunogenicity of *H. paragallinarum* vaccines.

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要 約

Haemophilus paragallinarum 血清型 1 菌株の赤血球凝集素: 澤田 章・久米勝巳・中井豊次 (北里研究所附属家畜衛生研究所) ——*Haemophilus paragallinarum* 血清型 1 菌株の各変異型菌を処理して得られた変性細菌細胞のニワトリ, ヒト O 型, ウサギの各新鮮血球ならびにグルタルアルデヒド固定血球に対する凝集性を調べたところ, 赤血球凝集性および生物学的性状の異なる 3 種の凝集素 (HA-L, HA-HL, 及び HA-HS) が認められた。すなわち, 易熱性, トリプシン感受性の HA-L は供試全血球を凝集したが, トリプシン耐性で易熱性の HA-HL または耐熱性の HA-HS は新鮮成鶏血球および新鮮ウサギ血球を凝集し, 他の血球は凝集しなかった。莢膜保有変異型菌の未処理細胞は全血球に対する凝集性を欠いたが, ヒアルロニダーゼで莢膜を除去すると HA-L 抗原が発現し, すべての赤血球を凝集した。HA-L 抗原をさらにトリプシンまたは 121°C で 2 時間加熱すると HA-HL または HA-HS が発現することから, HA-L 抗原は莢膜下の菌体最表層に存在する抗原と考えられた。赤血球凝集反応で電顕により変性細菌細胞外膜が赤血球表面に直接附着する像が認められたことから, 本菌のもつ 3 種の赤血球凝集素はいずれも細菌細胞外膜位にあると考えられた。