Haemagglutination profiles of *Helicobacter* species that cause gastritis in man and animals

NANCY S. TAYLOR, ANNA T. HASUBSKI, J. G. FOX and A. LEE*

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
and * School of Microbiology, University of New South Wales, Kensington, Sydney, Australia

Summary. Thirty-five *Helicobacter pylori* isolates, 21 *H. mustelae* isolates and four strains of *H. felis* were compared for their ability to agglutinate red blood cells (RBCs). Isolates were examined in a slide haemagglutination assay with RBCs from 11 animal species, including rodents, carnivores and primates, as well as man. RBCs were agglutinated by 65–90 % of *H. mustelae* isolates and 16–57 % of *H. pylori* isolates. Treatment of *H. mustelae* with pronase and heat inhibited haemagglutination (HA) whereas heating only of *H. pylori* inhibited HA. Treatment of all strains of *H. mustelae* with trypsin inhibited agglutination of human RBCs; 75 % of the treated strains did not agglutinate ferret RBCs. These results suggested that protein(s) may be important haemagglutinins for these bacteria. Variable HA profiles together with varying results after treatment of RBCs with fetuin, D-mannose, and neuraminidase suggested that multiple receptors may be involved in HA reactions with *H. pylori* and *H. mustelae*. The observation that *H. mustelae* and *H. pylori* agglutinated RBCs of several species and closely adhered to gastric epithelium supported the hypothesis that adherence plays a role in the colonisation and pathogenicity of *H. mustelae* and *H. pylori*. *H. felis* did not adhere to gastric epithelium and did not agglutinate RBCs of any species; nevertheless, *H. felis* can readily colonise and produce gastritis in several mammals.

Introduction

Helicobacter pylori is now firmly established as a causative agent of gastritis and, possibly, peptic ulcers in man.^{1,2} *H. pylori* are seen adhered closely to gastric epithelium, between intracellular junctions and in the mucus layer of the stomach.³ Similarly, *H. mustelae* causes gastritis, and possibly ulcers, in the stomach and duodenum of ferrets; it adheres to the gastric epithelium and is endocytosed.^{4,5} Like *H. pylori*, the ferret gastric organisms are found in the mucus layer of the stomach. *H. felis* has been isolated from the stomachs of cats and dogs.^{6,7} An active, chronic gastritis in germ-free mice and rats, and chronic inflammation in gnotobiotic dogs has been produced by oral inoculation of *H. felis*.^{8–10} The acute inflammatory response seen in gnotobiotic rodents is similar to that described in the early stages of *H. pylori* infection.^{8,9}

The observation that most bacterial adhesins are haemagglutinins is supported by the fact that bacterial adhesins share properties of moderate to strong hydrophobicity and the ability to recognise specific receptors.^{11,12} The chemical composition of the membranes of RBCs from various species differs and may give some clue to the receptor and adhesin necessary

for bacterial adherence. For example, the sialic acid content is high in guinea-pig and human RBCs, whereas O-acetyl derivatives of sialic acid are almost undetectable in RBCs of these species.^{13–16} In human RBCs, 95 % of the sialic acid is present as N-acetyl neuraminic acid.¹⁵ Human RBCs also contain the binding receptor for the fimbrial adhesin of *Escherichia coli* J96.¹⁷ Sheep RBCs contain the Forssman antigen that is also a receptor for *E. coli* J96.^{17,18}

Studies on haemagglutination (HA) profiles have suggested that adherence may be important in the colonisation and pathogenesis of *H. pylori* strains.^{19–21} Potential receptors for *H. pylori* have been purified and partially characterised; one is a glycerolipid, and another is N-acetyl neuraminyl lactose receptor for the *H. pylori* fibrillae.^{19, 21, 22} If HA of RBCs is related to adherence and, possibly, virulence, *H. mustelae*, an organism with similar pathogenicity to *H. pylori*, should also agglutinate RBCs. Although *H. felis* can cause gastritis, it does not adhere to gastric epithelium and should not agglutinate RBCs. The aim of this study was to compare the HA activity of *H. pylori* with the HA patterns of newly recognised gastric *Helicobacter* spp. in animals and to relate this HA to colonisation *in vivo*.

Materials and methods

Bacterial strains

All isolates were characterised as *H. pylori*, *H. mustelae*, or *H. felis* as described previously.^{1,2,4,6} Eleven *H. pylori* isolates were obtained from patients attending a gastroenterology clinic at Boston City Hospital. The other 24 isolates were obtained from the gastroenterology clinic at the Charity Hospital in New Orleans, LA. All 21 *H. mustelae* isolates were obtained from gastric biopsies of ferrets maintained in the Division of Comparative Medicine at Massachusetts Institute of Technology (MIT) over a 5-year period. *H. felis* type strain ATCC 49179 was obtained from a gastric biopsy of a cat at the University of New South Wales in Sydney, Australia. A further three strains of *H. felis* were obtained from stomachs of one cat and two dogs housed at the animal facility at the University of New South Wales.

Bacterial cultures

For the HA assay, *H. mustelae* and *H. pylori* were grown micro-aerophilically on Trypticase Soy Agar plates containing sheep blood 5% (Remel, Lenexa, KA, USA) for 48–72 h at 37°C. *H. felis* was grown on Blood Agar Base No. 2 (Oxoid) plates containing lysed horse blood 5%. The bacteria were harvested from the plates into phosphate-buffered saline (PBS) and washed three times with PBS. The suspension was adjusted spectrophotometrically (OD₆₆₀) to a density of *c.* 5×10^8 bacteria/ml.

Red blood cells

RBCs were obtained from various animal species housed at MIT. Group A and group B RBCs were obtained from human volunteers at the Division of Comparative Medicine. Blood was collected into heparinised tubes and centrifuged at 3000 rpm (Damon/IEC centrifuge model CRV 5000) for 10 min. RBCs were washed three times in PBS and used within 4 days of collection. RBCs were stored at 4°C at a concentration of 0.25% v/v in PBS.

HA assay

The HA assay was performed according to the method of Arthur *et al.*²³ A 10- μ l volume of RBC 0.25% suspension was thoroughly mixed with 10 μ l of bacterial suspension on a chilled glass slide. The slide was rotated at 60 rpm for 10 min on a rotary platform (American Rotator V, American Dade, Miami, FA, USA). The assay was modified to read the slides microscopically for evidence of HA. Isolates that agglutinated RBCs were diluted serially two-fold to determine the titre.

Inhibition assay

The HA assay was performed with pre-treated bacteria and non-treated RBCs or with pre-treated

RBCs and non-treated bacteria. Bacteria were pre-treated with pronase 0.02% or trypsin 0.025% at 37°C for 45 min before assay. Bacteria were also heated at 56°C for 45 min before assay. RBCs were pre-treated with fetuin 0.5 mg/ml, neuraminidase 4.0 μ g/ml or D-mannose 0.5% at 37°C for 1 h before assay. Trypsin was obtained from Whittaker MA Bioproducts, Walkersville, MD, pronase from Boehringer Mannheim, Indianapolis, IN, and fetuin, D-mannose and neuraminidase from Sigma Chemical Company, St Louis, MO, USA.

Comparison of HA assays

HA assays were performed on 14 strains of *H. pylori* by the assays described above and by the assays described previously.^{19,20} For the slide assay, 10 μ l of RBCs 2% v/v suspension was mixed with 10 μ l of bacteria at *c.* 5×10^7 cells/ml at room temperature. The assay was read visually after mixing for 1 min.¹⁹ For the microtitration plate assay, 20 μ l of RBCs 2% were added to 20 μ l of bacteria in the microtitration wells and the mixture was incubated at 4°C overnight before the HA patterns were read.²⁰ The assay was modified by adding bovine serum albumin (BSA), 1% v/v to the buffer in an attempt to reduce false positive reactions.

Results

HA assay

Neither *H. mustelae* nor *H. pylori* uniformly agglutinated RBCs of any one species tested in the cold slide agglutination assay. Of the 21 *H. mustelae* strains, 66–90%, and of the 35 *H. pylori* strains, 15–57%, agglutinated the RBCs of the species tested (table I).

Table I. RBC HA patterns of (%) *H. pylori* and *H. mustelae* isolates

Source of RBCs	Number (%) of strains that agglutinated RBCs	
	<i>H. mustelae</i> (21)	<i>H. pylori</i> (35)
Man*	19 (90.5)	10 (28.6)
Rabbit	16 (76.2)	13 (37.1)
Cat	14 (66.7)	7 (20.0)
Ferret	15 (71.4)	15 (42.9)
<i>Macaca mulatta</i>	14 (66.7)	ND
Dog	14 (66.7)	ND
<i>Macaca fascicularis</i>	17 (80.1)	ND
Sheep	17 (80.1)	ND
Guinea-pig	18 (85.7)	ND
Mouse	ND	5 (14.3)
Rat	ND	15 (42.9)
<i>Saimiri sciureus</i>	ND	20 (57.1)

H. felis did not agglutinate RBCs from any of the 12 species tested.

* Six strains (five *H. pylori* and one *H. mustelae*) were tested with human blood groups A and B. No difference in results was seen. ND = not done.

Figure. Inhibition by various treatments of agglutination of (a) human and (b) ferret red blood cells (RBCs) by *H. pylori* (▨) and *H. mustelae* (■).

Table II. Comparison of HA assays

<i>H. pylori</i> strain no.	Origin of RBCs	Slide assay	Micro-titration assay	<i>H. pylori</i> strain no.	Origin of RBCs	Slide assay	Micro-titration assay
C125	Cat	—	—(—)	C057	Cat	—	—(—)
C140	Cat	—	—(—)	C057	Mouse	—	—(+)
C140	Mouse	+	+(+)	BU22	Cat	—	—(—)
C140	Rabbit	—	—(+)	BU22	Mouse	—	—(—)
C140	Rat	+	+(+)	BU22	Rabbit	—	—(—)
C140	Man	+	+(+)	BU22	Rat	—	—(—)
BU7	Cat	—	—(+)	BU22	Man	—	—(—)
BU7	Mouse	—	—(+)	BU14	Cat	—	—(+)
BU7	Rabbit	+	+(+)	BU14	Mouse	+	+(+)
BU7	Rat	—	—(+)	BU19	Cat	—	—(+)
CO77	Cat	—	—(+)	BU19	Mouse	—	—(+)
CO77	Mouse	—	—(+)	J19	Cat	—	—(+)
CO77	Rabbit	—	—(+)	CO59	Cat	—	—(—)
CO77	Rat	—	—(+)	J11	Cat	+	+(+)
C144	Cat	—	+(+)	J11	Mouse	+	+(+)
C144	Mouse	—	—(+)	J11	Rabbit	+	+(+)
CO16	Cat	+	+(+)	J11	Rat	+	+(+)
BU21	Cat	—	—(—)	J11	Man	+	+(+)

Results were obtained by microscopic reading (100×) of slide assay and microtitration plate assay.

(+). Results obtained by visual reading of the microtitration plate.

The highest HA titre obtained from all strains was 128 but this was not consistent for the RBCs of any one species. All five of the *H. pylori* isolates and one strain of *H. mustelae* tested agglutinated human RBCs of

both groups A and B. The type strain of *H. felis* did not agglutinate RBCs of any of the 12 species tested, nor did three other strains of *H. felis* agglutinate RBCs of five different species.

Inhibition of HA

Inhibition of HA after various treatments of RBCs and bacteria to block or destroy potential receptors gave variable results (figure). Four strains of *H. mustelae* with titres against human RBCs and four *H. mustelae* strains with titres against ferret RBCs were examined for HA inhibition. Similarly, six isolates of *H. pylori* that agglutinated human RBCs and four isolates that agglutinated ferret RBCs were examined for HA inhibition. When bacteria were pre-treated with pronase and heat, HA of *H. mustelae* was uniformly inhibited with both human and ferret RBCs. Trypsin treatment of bacteria inhibited HA of all *H. mustelae* isolates and human RBCs, and three isolates did not agglutinate ferret RBCs. Heat uniformly prevented HA of *H. pylori* with both ferret and human RBCs. Of the *H. pylori* strains treated with trypsin and pronase, 75 % failed to agglutinate ferret RBCs. HA of human RBCs by *H. pylori* was less affected by these enzymes (figure).

D-Mannose, fetuin and neuraminidase inhibited agglutination of human RBCs with 50 % of the *H. pylori* and *H. mustelae* strains. These three agents were somewhat more effective in inhibiting HA of ferret RBCs by both *H. mustelae* and *H. pylori*. However, none of these compounds was effective in eliminating HA of all strains tested.

Comparison of HA assays

The results of two different methods for testing HA are shown in table II. In comparing 36 individual HA assays, the results of the slide assay, performed at room temperature and examined microscopically, agreed with the results obtained with the cold slide HA assay. However, the microtitration-plate assay was positive in 26 (72 %) of 36 assays on visual observation compared with the cold slide HA assay which was positive in 11 (30 %) of 36 tested. Results of 35 (97 %) of 36 tests examined microscopically agreed with the cold slide HA assay. The addition of BSA to the buffer did not reduce the number of reactions positive by visual examination.

Discussion

In our experience, the slide HA assay of Evans *et al.*¹² was difficult to read visually and microscopically, due to the high concentration of RBCs that was 2 % as compared with 0.25 % in the cold slide assay.¹⁹ However, in our study the results of 36 tests examined microscopically agreed with those of the cold slide HA assay. There were more positive results (72 %) with the visual microtitration plate HA assay than with the cold slide assay (30 %). However, more careful examination of the HA pattern by microscopy revealed many (39 %) false positive results emphasising some inherent inaccuracy in the former method.

Both *H. mustelae* and *H. pylori* agglutinated RBCs

of various species in the cold slide HA assay. Similar results have been shown previously with clinical isolates of *H. pylori*.^{19, 20, 24, 25} *H. mustelae* and *H. pylori* had variable patterns of HA with the RBCs of the 12 species examined. Other investigators have also reported variable HA profiles for *H. pylori* with RBCs of different species. However, there is some disagreement with respect to the HA profiles with different *H. pylori* isolates. Several investigators reported that all, or the majority of, their *H. pylori* strains agglutinated human RBCs.^{19, 20, 25, 26} Three HA patterns with human, sheep and rabbit RBCs have been observed with isolates of *H. pylori*.^{24, 27} The different results in HA patterns could be due to different assay conditions, different culture conditions, the length of storage of the isolates, serial passage of the bacterium, different receptors and haemagglutinins amongst *H. pylori* isolates.

For example, a novel glycerolipid was found in lipid extracts of human RBCs and human and pig stomach tissues and was recognised by viable *H. pylori*.²¹ Others have also suggested that a soluble haemagglutinin produced by *H. pylori* has a glycolipid receptor.²⁶ Another receptor identified for *H. pylori* on RBCs was N-acetyl neuraminylactose, that bound *H. pylori* fibrillar structures.¹⁹ Recent evidence has suggested that fibrillae were probably not involved in HA of the *H. pylori* strains assayed.²⁴ Several studies have implicated receptors for *H. pylori* based solely on HA data.^{19, 24, 27} Some authors have suggested that *H. pylori* possessed specific sialic acid receptors. Other studies have suggested that different receptors are also present.^{19, 24, 25, 27-29} Indeed, one report stated that strains of *H. pylori* and *H. mustelae* had common haemagglutinins that did not recognise sialic acid receptors.²⁰ Since HA was shown to be inhibited by treatment of the bacteria with heat and trypsin,²⁵ proteins may also be likely receptors for *H. pylori*.

Our data have indicated that multiple haemagglutinins and receptors may play a role in HA of RBCs by *H. pylori* and *H. mustelae*. The finding that 90.5 % of 21 *H. mustelae* isolates agglutinated human RBCs and 80 % and 85 % of *H. mustelae* agglutinated sheep and guinea-pig RBCs, respectively, implicates a sialic acid receptor. However, blocking assays with fetuin and neuraminidase treatment of human RBCs did not inhibit HA of all isolates, indicating that not all *H. mustelae* and *H. pylori* recognised sialic acid receptors on human RBCs. Blocking of HA by trypsin, heat and pronase suggested that a protein haemagglutinin may be important for HA of *H. mustelae*. However, it was not uniform among the *H. mustelae* strains with human and ferret RBCs (figure).

We conclude that *H. mustelae*, like *H. pylori*, can agglutinate the RBCs of a variety of species and that multiple receptors and haemagglutinins may be involved. These findings correlate with the close adherence of *H. mustelae* to the gastric mucosa.⁴ *H. felis*, that does not adhere to gastric mucosa, did not agglutinate the RBCs of any species tested. This

suggests that *H. felis* does not need to adhere to the gastric epithelium to colonise mucus and produce gastritis as observed in stomachs colonised by the adherent *H. pylori* and *H. mustelae*.^{8,9}

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