THE DETECTION OF ANTIBODY RESPONSE DURING IMMUNIZATION WITH *HELICOBACTER PYLORI* IN RABBITS BY INDIRECT IMMUNOFLUORESCENT ASSAY (IFA)

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Abstract. Detection of antibodies against *Helicobacter pylori* by indirect immunofluorescent assay (IFA) in rabbits has been developed as a non-invasive screening method. This study aimed to detect the antibody response by IFA after immunization with *Helicobacter pylori* in rabbits. Six healthy New Zealand white female rabbits were immunized subcutaneously with *Helicobacter pylori*, 4 times, on days 0, 14, 28 and 42, were used for IFA evaluation. Blood samples were then collected from each rabbit on day 0 and weekly, for twelve weeks. *H. pylori* were coated on slides and fixed with absolute methanol and air dried. The coated slides were incubated with sample rabbit sera for 50 minutes at room temperature and washed with PBS. The slides were incubated with goat anti-rabbit IgG antibody conjugated with FITC for 50 minutes at room temperature. The slides were evaluated by fluorescent microscopy. The rabbit sera had evaluated IFA titers starting from week 4 with a peak at week 8. Antibodies were constantly detected until the end of observation (week 12). The means $\log_2$ *H. pylori* antibody titers were 10.50 ± 1.32, 11.83 ± 1.065, 13.33 ± 0.19, 14.00 ± 0.23, 14.83 ± 0.37, 15.00 ± 0, 15.00 ± 0, 15.00 ± 0, and 14.83 ± 0.90 (mean ± SE, n = 6), respectively. Given the ability to detect *H. pylori* antibody in rabbits using IFA, the results of this experiment may be useful for evaluating the epidemiology and diagnosis of *H. pylori* infection in veterinary public health studies.

INTRODUCTION

Helicobacter pylori is a common bacterial infection in humans that is responsible for a variety of gastroduodenal pathologies, including peptic and gastric ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (Eck *et al*, 1997; Forman, 1998; Kabir, 2003; Suerbaum and Michetti, 2002). *H. pylori* infection can be diagnosed by tests requiring upper gastrointestinal endoscopy for the retrieval of a gastric biopsy specimen (microbiological culture, histological examination, and rapid urease tests) (Graham and Qureshi, 2001). During recent years, noninvasive diagnostic tests for *H. pylori* infection have gained in significance (Vaira and Vakil, 2001). Immunodiagnosis of *H. pylori* infection is attractive in comparison to other noninvasive diagnostic methods for the investigation of upper gastrointestinal symptoms (Newell and Stacet, 1993; Attallah *et al*, 2004). It has been suggested that animals are a reservoir of *H. pylori*, which may be of importance in human infection, and the role of *Helicobacter* spp in gastrointestinal diseases in dogs and cats is uncertained (Vaira *et al*, 1992). It has been known for years that gastric helicobacter-like organisms (HLO) are commonly present in the stomach of dogs but the relationship between these organisms and gastric diseases has never been resolved (Henry *et al*, 1987; Geyer *et al*, 1993; Hermanns *et al*, 1995; Eaton *et al*, 1996; Happonen *et al*, 1996; Yamazaki *et al*, 1998). Invasive *Helicobacter* spp infection diagnosis by histopathology and PCR have been investigated in necropsied dogs (Sailasuta *et al*, 2005). Indirect immunofluorescent assay (IFA) is easy to perform, has a high sensitivity and is inexpensive (Chan *et al*, 2003). It has been wildely used to screen for various infectious diseases, such as leptospirosis (Appassakij

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et al, 1995; Pradutkanchana *et al*, 2003) as well as autoimmune diseases, such as bullous pemphigoid (Chan *et al*, 2003). A *Helicobacter pylori* antibody detection in rabbit serum samples by indirect immunofluorescent antibody assay has been developed (Sailasuta *et al*, 2006). This study aimed to detect antibody response by IFA in rabbits after immunization with *H. pylori* evaluate serodiagnosis as a screening technique for *H.pylori* infection in domestic animals.

MATERIALS AND METHODS

Preparation of *H. pylori* cell lysate

The *H. pylori* specimens were kindly obtained from the Department of Microbiology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The bacterial cells were harvested, washed three times in phosphate-buffered saline (PBS; pH 7.2), and disrupted by a sonicator three times at 4 ˚C for 15 seconds each time (modified from Attallah *et al*, 2004). After centrifugation at 600*g* for 10 minutes at 4 ˚C, the protein content of the supernatant solution was determined with the use of bovine serum albumin as a standard (Lowry *et al*, 1951). The supernatant was split into aliquots and stored at -20 ˚C until used.

Production of anti-*H. pylori* antibody

A group of six healthy New Zealand female rabbits were immunized subcutaneously and intramuscularly at four different injection sites: both scapula regions of the fore limbs and the thigh muscle of hind limbs. Five hundred microliters of *H. pylori* cell lysate was diluted (by volume) with Freund's complete adjuvant and 0.5 ml was used for each injection site. Immunization took place 4 times, on days 0, 14, 28 and 42. The blood samples were then collected from all rabbits at week 0 and weekly for twelve weeks. The serum samples were then separated and stored at -20 ˚C until tested.

Indirect immunofluorescence assay

For IFA, xylane coated-slides were fixed with *H. pylori* at 108 cells/ml in cold methanol (-10 ˚C) for 15 minutes and air dried. The slides were reacted with 5 µl per well of rabbit sera at dilution factors of 2, 4, 8, and 16, for 30 minutes at room temperature. The slides were washed twice with 0.15 M phosphate-buffered saline (PBS, pH 7.2) for 30 minutes, stained with a 1:50 dilution of goat anti-rabbit immunoglobulin conjugated with FITC (Dako®, Denmark) for 50 minutes at room temperature. The slides were then washed twice in PBS for 5 minutes, mounted with buffer glycerol at pH 8.0 and observed under a fluorescent microscope. The detection of apple-green color in the spiral organism was scored as 2+, 3+, or 4+ in positive cells and 0 in negative cells. When the serum sample was positive, it was then rediluted for detection of the titer. The polyclonal anti-*H.pylori* antibody (Dako®, Denmark) and

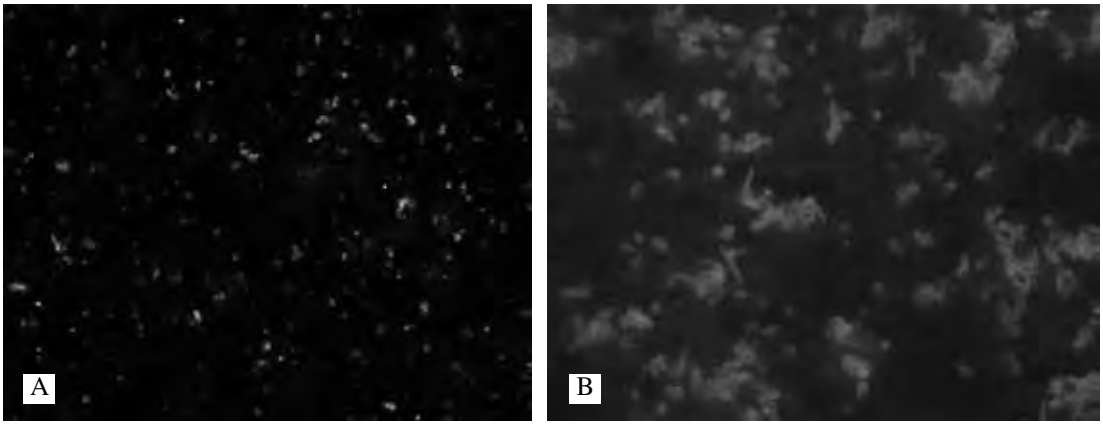

Fig 1- A: *H. pylori* under dark field microcopy (x400). B: Indirect immunofluorescence assay (IFA); *H. pylori* were positive in rabbit serum at 8 weeks after immunization, fluorescein-labeled antibody to rabbit IgG, Fluorescent microscope (x400).

Table 1
Geometric antibody titers against *H. pylori* in rabbits sera using IFA (n=6).

No	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6	Wk7	Wk8	Wk9	Wk10	Wk11	Wk12
Rabbit 1	0	0	0	13.00	13.00	13.00	14.00	15.00	15.00	15.00	15.00	15.00
Rabbit 2	0	0	0	7.00	13.00	13.00	14.00	14.00	15.00	15.00	15.00	15.00
Rabbit 3	0	0	0	13.00	13.00	14.00	14.00	15.00	15.00	15.00	15.00	15.00
Rabbit 4	0	0	0	5.00	6.00	13.00	15.00	15.00	15.00	15.00	15.00	16.00
Rabbit 5	0	0	0	13.00	13.00	14.00	14.00	15.00	15.00	15.00	15.00	13.00
Rabbit 6	0	0	0	12.00	13.00	13.00	13.00	15.00	15.00	15.00	15.00	15.00
Average	0	0	0	10.50	11.83	13.33	14.00	14.83	15.00	15.00	15.00	14.83
SD	0	0	0	3.25	2.61	0.47	0.58	0.37	0.00	0.00	0.00	0.90
SE	0	0	0	1.3281	1.065	0.1925	0.2357	0.1521	0	0	0	0.3664

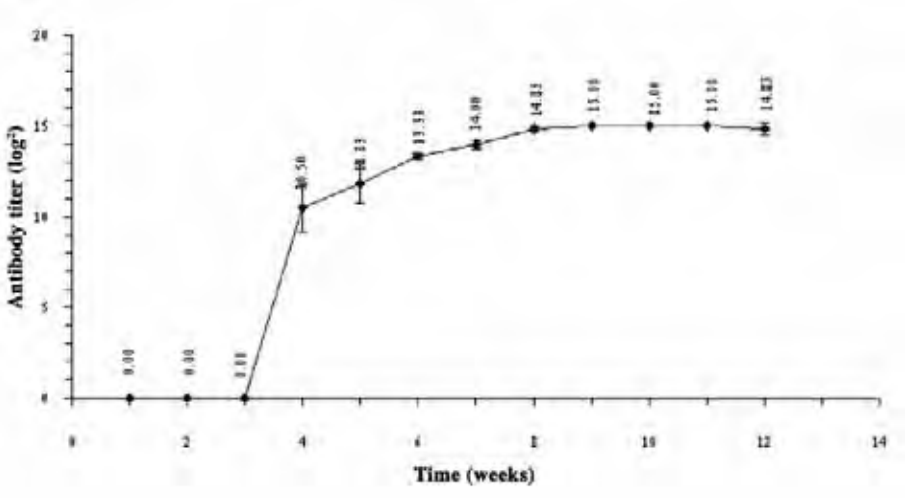

Fig 2- Mean of the $\log_2$ *H. pylori* antibody titer in rabbit sera determined by IFA during 12 weeks of observation. Serum samples collected from rabbits each week. The rabbits were immunized at days 0, 14, 28 and 42.

normal rabbit serum were used for positive and negative controls, respectively. The geometrical mean titers were used for evaluation of the titers each week in the six rabbits.

RESULTS

Reactivity of the developed anti-*H. pylori* antibodies by IFA

H. pylori lysate was observed on the fixed slide under a dark field microscope, and spiral and coccoid shapes were noted (Fig 1A). The serum samples that were collected weekly for 12 weeks. The results for the 72 serum samples examined are shown in Table 1. The means for the log2 of the *H. pylori* antibody titers were 10.50 ± 1.32, 11.83 ± 1.065, 13.33 ± 0.19, 14.00 ± 0.23, 14.83 ± 0.37, 15.00 ± 0, 15.00 ± 0, 15.00 ± 0, and 14.83 ± 0.90 (mean ± SE, n = 6), respectively. The *H. pylori* antibody titers in the rabbit sera was positive by IFA starting from week 4. The peak of the antibody titer was demonstrated at week 8 and was constantly detected until the end of observation (week 12) (Fig 2). Sera collected from the rabbits, immunized with the *H. pylori* lysate, at the week 8th had fluorescein-labeled antibody to rabbit Ig under fluorescent microscope (Fig 1B).

DISCUSSION

The IFA has been used in diagnostic laboratories successfully for 2 decades and the potential utility of IFA for the detection and quantification of leptospirosis antibody has been well documented (Appassakij *et al*, 1995; Pradutkanchana *et al*, 2003). Similarly, the capability of IFA for the detection of *H. pylori* infection in serum as a non-invasive method has been reported (Attallah *et al*, 2004). A panel of 72 serum samples showed a classical IgG immune response to *H. pylori* (Anderson *et al*, 1986; Boonpucknavig and Doungchawee, 1997). In this experiment, the sera were then examined for IFA, and the specificity of any positive reactions was checked against the control well. IFA does give some false-positive results due to unwanted positive protein in the serum (Boonpucknavig and Doungchawee, 1997). It has been reported that *H. pylori* in humans crossreacts with several antigens from *P. aeruginosa*, *H. influenzae* and *C. jejuni* (Johansen *et al*, 1995). Thus, the high antibody titers in the experiment rabbits should be reconsidered depending on the prevalence of infection. The antibody response in all six rabbits in this study were synchronized. The sera obtained from this study can be used as *H. pylori* antiserum for in-house diagnosis. The IFA described appears to be sufficiently sensitive and specific to be useful for the detection of *H. pylori* antibody. This method is an alternative, is easy-to-use, and can be applied as a non-invasive test for the detection of *H. pylori* infection in domestic animals as a benefit for veterinary public health.

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