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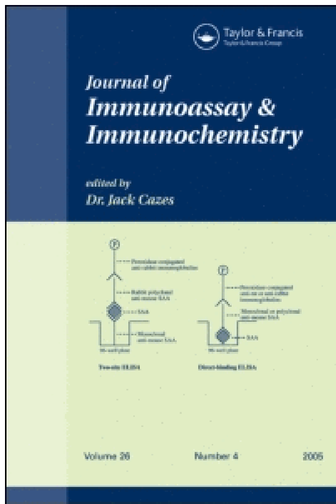

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Antibody Response to *Helicobacter pylori* Excretory Antigen and the Cross Reaction Study

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Abstract: *Helicobacter pylori* is recognized as a major case of gastritis and peptic ulcer and a key factor in the development of gastric cancer, gastric lymphoma, and non-ulcerative dyspepsia in man. The detection of antibodies specific for strains of *H. pylori* has demonstrated the value of serology for providing evidence of infection. The present study was conducted to detect the antigenic proteins of excretory antigen of *H. pylori* with Western blotting and examine whether anti-*H. pylori* IgG and IgA antibodies from *H. pylori* positive patients cross-react with antigens from other common bacterial pathogens. By using SDS-PAGE, 20 different proteins were found in the excretory antigen. By Western blotting and absorption studies, there were indications that anti-*H. pylori* IgA antibodies directed against 54 kDa, 50 kDa and 27 kDa cross-reacted with antigens from other bacteria, and that *H. pylori* proteins of 99 kDa, 88 kDa and 81 kDa possibly shared similar epitope with antigens of other pathogens not tested in the absorption studies. The cross-reactivity occurred in this study was not significantly affect the performance of the in-house ELISA.

Keywords: Excretory proteins, *Helicobacter pylori*, In-house ELISA, Western blotting

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INTRODUCTION

Helicobacter pylori is recognized as a major case of gastritis and peptic ulcer and a key factor in the development of gastric cancer, gastric lymphoma, and non-ulcerative dyspepsia in man.[1,2] Patients infected with the organisms have been shown to produce serum antibodies to *H. pylori* antigens. The detection of antibodies specific for strains of *H. pylori* has demonstrated the value of serology for providing evidence of infection. A number of different serological techniques have been used to detect patient antibodies, including haemagglutination, complement fixation, coagulation, indirect immunofluoresence, and latex agglutination.[3]

However, ELISA and immmunoblotting have emerged as the two most frequently used techniques. In our laboratory we established our own in-house ELISA to detect the anti-*H. pylori* antibody. Antigen played a major role in the serological test. The specificity of our in-house ELISA with secretary antigen was highest. Hence, with Western blotting, we can prove that the antigen we used in our in-house ELISA was specific for anti-*H. pylori* antibody and absorption of positive serum with *H. pylori* alone or *H. pylori* and with cocktail of other pathogens will not show a positive results. In spite of that, absorption of positive serum with cocktail of pathogens alone showed positive results. With that, we can identify that all proteins in the excretory antigen which only react with *H. pylori* antigen and not with antigen of other pathogens. The present study was designed to use SDS-PAGE, in-house ELISA, and an immunoblot assay to investigate the number of proteins which react as an antigenic protein, with particular emphasis on whether cross reaction could occur with *H. pylori* antibody and antigens of other pathogens in an in-house ELISA. The results that we obtained from this study were important to understand the cross reaction which might be happen and how this can influence the in-house ELISA.

EXPERIMENTAL

Study Population

Blood samples were collected from 10 *H. pylori* positive subjects and 10 *H*. pylori negative subjects, confirmed by endoscopic investigation and showed the highest OD_{490nm} for positive subjects and lowest OD_{490nm} for negative subjects by an in-house ELISA. This serum was pooled so that there was enough serum sample for the whole experiment. The pooled positive and negative sera were absorbed with *H. pylori*, or *H. pylori* and ten other bacterial cells, and pooled sera without absorption were used for ELISA study.

Microorganisms

Helicobacter pylori strain 47 was used for the preparation of excretory antigen. The strain was cultured on blood agar plates (Eugonagar; BBL,Cockeysville, MD.,USA) containing 10% (v/v) human blood, incubated in a incubator (Shel Lab) under microareobic (10% CO_2 atmosphere conditions) at 37°C for 96 h. *Escherichia coli, Pseudomonas stutzeri, Proteus vulgaris, Salmonella typhi, Shigella dysenteriae, Vibrio cholerae*, and *Vibrio parahaemolyticus* were cultured on blood agar plates and incubated at 37°C for 18 h, while *Campylobacter jejuni, Campylobacter coli*, and *Campylobacter fetus* were cultured on Muller-Hinton agar at 37°C and incubated under microareobic (10% CO_2 atmosphere) conditions at 37°C for 3 days.

Preparation of Excretory Antigen

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Bacterial cells were harvested and placed into preweighed, screw-capped Eppendorf tubes. Bacteria were sedimented by centrifugation (3,000 rpm, 15 min) (Mini Spin Plus, Germany); we removed the supernatant and pipetted 1 mL dH_2O and vortexed vigorously to mix. The suspended cells were incubated at 37°C for 5 h. After that, centrifuge (3,000 rpm, 15 min) again. Transfer the supernatant containing the excretory antigen into a clean 1.5 mL micro-centrifuge tube. The aliquots were stored at –70°C until required for SDS-PAGE, in-house ELISA, and immunoblotting.

SDS-PAGE

The SDS-PAGE profiles of excretory antigen were prepared using the method as described by the Instruction Manual (170-3930; Bio-Rad Laboratories, USA). Gels comprised a 4.5% (w/w) acrylamide stacking gel and 12.5% (w/w) acrylamide separation gel. Samples were applied to gels alongside protein molecular weight standards (MBI, Fermentas, USA). Electrophoresis was performed using a Protean II xi cell (170-3930; Bio-Rad Laboratories, USA) with a constant current of 25 mA for 1.5 h. Gels, were either stained with Coomassie Blue[4] or were used for immunoblotting.

Immunoblotting

The SDS-PAGE protein profiles were transferred onto nitrocellulose sheets (0.10 μm, Biometra B. Braun Biotech) using an electro transfer apparatus ((Bio-Rad Richmond CA, Mini Trans–Blot Electrophoretic

Transfer Cell) for 1 h at 100 V. Individual protein profiles were prepared by cutting the nitrocellulose sheets into strips with pinking shears to enable realignment of one part with protein molecular weights standards and protein profiles for amido black staining (Fluka, Chemie, Switzerland). After blocking with 10% (w/v) skimmed milk in phosphate-buffered saline (PBS; 140 mmol-L NaCl, 2.7 mmol-L KCl, 8 mmol L^{-1} Na_2HPO_4, 1.5 mmol-L KH_2PO_4) for 12 h at 4°C and washing (three times) with PBS containing 0–05% Tween 20 (PBS-Tween); strips were incubated with pooled sera (500 μL/ stripe, 1:200 dilution with PBS) for 90 min at room temperature. Following washing (three times) with PBS-Tween, antibody-antigen complexes were detected with anti-human-IgA and IgG (1:1,000 dilution with PBS; Sigma Chem., USA) antibody conjugated with peroxidaes. Strips were washed as above before adding the enzyme substrate comprising 0.015 g peroxide horseradish (Bio-Rad Richmond Ca) diluted in 5 mL methanol (Mallinckrodt), 25 mL PBS, and 5 μL hydrogen peroxide (Merck KgaA, Darmstadt, Germany). Color development was carried out for 10 min. The enzyme reaction was stopped by washing strips with deionized water.

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ELISA

An in-house ELISA was used as described previously.[5] The pooled positive and negative serum samples were diluted (1:100) in PBS 500 μL added to ELISA plate wells. After incubation for 90 min at 37°C, plates were washed (three times) with the wash buffer PBS-Tween, which was then followed by the addition of 50 mL peroxidae-conjugated antibody specific for human IgG and IgA immunoglobulins and reincubation for 90 min at 37°C. Unbound antibodies were removed by washing as above and 50 μL substrate solution was then added. Colour development was carried out in the dark for 10 min prior to the addition of 50 μL (2 M, H_2SO_4) stop solution. The absorbance was read within 10 min at 490 nm. Absorbance values of 1.0 or above for IgG and 0.1 or above for IgA were considered as indicative of significant levels of antibody according to our in-house ELISA. The sensitivity, the specificity, positive predictive value, and negative predictive value were 90%, 92%, 69%, and 98% for IgG-ELISA, 69%, 98%, 88%, and 94% for IgA-ELISA, and 86%, 97%, 85%, and 97% for both IgG and IgA, respectively.

RESULTS

The OD_{490nm} value results for pooled positive and negative sera absorbed with *H. pylori* or; *H. pylori* and ten other bacterial cells or; ten other

bacterial cells and the pooled serum without absorption for ELISA are shown in Table 1. All the pooled negative sera gave OD_{490nm} values less than the cut-off value, but the differences of OD_{490nm} values for pooled positive sera was significantly different ($F = 474.824$ for IgG and $F = 410.895$ for IgA, $p < 0.05$) and greater than the cut-off value. The decreasing of titer/OD_{490nm} values below the cut off value for IgG-ELISA and IgA-ELISA was observed for pooled positive sera absorbed with *H. pylori* and *H. pylori* and ten other bacterial cells. But, pooled positive sera absorbed with the ten other bacterial cells didn't show such a decrease in titer/OD_{490nm} value compared to the positive control sera (Table 1). Although the OD_{490nm} values varied between pooled positive control sera and pooled sera absorbed with the ten other bacterial cells, but the values were higher than the cut off value for IgG-ELISA and IgA-ELISA.

SDS-PAGE of EA proteins consisted of 20 types of proteins as detected by staining with Coomassie brilliant blue (Fig. 1). The strength of band 99 kDa, 88 kDa, 81 kDa, 72 kDa, 64 kDa, 39 kDa, 35 kDa, 33 kDa, 27 kDa, 17 kDa, and 15 kDa with Coomassie brilliant blue were weak and they were minor protein bands. Meanwhile, protein of 113 kDa, 62 kDa, 59 kDa, 54 kDa, 50 kDa, 32 kDa, 30 kDa, 25 kDa, and 22 kDa were recognized as major protein bands and this finding was comparable with others works.[6–8]

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Table 1. OD_{490nm} value for pooled positive and negative sera for in-house-ELISA

OD_{490nm} value for IgA-ELISA				OD_{490nm} value for IgG-ELISA			
A[a]	B[b]	C[c]	D[d]	A	B	C	D
0.698	0.003	0.662	0	2.12	0.195	1.557	0.221
0.648	0	0.522	0	2.311	0.196	2.043	0.638
0.744	0	0.714	0.001	2.207	0.08	2.128	0.497
0.639	0	0.47	0	1.96	0.018	1.749	0.532
0.498	0.006	0.474	0	1.941	0.179	1.846	0.534
0.641	0.001	0.616	0	1.837	0.14	1.507	0.336
0.613	0.004	0.541	0	1.973	0.211	1.972	0.56
0.773	0	0.606	0.009	2.012	0.317	1.726	0.215
0.862	0	0.526	0.009	2.024	0.115	2.000	0.218

[a]Sera without absorption as control.
[b]Absorption with *H.pylori*.
[c]Absorption with other bacterial cells.
[d]Absorption with *H.pylori* and other ten bacterial cells.
$P = 0.0001$ for IgA-ELISA.
$P = 0.0001$ for IgG-ELISA.

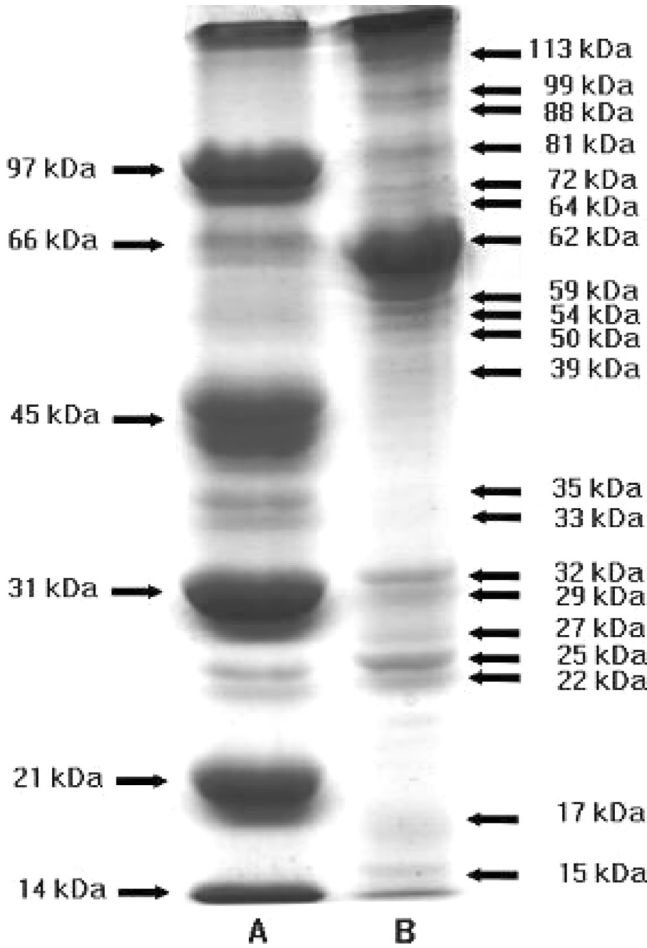

Figure 1. The SDS-PAGE profiles of excretory antigen with numerous proteins as detected by staining with Coomassie brilliant blue. A) Protein molecular weight standards; B) Excretory antigen.

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The replicate profiles were transferred onto nitrocellulose membranes and reacted with pooled positive and negative sera. The intensity of the immunoblot reaction was designated as +2, +1 or –VE for negative reaction, on the basis of the strength of the antibody reaction observed. In this study, the IgG and IgA anti-EA antigen antibody gave a different pattern of reaction.

IgG anti-EA antigen antibody tested with pooled positive sera absorbed with the cocktail of ten other bacteria cells and pooled positive control sera were reacted with 18 protein bands among the 20 proteins

bands. The 99 kDa, 81 kDa, 59 kDa, 39 kDa, 35 kDa, 33 kDa, and 32 kDa protein bands (Strip A and D; Fig. 2) gave a +2 reaction compared to other protein bands. IgA anti-EA antigen antibody gave a positive reaction with the protein of 113 kDa, 99 kDa, 88 kDa, 81 kDa, 72 kDa 63 kDa, 62 kDa, 54 kDa, 50 kDa, and 27 kDa. All the protein bands gave a +2 reaction for pooled positive control, but for pooled positive sera absorbed with ten other bacterial cells, it gave a +2 reaction with the protein of 54 kDa, 50 kDa and 27 kDa compared to other protein bands. (Strip A and D; Fig. 3).

Nevertheless, pooled positive sera absorbed with *H. pylori* or; *H. pylori* and other ten bacterial cells gave a –Ve reaction (Strip B and C; Fig. 2; Strip B and C; Fig. 3). Pooled negative sera tested with IgA anti-EA antigen antibody revealed a –VE reaction for all strips (Fig. 5) but, for IgG anti-EA antigen antibody, it gave a positive reaction with the 99 kDa, 88 kDa, and 81 kDa protein bands (Strip A and D; Fig. 4) and protein bands 81 kDa gave a +2 reaction compared to +1 by the other two protein bands.

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DISCUSSION

The aim of the present study was to evaluate the reliability of an in-house ELISA for the detection of human antibodies against *H. pylori* infection as a means of providing evidence of an interspecies antigenic cross-reactivity and the maintenance of the specificity of the in-house ELISA. In this study, a number of proteins exist in the excretory protein of *H. pylori* was identified by electrophoresis SDS-PAGE and Coomasie blue stain. The protein profile shown by the excretory antigen was comparable to the previous studies reported by other researchers.[6–8] A Western blotting technique was used to identify antigenic protein components present in the excretory antigen. In addition, the in-house ELISA, Western Blotting, and an Absorption Study were used to determine the interspecies antigenic cross-reactivity.

The pooled positive and negative sera were absorbed with *H. pylori* or; *H. pylori* and ten other bacterial cells and tested by the in-house ELISA to determine whether anti-*H. pylori* antibody is able to react with the antigens of other pathogens. The pooled sera without absorption acts as control. Predictable results were obtained. The OD_{490nm} value decreases lower than the cut-off point for IgG-ELISA and IgA-ELISA when the pooled positive serum was absorbed with *H. pylori* or *H. pylori* with the cocktail of the other ten bacterial cells. The result has suggested that the absorption is complete and adequate. The OD_{490nm} values obtained in this study were higher than the cut off value, although they were lower than the positive control serum for IgG-ELISA and IgA-ELISA when positive serum was absorbed with the cocktail of other

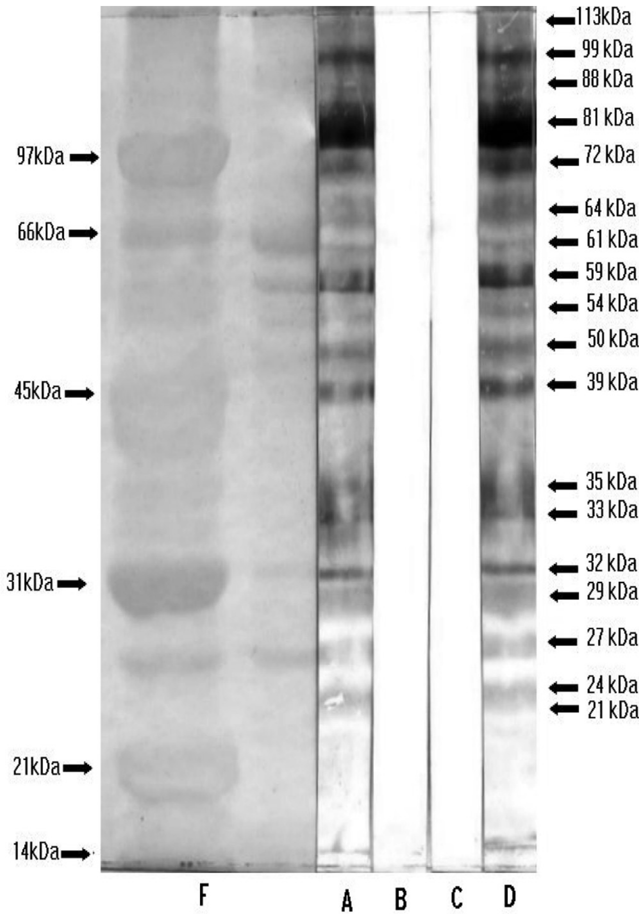

Figure 2. Western blotting of excretory antigen and the reaction with IgG antibody of pooled positive sera. A) Pooled positive sera without absorption as control; B) Absorption with *H. pylori*; C) Absorption with *H. pylori* and other ten bacterial cells; D) Absorption with other ten bacterial cells; E) Protein molecular weights standards and excretory antigen were transferred onto nitrocellulose sheets and detected by staining with amido black.

bacterial cells. The result suggested that the cross-reactions happened between *H. pylori* positive serum and the other bacterial antigens. However, decreasing the amount of antibodies which caused the cross-reactions when the pooled positive serum was absorbed with the cocktail of other bacterial cells did not affect, significantly, the in-house ELISA result ($p < 0.05$) in this study. For the negative pooled sera, the OD_{490nm} values obtained for all types of absorption and negative control serum

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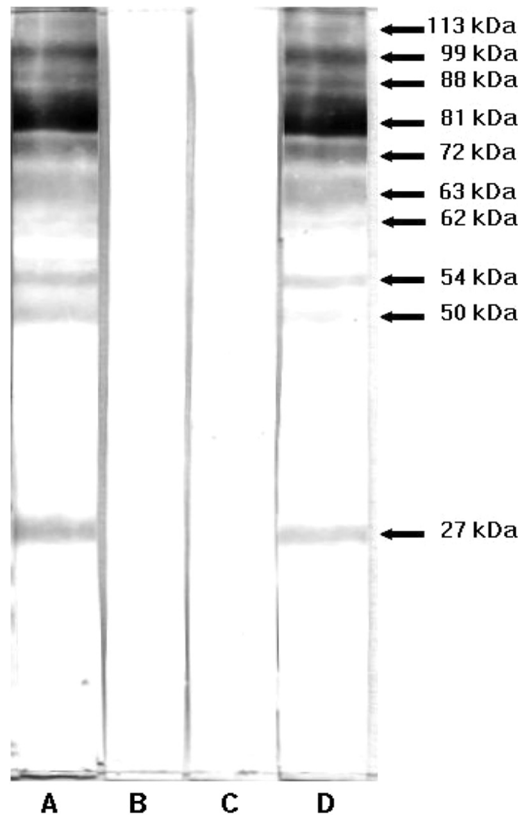

Figure 3. Western blotting of excretory antigen and the reaction with IgA antibody of pooled positive sera. A) Pooled positive sera without absorption as control; B) Absorption with *H. pylori*; C) Absorption with *H. pylori* and other ten bacterial cells; D) Absorption with other ten bacterial cells.

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was lower than the cut-off value for IgG-ELISA and IgA-ELISA, as predicted.

Twenty different proteins were identified in EA by using SDS-PAGE. The Western blotting study with the pooled positive sera showed that, amongst the 20 proteins of EA, only 18 reacted with IgG, whereas only 10 reacted with IgA. Pooled positive sera that absorbed the cocktail of the other bacterial cells exhibited a weak reaction with the proteins of 54 kDa, 50 kDa, and 27 kDa in comparison to the control sera. These finding suggested that there was a cross-reaction between *H. pylori* positive sera with the antigens of other pathogens. However, the cross-reaction which occurred in this study was not significant because there were other proteins of EA still reactive with *H. pylori* positive serum antibody.

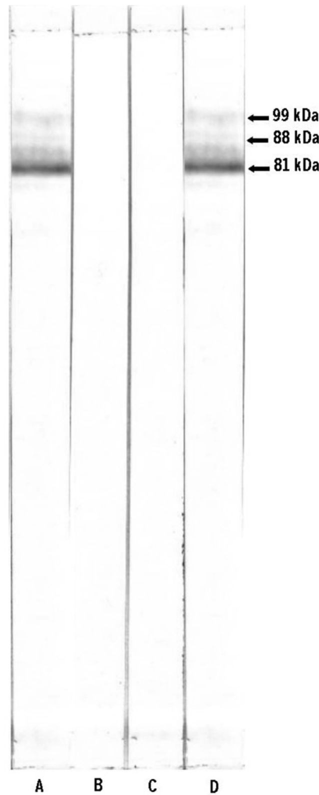

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Figure 4. Western blotting of excretory antigen and the reaction with IgG antibody of pooled negative sera. A) Pooled negative sera without absorption as control; B) Absorption with *H. pylori*; C) Absorption with *H. pylori* and other ten bacterial cells; D) Absorption with other ten bacterial cells.

For the pooled *H. pylori* negative sera, 3 proteins (99 kDa, 88 kDa, and 81 kDa) reacted with IgG. But, such a reaction did not occur with IgA. There was a report which stated that *H. pylori* negative serum were reacted with high molecular weight antigen.[9] The results of this study suggested that the negative serum absorbed with the cocktail of other bacterial cells and the *H. pylori* negative serum interacted with 99 kDa, 88 kDa, and 81 kDa molecular weight proteins. The cross-reaction might be due to the sharing of an epitope with antigens of other pathogens, which was not studied in this research.[10] The finding also suggested why the OD_{490nm} value for the pooled negative serum and pooled positive serum, which was absorbed with *H. pylori* or the mixture of other bacterial cells with *H. pylori*, was still giving a reading which was not zero. Even though the cross-interaction happened in this study, the detected

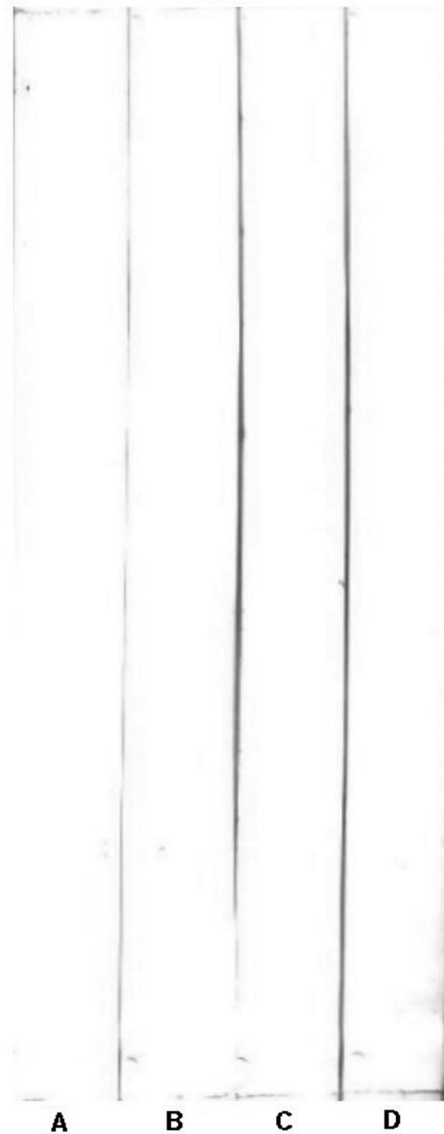

Figure 5. Western blotting of excretory antigen and the reaction with IgA antibody of pooled negative sera. A) Pooled negative sera without absorption as control; B) Absorption with *H. pylori*; C) Absorption with *H. pylori* and other ten bacterial cells; D) Absorption with other ten bacterial cells.

sensitivity and specificity values of the in-house ELISA was still high. This was due to a very weak cross-reaction which did not give a significant effect on the specificity of the in-house ELISA.

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In conclusion, the cross reaction which happened between *H. pylori* antigen and the antigen of other pathogens did not affect the performance of the in-house ELISA.

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