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Immunoproteomics of *Helicobacter pylori* infection and relation to gastric disease

The Gram negative bacterium *Helicobacter pylori* is a human pathogen which infects the gastric mucosa and causes an inflammatory process leading to gastritis, ulceration and cancer. A systematic, proteome based approach was chosen to detect candidate antigens of *H. pylori* for diagnosis, therapy and vaccine development and to investigate potential associations between specific immune responses and manifestations of disease. Sera from patients with active *H. pylori* infection (n = 24), a control group with unrelated gastric disorders (n = 12) and from patients with gastric cancer (n = 6) were collected and analyzed for the reactivity against proteins of the strain HP 26695 separated by two-dimensional electrophoresis. Overall, 310 antigenic protein species were recognized by *H. pylori* positive sera representing about 17% of all spots separated. Out of the 32 antigens most frequently recognized by *H. pylori* positive sera, nine were newly identified and 23 were confirmed from other studies. Three newly identified antigens which belong to the 150 most abundant protein species of *H. pylori*, were specifically recognized by *H. pylori* positive sera: the predicted coding region HP0231, serine protease HtrA (HP1019) and Cag3 (HP0522). Other antigens were recognized differently by sera from gastritis and ulcer patients, which may identify them as candidate indicators for clinical manifestations. The data from these immunoproteomic analyses are added to our public database (http://www.mpiib-berlin.mpg.de/2D-PAGE). This platform enables one to compile many protein profiles and to integrate data from other studies, an approach which will greatly assist the search for more immunogenic proteins for diagnostic assays and vaccine design.

Keywords: Antigen / Pathogenesis / Proteome analysis / Immunoblotting / Two-dimensional electrophoresis
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1 Introduction

The Gram negative bacterium *Helicobacter pylori* is a human pathogen, which infects the gastric mucosa, the prevalence is as high as 50% of the worlds population and 35% of the European population [1]. It has been shown recently that *H. pylori* is the cause for an inflammatory process leading to gastritis, duodenal or gastric ulcer and in some cases even to mucosa-associated lymphoid tissue lymphoma or gastric carcinoma [2–6]. The WHO declared *H. pylori* as a definitive carcinogen and 1 in 34 infected men and 1 in 60 infected women die of the consequences of *H. pylori* infection [7].

Diagnosis of *H. pylori* can be obtained by invasive and noninvasive methods [8]. Gastroduodenal endoscopy is performed in the case of suspected ulcerations or cancer, and gastric biopsies are taken for further analysis. These biopsies can be investigated for the presence of *H. pylori* using the enzymatic urease test, histology, direct microscopy, culture, and PCR. The genes *cagA*, *vacA*, *ureA* and *ureC* have been detected directly in biopsies by specific PCR [9–11]. A noninvasive test for the presence of *H. pylori* is the ^{13}C-urea breath test which also allows monitoring of the infection. Serological tests such as enzyme-linked immunosorbent assay (ELISA) and immunoblots are being used to characterize *H. pylori* protein recognition, but were so far not efficient in detecting early or low humoral responses and in monitoring the effect of therapy. Several diagnostically relevant antigens like the 26 kDa antigen and the 35 kDa antigen were characterized only by their molecular mass using one-dimensional SDS-PAGE immunoblots [12–14] and there is a need for more and precisely characterized antigens of diagnostic value.

H. pylori infection can be treated by a "triple therapy" combining a proton pump inhibitor with two antibiotics or, if not successful, by quadruple therapy [15]. However, the high rate of persistent infections, increasing antibiotic

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Abbreviation: MALT, mucosa associated lymphoid tissue

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resistance and the potential benefit from therapeutic immunization are the rationale to develop a simple low cost vaccine against *H. pylori* [16]. Although protection against *H. pylori* has been shown in a gene deficient mouse model to be mediated by $CD4^+$ T cell responses, the protective antigens tested in animal models so far are also seen by antibodies [17–23]. In this respect, antibody recognition is a good tool to identify vaccine candidates. Immunoproteomics is a very sensitive method, as antigens including CagA, catalase, HpA, HspA, SodB, urease A and B can be easily detected by two-dimensional electrophoresis and immunoblot analysis [24–26]. The advantage of 2-DE combined with MALDI-MS is the combination of high sensitivity and clear characterization of antigens not only by a rough molecular mass estimation, but by a distinct assignment to amino acid sequences [24, 27] and further allows the analysis of complex immunoreactivity patterns. As *H. pylori* shows a high degree of variation between different strains, vaccine candidates should be conserved and the only way to screen a large number of possible antigens is based on the selection of a defined *H. pylori* strain.

Here we present data obtained from a comprehensive immunoproteomics study using the *H. pylori* strain 26695, which has a completely sequenced genome thus facilitating exact identification of candidate antigens [28]. In order to evaluate differences in immunoreactivity patterns, antibody responses against this strain were analyzed in sera from patients with different clinical manifestations. A variety of different immunoreactivity patterns was found by comparing sera from patients with active *H. pylori* infection and sera from controls with nonrelated gastric disorders. Several further differences in immune recognition patterns were seen between sera from patients with *H. pylori* related ulcer and gastritis.

2 Methods

2.1 Patients

In this study, patients with gastric disorders who underwent gastroduodenal endoscopy in the Medical Center, Charité, Campus Virchow-Klinikum in Berlin were tested for active *H. pylori* infection. Clinical parameters leading to diagnosis, history of previous *H. pylori* infection, treatment of *H. pylori* infection, clinical disorders, further medication and diseases were recorded. The study has been approved by the ethical committee of the Charité, Campus Virchow-Klinikum. Clinical diagnosis of *H. pylori* was obtained from gastric biopsies by evaluating culture, histology and quick urease assay (CLO test). According to these criteria, only 24 patients showed active *H. pylori* infection and were not treated at the time of evaluation. One of these 24 patients, for whom *H. pylori* culture was not done, had positive histology, CLO test and high serum antibodies against *H. pylori* in ELISA (Behring, Marburg, Germany) and was thus also included. Twelve patients with negative results in all tests, did not report or show any sign of prior infection and were considered negative for *H. pylori*. From the six cancer patients, five had gastric cancer and one had MALT (mucosa associated lymphoid tissue) lymphoma of the upper gastrointestinal tract. One of these patients was *H. pylori* positive and four had positive CLO tests, which could however also be related to other bacterial infections. Based upon the criteria mentioned above, active *H. pylori* infection could not be clearly defined for the majority of the cancer patients and they were considered as a separate group.

2.2 *Helicobacter pylori* growth conditions

H. pylori 26695, a strain with an entirely sequenced genome [28] was grown on serum plates [29] at 37°C in a microaerobic atmosphere (5% O_2, 85% N_2, and 10% CO_2) for two days. The bacteria were harvested, washed twice in ice-cold PBS containing proteinase inhibitors (1 mM PMSF, 0.1 μM pepstatin, 2.1 μM leupeptin, 2.9 mM benzamidin) and lysed by resuspension in half a volume of distilled water. The resulting volume in μl was multiplied by (i) 1.08 to obtain the amount of urea in mg to be added; (ii) 0.1 to obtain the volume of 1.4 M DTT and 40% Servalyte (Serva, Heidelberg, Germany) p*I* 2–4 to be added. CHAPS was included to obtain an end concentration of 1%. The end concentrations of DTT and urea were 70 mM and 9 M, respectively. Solubilization of the proteins occurred within 30 min at room temperature. A protein concentration of 15 μg/μL +/− 25% was obtained.

2.3 Two-dimensional electrophoresis

H. pylori proteins were resolved using a 7 cm × 8.5 cm 2-DE gel system [30] with a resolution power of about 1 000 protein species. Twenty μg of protein were applied to the anodic side of the IEF gel. In the second dimension 1.5 mm thick gels were used. The proteins were detected by silver staining optimized for these gels [30]. For the identification of proteins and immunoblotting, 20 μg of protein were applied. The proteins were stained in gels by Coomassie Brilliant Blue R250 [31] or G250 as described [32].

2.4 Peptide mass fingerprinting

Peptide mass fingerprinting was performed as previously described [24, 33]. Optimized conditions included the use of volatile buffer, decreased trypsin concentrations,

and reduction of volumes below 20 μL allowed the identification of weakly stained Coomassie Blue G-250 protein spots starting with only one excised spot. The peptide solution was mixed with an equal volume of a saturated α-cyano-4-hydroxy cinnamic acid solution in 50% acetonitrile, 0.3% TFA and 2 μL were applied to the sample template of MALDI mass spectrometer (Voyager Elite, Perseptive Biosystems, Framingham, MS, USA). Data were obtained using the following parameters: 20 kV accelerating voltage, 70% grid voltage, 0.050% guide wire voltage, 100 ns delay, and a low mass gate of 500.

Peptide mass fingerprints were searched using the program MS-FIT (http://prospector.ucsf.edu/ucsfhtml/msfit.htm) by reducing the proteins of the NCBI database to the *Helicobacter* proteins and to a molecular mass range estimated from 2-DE 20% and allowing a mass accuracy of 0.1 Da for the peptide mass. In the absence of matches, the molecular mass window was extended. Partial enzymatic cleavages leaving two cleavage sites, oxidation of methionine, pyroglutamic acid formation at *N*-terminal glutamine and modification of cysteine by acrylamide were considered in these searches.

2.5 Immunoblotting

For immunostaining the proteins were transferred from the 2-DE gels onto PVDF membranes (ImmobilonP, Millipore, Eschborn, Germany) by semidry blotting [34] using a blotting buffer containing 100 mM borate, 20% methanol, pH 9.0. The blotting time was 2 h with a current/area of 1 mA/cm^2. Next, the membrane was blocked with 5% skim milk, 0.05% Tween-20 in PBS for at least 1 h at room temperature. After blocking the membrane was washed 3 times for 5 min. All washing steps were performed with PBS, 0.05% Tween 20. Antigens were detected by incubation of the membranes with human sera for one hour in a dilution of 1:200, followed by a secondary antibody (anti-human polyvalent immunoglobulins, G, A, M, peroxidase conjugated, Sigma A-8400, Deisenhofen, Germany) at a dilution of 1:10 000. Before and after addition of the secondary antibody the membranes were washed 4 times for 15 min in PBS, 0.05% Tween-20. To visualize the results, the washed membrane was incubated with 30 mL/membrane of a 1:1 mixture of Enhanced Luminol Reagent and Oxidizing Reagent for 1 min (Renaissance Western blot chemiluminescence reagent for ECL immunostaining (NEN, Köln, Germany)). The detection reagent was drained off and the membrane wrapped in plastic foil was then exposed to Kodak BioMax MR1 film for 5 min.

2.6 Evaluation of blots and gels

Initial analysis was performed as previously described [35]. Briefly, the silver stained pattern of a small gel was used as a master pattern, with each of the spots numbered. The protein pattern corresponded to the silver stained pattern of larger gels described previously [24].

Spot detection was performed by the TopSpot evaluation program (Algorithmus, Berlin, Germany). After automatic segmentation and spot detection, all additional visually detected protein spots were introduced into the master pattern interactively. Each spot that reacted with the different sera was numbered and compared to the silver stained spots for further identification. The optical density of the immunoreactive spot was classified into five categories: not detectable –; very low staining 0.5; low staining 1; staining 2; intensive staining 3; and very intensive staining 4. For each group of sera a signal frequency in % was calculated as the sum of intensity grades (0.5, 1, 2, 3, 4) of each serum divided by the maximal reachable value $(n \times 4) \times 100$, where n = number of sera. Spots with a signal frequency >10 were analyzed in more detail. Statistical analysis was performed using the software SPSS for Windows. For the analysis of nominal data and rates, chi-square analysis was used. Mean intensities of spots in the different groups of immunoblots were compared using the Kruskal-Wallis test and significance was as indicated ($p < 0.05$).

3 Results

3.1 Heterogeneous immunoreactivity pattern revealed by 2-DE immunoblots

We compared sera from infected patients and from a control group of patients with *H. pylori* unrelated gastric disorders. We further included sera from a group of cancer patients, who did not show active *H. pylori* infection. Sera from individual patients were used for immunoblot analysis to detect antigens of the *H. pylori* strain 26695. The immunoblot patterns of protein spots recognized on these immunoblots were compared with a standard silver stained 2-DE pattern. Immunoreactive spots that could be identified are marked with a code consisting of a number (1–6) that represents the corresponding sector on the standard gel and an attributed number (Fig. 1). Identification of detected protein spots was performed by in-gel digestion and MALDI-MS analysis. The identified protein species were listed in a database and named according to the classification of *H. pylori* proteins in the TIGR database [28]. Protein species can be accessed by name or by spot number using our 2-DE database (http://www.mpiib-berlin.mpg.de/2D-PAGE).

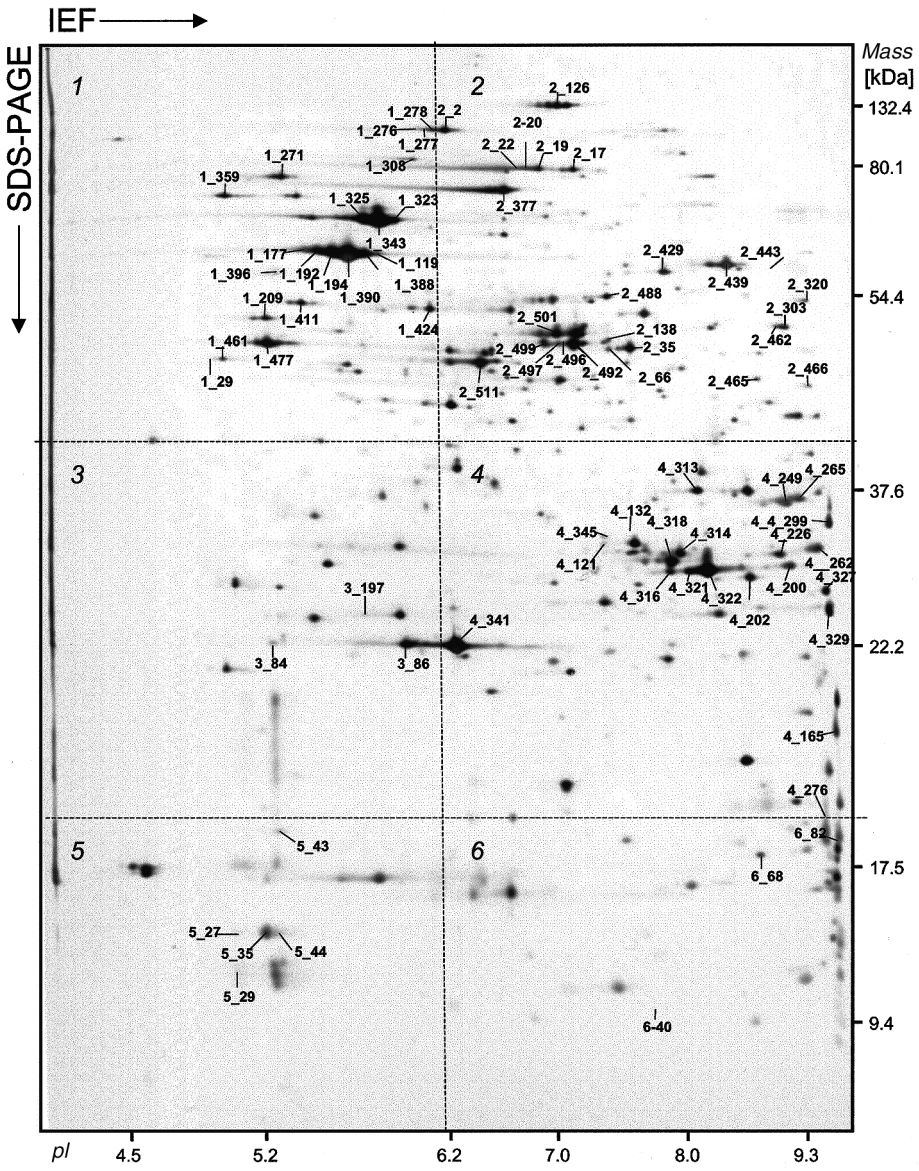

Figure 1. 2-DE gel of cellular proteins from *H. pylori* 26695 detected by silver staining. Six sectors (1–6) are separated by dashed lines. Spots that have been identified as immunogenic are marked with numbers corresponding to protein identities in the 2-DE database and consist of the number for the sector in the gel and a number for identification.

Similar to the silver stained gel pattern, individual spots on the immunoblots were distributed over the whole p*I* range of 4–10 and the whole molecular mass range of 5–150 kDa. We observed that some immunoreactive material accumulated in the low p*I* range and did not migrate into the IEF gel. The significance of this observation is not yet clear and needs further investigation. Examples for individual immunoreactive patterns are shown in Fig. 2 and include sera from a *H. pylori* negative patient (Fig. 2A), from patients with *H. pylori* related gastritis or ulcer (Figs. 2B and C) and a serum of a cancer patient (Fig. 2D). Between three and 153 spots were detected by individual sera on these immunoblots. Although the patterns were quite heterogeneous, two features were obvious: (i) most protein spots that were reacting with sera from gastritis, ulcer or cancer patients were clearly more intense than those detected by sera from *H. pylori* negative patients and (ii) a higher number of spots was detected. Together this is indicative of higher antibody titers directed against a greater number of antigens. Spots that could be assigned to particular ORFs are marked in the figures, tables and in the text. As an example, spot 5_35 containing 50S ribosomal protein L7/L12 is found in segment 5 of the silver gel and on all four immunoblots shown (Figs. 1 and 2).

We detected seven series of spots that were prominent in the silver stain and corresponded to major antigens of *H. pylori* recognized by sera on the immunoblots in Fig. 2. The series corresponding to species of GroEL proteins

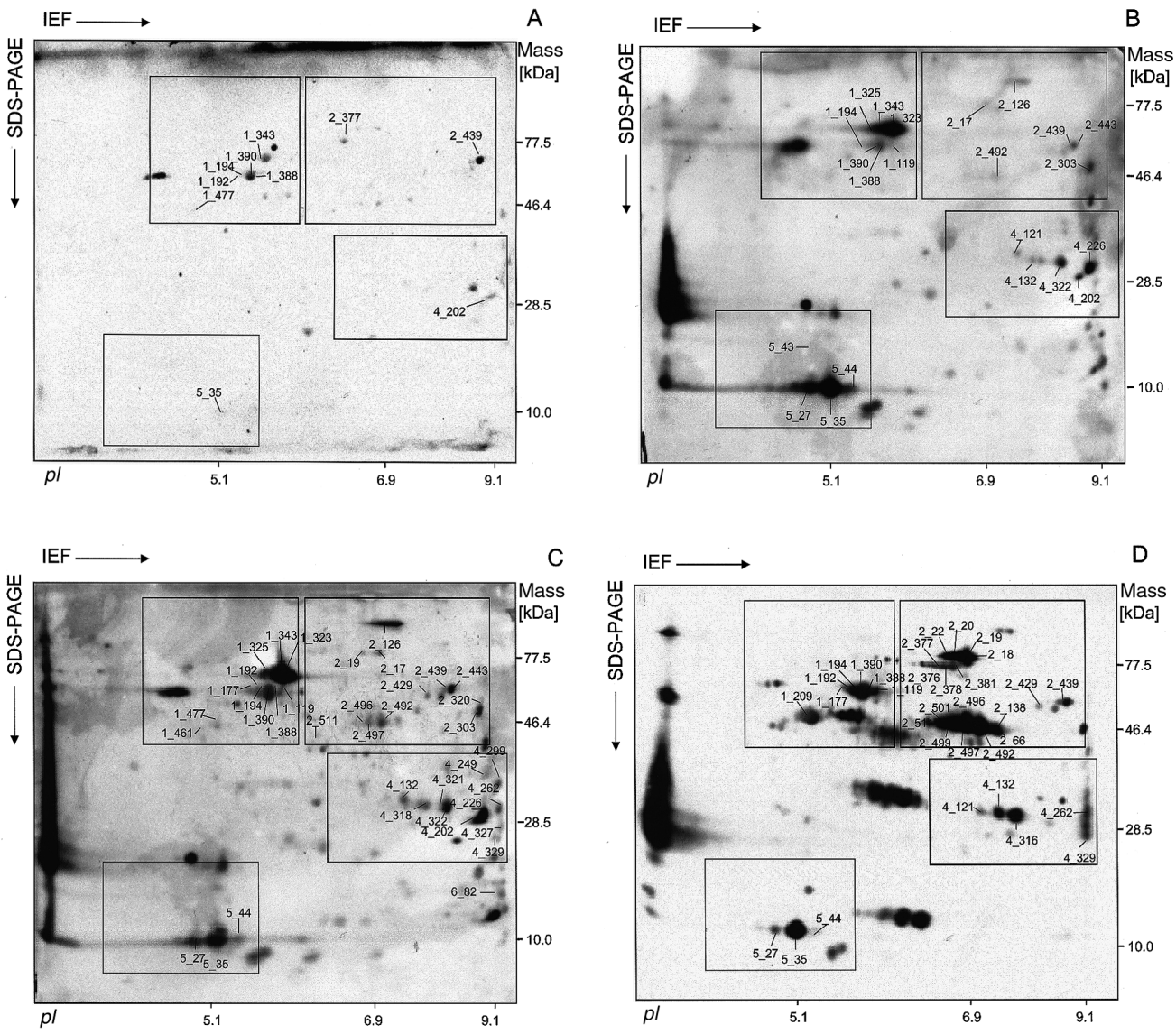

Figure 2. *H. pylori* 26695 antigens detected by immunostaining on 2-DE blots with sera from patients with gastric disorders: A, *H. pylori* unrelated gastritis; B, *H. pylori* gastritis; C, *H. pylori* gastric ulcer; D, gastric cancer. Spots that have been identified are marked with numbers corresponding to numbers in the 2-DE database and consist of the number for the corresponding sector 1–6 in the gel (see Fig. 1) and a number for identification.

(HP0010, main spot 1_390), 50S ribosomal proteins L7/L12 (HP1199, main spot 5_35) and catalase (HP0875, main spot 2_439) reacted with sera from all four patients shown (Figs. 2 A–D). The urease B subunit series (HP0072, main spot 1_343) reacted with the negative, gastritis and ulcer sera, whereas the series from Cag 26 (HP0547, spot 2_126) was stained with the gastritis or ulcer sera (Figs. 2B and C). Isocitrate dehydrogenase (HP0027, main spot 2_492) and the putative neuraminyl-lactose-binding protein HpaA (HP0410, main spot 4_132) reacted with the gastritis, ulcer and cancer sera, respectively (Figs. 2B–D). These series of spots were detected on most of the immunoblots tested and were also found useful for orientation in the 2-DE silver stain (Fig. 1).

3.2 Comparison of antigens detected by sera from *H. pylori* positive and negative patients

In order to detect highly immunogenic antigens of *H. pylori*, we first focused on proteins that were strongly recognized by sera from the 24 *H. pylori* positive and 12 negative

patients. Identification of protein species was then performed by comparison to the spots detected in the silver stained gels that were already assigned to particular ORFs [24]. The intensity of a particular spot was classified on a stepwise scale between 0.5 and 4. In addition, a signal frequency was calculated for each group of sera that takes into account the occurrence of recognition and the intensity of the spot for semiquantitative comparison as defined in Section 2.6 and in a former study [35]. Table 1 lists spots and corresponding proteins recognized by sera of *H. pylori* positive patients with a signal frequency exceeding a threshold of 10. A statistical analysis was performed to compare occurrence or intensity of recognition by sera from the *H. pylori* positive compared to the negative group and significantly higher occurrence or intensity of recognition by the former ($p < 0.05$) is marked. In total, 310 different protein spots were reacting with *H. pylori* positive sera. From these spots, 116 were only recognized by positive sera, while 156 spots were recognized with a higher signal frequency compared to the negative sera and 38 spots were recognized with an equal frequency. The number of spots stained by individual positive sera was widely spread (range 7 to 153) with a median of 52, while negative sera stained with a range from 3 to 66 and a median of 20 spots. Forty-two protein species representing 32 genes were recognized by positive sera with a signal frequency over 10. Four antigens included series of several spots recognized with a signal frequency $>$ 10. The antigens with the highest signal frequencies ($>$ 20) recognized by sera from *Helicobacter* positive patients were the 50S ribosomal protein L7/L12 (HP1199, spot 5_35), catalase (HP0875, spot 4_439), GroEL(HP0010, spot 1_390), Cag 16 (HP0537, spot 2_466), Cag 26 (HP0547, spot 2_126), urease A (HP0073, spot 4_322), urease B (HP0072, spot 1_343), hemolysin secretion protein precursor HylB (HP0599, spot 1_424), signal recognition particle protein Ffh (HP1152, spot 2_320) and a *H. pylori* predicted coding region (HP0231, spot 4_226). Except Cag16, HylB and Ffh, these antigens are also stained on blots B and C (Windows of sector 1_, 2_, 4_ and 5_ in Fig. 2). Ffh is stained on blot C in Figure 2. In sera from *H. pylori* negative patients, only seven antigens were recognized with a signal frequency $>$ 10. A signal frequency of more than 20 was achieved for the 50S ribosomal protein L7/L12, urease A, catalase and the conserved hypothetical secreted protein HP1098 (spot 4_262).

Six identified proteins were only recognized by positive sera: the predicted coding region HP0231, the serine protease HtrA, Cag 26 (HP0547, spot 2_126), Cag 3 (spot 2_443), ClpB (HP0264 spot 1_308) and the trigger factor HP0795 (spot 1_411). From isocitrate dehydrogenase IcD and 50S ribosomal protein RpL7/L12 several protein species were recognized. Two protein species of isocitrate dehydrogenase: 2_496 (not shown) and 2_497 (Table 1) were only recognized by positive sera, whereas the main spot 2_492 was recognized by both positive and negative sera (not shown). One of the protein species from RpL7/L12 (spot 5_44) was also only recognized by positive sera. In addition, six identified protein species were recognized by only one *H. pylori* negative serum with an intensity of 0.5, but in different combinations by the positive sera (Table 1). The following relative specifically recognized protein species were detected by the highest number of sera (Table 1): the Cag 26 protein, a typical marker for the subgroup of type I *Helicobacter* strains was recognized by 14/24 positive sera, a predicted coding region (HP0231, spot 4_226) and ATP-synthase α chain (HP1134, spot 1_396) were recognized by 11/24 positive sera, respectively, the serine protease HtrA (HP1019, spot 2_429) by 9/24 positive sera and fumarate reductase (HP0192, spot B17) was recognized by 8/24 positive sera. The so far unidentified protein spot 5_53 was also only recognized in one negative serum (not shown). Different combinations of these proteins are recognized on individual blots (Fig. 1). Other proteins as the CLPB (HP0264 spot 1_308) and the trigger factor HP0795 (spot 1_411) were recognized in combination with fumarate reductase (spot 2_17), 30S ribosomal protein S5 (spot 6_82), ATP-synthase α chain (spot 1_396), Cag 26 (spot 2_126) and Cag 3 (spot 2_443) by other sera from gastritis or ulcer patients (not shown).

In conclusion, antibodies from *H. pylori* positive patients recognized more protein species with higher intensity in different patterns compared to the control group, and a few identified antigens appear to be specifically recognized.

3.3 Differences in antigen recognition associated with disease manifestation

The *H. pylori* infected patients were diagnosed either with gastritis or ulcer. Antibodies from infected ulcer patients recognized more protein species with higher signal intensities than did those from most of the gastritis patients. In order to test the hypothesis of a correlation between antigen recognition and clinical manifestation, a statistical analysis was performed on the antigen pattern of sera from the 15 patients with *H. pylori* associated gastritis and the nine patients with either gastric or duodenal ulcer and revealed 23 protein species of 19 proteins with significant differences ($p < 0.05$) with respect to occurrence or signal intensity (Table 2). The signal frequencies that take into account both parameters are also shown. As only three patients had duodenal ulcer, no further statistical analysis was possible to distinguish between gastric and duodenal ulcer.

Table 1. *H. pylori* antigens recognized by sera from infected individuals with a signal frequency >10 or with significant differences of recognition compared to *H. pylori* negative sera

Protein class		Spot	ORF	Identity	Short name	Hp positive ($n=24$)		Hp negative ($n=12$)	
						frequency[d]	occurrence	frequency	occurrence
B2.	2'-deoxyribonucleotide metabolism	2_488	HP 0829	Inosine-5'-monophosphatase dehydrogenase	GuaB	11	6	2	2
B4.	Central intermediary metabolism	4_322	HP 0073	Urease alpha-subunit (Urea Amidohydrolase)	UreA[a]	33	19	27	7
		1_343	HP 0072	Urease beta-subunit (Urea Amidohydrolase)	UreB[a]	28	14	15	8
D7.	Pyridoxine	4_314	HP 1582	Pyridoxal phosphate biosynthetic protein J	PdxJ	11	7	7	3
F3.	Anaerobic energy metabolism	2_17	HP 0192	Fumarate reductase flavoprotein subunit	FrdA[a]	17	8	1	1
F4.	ATP-proton motive force interconversion	1_209	HP 1132	ATP synthase beta chain	AtpB	16	8	1	1
		1_396	HP 1134	ATP synthase alpha chain	AtpB	15*	11*	1	1
F11.	TCA cycle	2_497	HP 0027	Isocitrate dehydrogenase	Icd[a]	7*	8*	0	0
		2_66+ 2_138		Protein associated with isocitrate dehydrogenase, n.i.[c]	. . .[a,b]	8	10	11	6
I2.	DNA-dependent RNA-polymerase	1_461	HP 1293	DNA-directed RNA polymerase alpha chain	RpoA	17	9	5	5
J3.	Ribosomal proteins: synthesis and modification	4_165	HP 1307	50S ribosomal protein L5	Rpl5	13	5	2	2
		4_299	HP 1201	50S ribosonial protein L1	Rpl1	17	10	6	3
		5_35	HP 1199	50S ribosomal Protein L7/L12	Rpl7/l12[a]		21	27	10
		5_44	HP 1199	50S ribosomal Protein L7/L12	Rpl7/l12[a]	6*	11*	0	0
		6_82	HP 1302	30S ribosomal protein S5	Rps5	14	6	1	1
J5.	Translation factors	1_477	HP 1205	Elongation Factor (EF-TU)	Tuf B[a]	18	9	3	3
K2.	Protein folding and stabilization	1_359	HP 0109	DnaK protein (Heat shockprotein 70)	DnaK[a]	11	6	1	1
		1_390	HP 0010	GroEL	GroEL[a]	37*	20	14	8
K3.	Protein and peptide secretion	2_320	HP 1152	Signal recognition particle protein (54 homolog)	Ffh	24	12	4	4
K4.	Degradation of proteins, peptides and glycopeptides	1_308	HP 0264	ClpB protein	ClpB	14	7	0	0
		2_303	HP 1350	Protease	. . .	30	14	8	7
		2_429	HP 1019	Serine protease	HtrA[a]	10*	9*	0	0
L1.	Other regulatory functions	4_265	HP 0400	Penicillin tolerance protein	LytB	13	7	1	1
M2.	Surface structures	4_132	HP 0410	Putative neuraminyl-lactose-binding hemagglutinin homolog	HpaA[a]	12	9	3	2
M5.	Cell envelope: others	4_202	HP 1564	Outer membrane protein	. . .	18	14	4	4
N3.	Detoxification	2_439	HP 0875	Catalase	. . .[a]	48	20	26	8
N6.	Cellular processes: pathogenesis	2_126	HP 0547	Cag 26	Ca 26	39*	14	0	0
		2_466	HP 0537	Cag 16	Cag 16	11	14	4	3
		2_443	HP 0522	Cag 3	Cag 3	14	7	0	0
N8.	Cellular processes: others	1_424	HP 0599	Hemolysin secretion protein precursor	HylB[a]	27*	12	2	2
P1.	Unknown function	1_411	HP 0795	Trigger factor	. . .	19	6	0	0
Q1.	Conserved hypothetical	4_318	HP 0318	Conserved hypothetical protein	. . .	13	9	7	3
		4_262	HP 1098	Conserved hypothetical secreted protein	. . .	14	8	24	6
Q2.	Hypothetical proteins	4_226	HP 0231	*H. pylori* predicted coding region HP 0231	. . .[a]	23*	11*	0	0
		4_276	HP 0305	Hypothetical protein	. . .	20	15	10	4

Nomenclature according to the TIGR database;

a) spot occurs in a series

b) determined by localisation on the gel

c) n.i. not yet identified

d) signal frequency calculated as the sum of intensity grades (0.5, 1, 2, 3, 4) of each serum divided by the maximal reachable value ($n \times 4$) $\times$ 100, where n = number of sera;

* significant difference compared to negative group, $p < 0.05$

Table 2. Sera from gastritis, ulcer and cancer patients react with *H. pylori* proteins in significantly different patterns

Protein class		Spot	ORF	Identity	Short name	Occurrence %			Mean intensity			Frequency[e]		
						G *n*=15	U *n*=9	C *n*=6	G *n*=15	U *n*=9	C *n*=6	G *n*=15	U *n*=9	C *n*=6
A6.	Amino acid biosynthesis	2_377	HP 0695	Hydantoin utilization protein A	HyuAr[a]	33	22	50.0	0.5	0.2	1,8°	13	5	45
B4.	Central intermediary metabolism	4_321	HP 0073	Urease alpha-subunit	UreA	20	55	50	0.3	1.3*	1.1	8	33	28
F3.	Anaerobic energy metabolism	2_17	HP 0192	Fumarate reductase flavoprotein subunit	FrdA	20	55	67*	0.5	0.9	1,8	13	23	45
F11.	TCA cycle	2_492	HP 0027	Isocitrate-dehydrogenase	Icd[a,b]	20	77*	67*	0.4	0.3	1	10	8	30
		2_496	HP 0027	Isocitrate-dehydrogenase	Icd[a,b]	20	55	50	0.3	0.3	1.1	8	8	28
		2_497	HP 0027	Isocitrate-dehydrogenase	Icd[a,b]	20	55	50	0.3	0.3	1.1	8	8	27
		2_499	HP 0027	Isocitrate-dehydrogenase	Icd[a,b]	20	55	67	0.3	0.3	1.8	8	8	45
		2_66+ 2_138		Protein associated with Isocitrate dehydrogenase in the 2-D gel, n.i.[d]	Icd[a,c]	26	66	83*	0.3	0.3	1.8*	8	8	45
I2.	DNA-dependent RNA polymerase	1_29	HP 1293	DNA-directed RNA polymerase A alpha chain (Transciptase alpha chain)	RpoA	0	0	33*,°	0	0	0.8*	0	0	20
J3.	Ribosomal proteins	5_35	HP 1199	50S ribosomal protein L7/L12	Ppl7/ l12[a,b]	33	66	16	0.2	0.3	0.1	5	8	3
		5_44	HP 1199	50S ribosomal protein L7/L12	Ppl7/ l12[a,b]	40	77*#	0	0.8	1.8#	0	20	45	0
		6_68	HP 0514	50S ribosomal protein L9	Rpl9	0	33*	0	0	0.6*	0	0	15	0
		6_82	HP 1302	30S ribosomal protein S5	Rps5	7	55*	33	0.3	1.1*	0.2	8	28	5
J5.	Translation factors	1_477	HP 1205	Elongation factor (EF-TU)	TufB[a]	20	66*	67*	0.4	1.3*	0.9	9	33	23
K3.	Protein and peptide secretion and trafficking	2_320	HP 1152	Signal recognition particle protein (fifty-four homolog)	Ffh	33	77*	50	0.5	1.8	0.8	13	45	20
K4.	Degradation of proteins, peptides, and glycopeptides	3_84	HP 0794	ATP-dependent Clp protease proteolytic subunit (Endopeptidase CLP)	ClpP	0	11	33*	0	0.1	0.2	0	1	5
M2.	Surface structures	4_121	HP 0410	Putative neuraminyl-lactose-binding hemagglutinin homolog	HpaA	6	0	33°	0.1	0	0.2	1	0	6
N6.	Cellular processes: pathogenesis	2_466	HP 0537	Cag 16	Cag 16	40	88*#	17	0.2	0.8*#	0.3	6	21	8
P1.	Unknown	2_35	HP 1104	Cinnamyl-alcohol dehydrogenase ELI3-2	Cad	6	22	67*	0.2	0.1	1.6*,[c]	5	3	40
Q1.	Conserved hypothetical	4_249	HP 0175	Hypothetical protein HP0 175 precursor	...	13	66*	50*	0.2	0.7*	0.3	5	18	8
		4_327	HP 1285	Conserved hypothetical protein	...	40	89*	50	0.6	0.4	0.8	15	11	20
Q2.	Hypothetical	4_276	HP 0305	Hypothetical protein	...	46	88*	50	0	1.3*	1.7*	1	32	42
		2_465		n.i.	...	7	27*	17	0.1	0.3	0.5*	2	8	13

Nomenclature according to the TIGR database;
a) spot occurs in a series;
b) series of spots with different recognition rate;
c) determined by localisation on the gel;
d) n.i. not yet identified;
e) signal frequency calculated as the sum of intensity grades (0.5, 1, 2, 3, 4) of each serum divided by the optimal reachable value $(n \times 4) \times 100$, where n = number of sera;
° significant difference compared to ulcer group, $p < 0.05$;
* significant difference compared to gastritis group, $p < 0.05$;
# significant difference compared to cancer group, $p < 0.05$

Table 3. *H. pylori* proteins preferentially recognized by sera of cancer patients with a frequency > 10

Protein class		Spot	ORF	Identity	Short name	Frequency[d]		
						Gastritis	Ulcer	Cancer
A6.	Amino acid biosynthesis	2_377	HP 0695	Hydantoin utilization protein A	HyuA[a]	13	5	45
D7.	Biosynthesis of cofactors: pyridoxine	4_314	HP 1582	Pyridoxal phosphate biosynthetic protein J	PdxJ	10	14	42
E4.	Central intermediary metabolism	4_200	HP 1186	Carbonic anhydrase	...	1	0	17
	Amino acids and amines	2_511	HP 0294	Aliphatic amidase	AimE	4	3	15
F3.	Anaerobic energy metabolism	2_17	HP 0192	Fumarate reductase flavoprotein subunit	FrdA	13	23	45
F11.	TCA cycle	2_2	HP 0779	Aconitate hydratase 2 (Citrate hydro-lyase-2)	AcnB	8	6	23
		2_492	HP 0027	Isocitrate dehydrogenase	Icd[a]	8	10	30
		2 66+ 2 138		Protein associated with Isocitrate dehydrogenase, n.i.[c]	Icd[a,b]	8	10	30
G4.	Transport and binding proteins: cations	4_313	HP 1562	Iron (III) ABC transporter, periplasmatic iron-binding protein	CeuE	4	4	21
I2.	DNA-dependent RNA polymerase	1_29	HP 1293	DNA-directed RNA polymerase alpha chain	RpoA[a]	0	0	20
K2.	Protein folding and stabilization	1_177	HP 0010	GroEL	GroEL[a]	4	6	19
M2.	Surface structures	4_132	HP 0410	Putative neuraminyl-lactose-binding hemagglutinin homolog	HpaA[a]	18	15	33
		4_121	HP 0410		HpaA	1	0	6[e]
M5.	Cell envelope: others	4_202	HP 1564	Outer membrane protein	...[a]	12	29	50
P1.	Unknown	2_35	HP 1104	Cinnamyl-alcohol dehydrogenase ELI 3-2	Cad	5	3	40
Q1.	Conserved hypothetical	4_262	HP 1098	Conserved hypothetical secreted protein	...	12	14	42

Nomenclature according to the TIGR database;
a) spot occurs in a series;
b) determined by localisation on the gel;
c) n.i., not yet identified;
d) signal frequency calculated as the sum of intensity grades (0.5, 1, 2, 3, 4) of each serum divided by the maximal reachable value ($n \times 4) \times 100$, where n = number of sera;
e) minor spot recognized

The following proteins were identified that showed significantly different recognition by sera from ulcer patients as compared to sera from gastritis patients ($p < 0.05$): a conserved hypothetical protein (HP1285, spot 4_327), a hypothetical protein precursor (HP0175, spot 4_249), a hypothetical protein (HP0305, spot 4_276), a signal recognition particle protein (HP1152, spot 2_320), the 50S ribosomal protein L9 (HP0514, spot 6_68) and the 30S ribosomal protein S5 (HP1302, spot 6_82), elongation factor TufB (HP1205, spot 1_477) Cag 16 (HP1133, spot 2_466) and the not yet identified protein species 2_465. Two protein species from the metabolic enzyme isocitrate dehydrogenase (spots 2_492 and 2_499) and one from the 50S ribosomal protein L7/L12 (HP1199, spot 5_44), which both occur in series of spots on the gels, were also more often recognized by sera from ulcer patients, while other species from these proteins did not show significant differences in recognition. From the 23 protein species showing significant differences in antigen recognition, eleven were significantly more often recognized ($p < 0.05$) by sera from ulcer patients compared to gastritis patients. Eight protein species showed a significantly higher mean intensity ($p < 0.05$) and seven fulfilled both criteria. Only a few antigens, such as the hydantoin utilization protein A (HP0695, spot 2_377) or the putative neuraminyl-lactose-binding protein HpaA (HP0410, spots 4_121 and 4_132) (all in Table 3) or the 26 kDa antigen (HP 1563, spot 4_341 in Fig. 1), which is known as a strong antigen in *Helicobacter* infection are less often or intensely recognized by sera from ulcer compared to gastritis patients.

We further detected similar recognition patterns between sera from the six cancer patients and sera from ulcer patients in many cases. The metabolic enzymes isocitrate dehydrogenase (HP0027, main spot 2_492) and fumarate

reductase (HP0192, spot 2_17), a conserved hypothetical protein (HP1285, spot 4_327), a hypothetical protein precursor (HP0175, spot 4_249), and elongation factor TufB (HP1205, spot 1_477), were recognized by at least half of the sera from ulcer and cancer patients (Table 2). Because only six patients with cancer who did not show active *H. pylori* infection were tested, the statistical analysis for the comparison of sera from this group with sera from the gastritis and the ulcer group is not very informative, but several differences were obvious. In some cases, the intensity of recognition by sera from cancer patients was higher compared to sera from ulcer patients, as shown for isocitrate dehydrogenase Icd spot B499 and a not yet identified protein associated with Icd by localization in the gel (spots 2_66 and 2_138) in Figs. 2C and D and for example the hypothetical protein (HP0305, spot 4_276) in Table 2. In addition, recognition of the metabolic enzyme cinnamyl-alcohol dehydrogenase (HP1104, spot 2_35) as well as the hydantoin utilization protein A (HP0695, spot 2_377) (Table 3) resulted in a much higher signal frequency for sera from cancer patients compared to ulcer or gastritis patients. A conserved hypothetical secreted protein HP 1098 (spot 4_262) and the outer membrane protein HP1564 (spot 4_202) were also recognized with a high signal frequency by cancer patients (Table 3).

To date, it has only been possible to test a small number of patient sera, and as a result we are only able to highlight tendencies linking candidate antigen recognition patterns with different disease manifestations. Therefore, we created an initial platform by adding the detected antigens and immunoreactive patterns to our database of identified proteins from *H. pylori* (http://www.mpiib-berlin.mpg.de/2D-PAGE). Proteins and antigens can be searched and analyzed in an interactive way using NCBI accession numbers or numbers defined in the TIGR database. This platform is open to the public and offers a basis for other groups to contribute more data in order to test the hypothesis of antigens related to disease.

4 Discussion

4.1 *H. pylori* specific antigens

In order to characterize candidate antigens of *H. pylori* for diagnosis and therapy, we have systematically analyzed differences in the *Helicobacter* 2-DE antigen recognition profiles of the strain 26695 by the use of sera from patients with different gastric disorders. Of 1800 protein spots from this strain detected by silver staining, 310 spots reacted with sera from *H. pylori* positive patients. Forty-two protein species comprising 32 proteins were identified from the antigens recognized with a signal frequency > 10. Among these proteins most frequently recognized by *H. pylori* positive sera, nine were newly identified and 23 were confirmed from our previous study and other 2-DE analyses [24–26].

As *H. pylori* strains show a high degree of variability, each approach to define antigens for diagnosis and vaccination has to cope with this problem. We have taken advantage of a 2-DE immunoblot analysis of the completely sequenced *H. pylori* strain 26695 from which we have already identified more than 150 abundant proteins [24]. This allowed us to identify new immunogenic antigens in this study. Further advantages of our proceeding are the evaluation of all protein species of the pathogen on one gel and clear separation of basic proteins. For example, the hypothetical protein HP0305, p*I* = 9.08, the outer membrane protein HP 1564, p*I* 8.83, Cag 16 (HP0537), p*I* 9.7, or the protease HP1350, p*I* = 9.45, were recognized by more than half of the sera from positive patients.

As an extensive screening was performed, the variations in affinity between antibody and individual antigen could not be taken into account. Bearing in mind the general and technical limitations of this approach with respect to genetic variation, affinity of the antibodies, conformational epitopes or some extracellular or membrane-bound antigens, our approach is the only way to achieve standardized high-resolution of individual protein patterns and exact identification of antigens recognized by the different sera tested. The characterization of conserved epitopes is beneficial for both diagnostic and vaccine purposes. So far, mainly conserved or very abundant antigens of *H. pylori* including urease A and B subunits, catalase, CagA, VacA, the GroES homologue HspA and NapA have been analyzed for their protective or therapeutic potential in animal studies and they were all recognized by serum antibodies. None have been shown to be protective in humans [22, 36–40]. Thus characterization of further antigens seems important to evaluate valuable combinations of antigens for vaccine purposes.

Several very abundant proteins from the *Helicobacter* proteome were recognized by *H. pylori* negative sera. For some of these antigens, corresponding proteins are known from other bacterial proteome analyses [41, 42]. They may thus be considered to be crossreactive antigens from other bacterial infections, e.g. *Campylobacter jejuni*, *Salmonella* or pathogenic *Escherichia coli*: 50S ribosomal protein L7/L12, catalase, and GroEL as examples. Interestingly, one protein species from the 50S ribosomal protein L7/L12 (spot 5_44) was only recognized by positive sera and may represent a post-translational modification of the protein. This is a further example for a differentiation of the immune response against different protein species of one protein, as has been described for

another ribosomal protein from *Salmonella* strains [43]. In addition, urease A and B were recognized by antibodies from *H. pylori* negative patients correlating with observations of low specificity of *Helicobacter* detection in diagnostic assays that contain recombinant urease [44]. At this time, the main problem of both commercial and scientific assays used for the detection of *H. pylori* infection is the relatively low correlation with disease and low specificity [44–46]. We have identified seven proteins, which were only detected by sera from patients with active *H. pylori* infection: a trigger factor HP0795, the stress protein ClpB (HP0364), Cag 26 (HP0547), the predicted coding region HP0231, the serine protease HtrA (HP1019) and Cag 3 (HP0522); the latter three seem to be new specifically recognized antigens. These antigens were recognized by sera from *H. pylori* positive patients in combinations of at least three antigens with the following proteins: the 30S ribosomal protein S5 (HP1302) and fumarate reductase (HP0192) which were newly identified and ATP-synthase α chain (HP1134) or the heat-shock protein DnaK (HP0109) which were also detected in former studies [25, 26]. All these proteins are among the 150 most abundant protein species of *H. pylori* [24] and may therefore represent very immunogenic candidates for the development of a diagnostic assay that may detect a variety of *H. pylori* strains. Outer membrane proteins are further candidates for diagnostic purposes and several were detected in a recent study in our laboratory (in preparation). The antigens detected in this study will now be tested by using a larger number of patients and several strains.

4.2 *H. pylori* antigens and relation to disease

It would be desirable to have serological markers to indicate different manifestations of gastric disease. Many studies in the past did not succeed to reveal such markers either because of limited resolution in one-dimensional protein analysis [14, 47–49] and reviewed in [50] or because of limited numbers of samples [51]. In our study, several antigens seemed to be recognized with a higher intensity by sera from ulcer patients compared with sera from gastritis patients. This may be due to persistence of strong inflammation and tissue damage leading to increased antigen presentation during the development of ulcer. There are also antigens that seem not to be recognized because of a general amplification of antigen recognition in ulcer. For example, four of these antigens which are better recognized than the dominant antigens catalase, GroEL or urease A and B are the hypothetical protein precursor HP 0175, fumarate reductase (HP0192), the 30S ribosomal protein S5 (HP1302) and a signal recognition particle protein HP 1152. They are recognized in patterns by sera from *H. pylori* infected ulcer patients. We are working to verify these encouraging tendencies by comparing a larger number of protein patterns. In addition the public database we have developed offers a standardized interactive platform that could be used by other interested groups to collaborate and contribute more data on serum recognition profiles in order to join efforts by analyzing complex protein profiles. This approach will ultimately allow us to define series of antigens which will be a great advance for diagnostic assays and vaccine design.

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