Comparison of ELISA antigen preparations alone or in combination for serodiagnosing Helicobacter pylori infections

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Abstract
The immunoglobulin G antibody response to Helicobacter pylori was assessed in 78 patients with non-ulcer dyspepsia using five different antigen preparations. All patients were endoscoped and biopsied. The H pylori state was determined histologically on at least two endoscopic biopsy specimens using a modified Giemsa stain. The ultracentrifuged cell sonicate, acid glycine extract, and 120 kilodalton protein antigens were specific in diagnosing infection (95–98%), but had only moderate sensitivity (70–84%). By mixing either of the two complex antigens with the 120 kilodalton protein, the sensitivity of the test was increased to 97% without affecting the high specificity. The combination of ultracentrifuged sonicate or acid glycine extract with the 120 kilodalton protein therefore seems to be superior to the individual antigen preparations and is particularly suitable for the serodiagnosis of H pylori infection.

The strong association between Helicobacter pylori colonisation and chronic antral gastritis and peptic ulceration is widely recognised. An increasing body of evidence suggests that the organism has a role in the pathogenesis of chronic gastritis, although exactly how is unclear. Diagnosis of colonisation usually relies on culture of biopsy specimen, histology, or urease testing. Such techniques are invasive, but non-invasive techniques such as urea breath tests and serology have been used. The former technique, using either 13C or 14C, is generally time consuming and relatively expensive compared with the latter. Several enzyme linked immunosorbent assays (ELISA) have been described, but the tests only partially meet the ideal of very high sensitivity and specificity. Little work has been carried out that directly compares the different techniques. The aim of this study was to compare three different antigen preparations and their combination in terms of sensitivity and specificity for diagnosing H pylori colonisation.

Methods
Sera were collected from 78 consecutive dyspeptic patients (excluding those with ulceration at endoscopy) attending an endoscopy clinic. Antral and body mucosal biopsy specimens were taken at endoscopy. The sera were stored at −20°C before assay.

The gastric biopsy specimens were formalin fixed and routinely processed. Sections stained with haematoxyline and eosin were used to assess the mucosal inflammation and a modified Giemsa stain was used to diagnose H pylori colonisation. The sections were assessed by a single pathologist who was unaware of the clinical diagnosis.

H pylori NCTC 11637 was used throughout for all antigen preparations. The strain was cultivated on Mueller Hinton agar (Oxoid), which was supplemented with 5% defibrinated sheep blood, for 72 hours at 37°C under microaerophilic conditions. Bacteria were harvested, resuspended, and washed (three times) in phosphate buffered saline (PBS), 0.01 M, pH 7.2. The resulting pellet was immediately processed or frozen at −25°C.

The ultracentrifuged cell sonicate was prepared as follows: The washed strain was inactivated at 60°C for 15 minutes and then sonicated for 10 seconds in a Branson ultrasonifier (microtip, position 5, 40% pulsed). The cell sonicate was then ultracentrifuged (100 000 × g for 60 minutes) in a fixed angle rotor (Centrikon—T 2060, Kontron Instruments) and the supernatant stored at −25°C.

To prepare an acid glycine extract, to every 100 mg (net weight) of bacterial cell mass 2.5 ml of glycine buffer (0.2 M, pH 2.2) were added; this suspension was gently mixed for 20 minutes at room temperature and centrifuged (1800 × g for 15 minutes at 4°C). The supernatant was neutralised with NaOH (1.0 M) to pH 7.0 and dialysed against distilled water (24 hours at 4°C). Insoluble particles were spun down and the supernatant stored at −25°C.

The high molecular weight 120 kilodalton protein was prepared by electroelution from sodium dodecyl sulphate polyacrylamide gels as described previously. All antigen preparations were diluted in sodium carbonate/bicarbonate buffer (0.05, pH 9.6). A protein concentration of 4 mg/l, found to be optimal in previous experiments, was used throughout. For mixtures of the ultracentrifuged cell sonicate or the acid glycine extract with the 120 kilodalton protein antigen, a concentration of 4 mg/l of each constituent was found to be optimal by checkerboard titration. Before each experiment round bottomed microtitre plates were coated with 0.1 ml/well of antigen preparation and then incubated for 16–18 hours at 4°C. To
detect class specific antibodies peroxidase conjugated anti-human IgG (goat; Fc specific; Nordic) was used at a dilution of 1/1000. Patients’ sera and conjugate were diluted in phosphate buffer (pH 7.4) supplemented with 2% Tween-20 and 1% bovine serum albumin; the plates were rinsed with phosphate buffer (pH 7.4) supplemented with 0.05% Tween 20. A substrate of 40 mg o-phenylene-diamine dihydrochloride (Sigma) in 100 ml phosphate/citrate buffer (pH 5.0) with added H₂O₂ (30%; 0.04 ml) was used. Coated plates were filled with 0.1 ml/well of log₂ dilutions of the test sera, starting from 1:150, and incubated at 37°C for two hours covered with a lid. After three washings plates were reincubated (37°C for two hours) with conjugate (0.1 ml/well). After another three washings 0.1 ml of substrate solution was added to each well and the plates were incubated at room temperature in the dark for 15 minutes. The enzymatic reaction was stopped by adding 0.05 ml/well sulphuric acid (2.5 M) and the plates were read by optical density (OD) measurements at 492 nm.

In an x-y diagram the OD values recovered from the ELISA readings were plotted against the log₂ serum dilutions for each antigen and serum used. For each individual patient serum, a titre was calculated by projecting the intersection point of the serum dilution curve with the horizontal line originating in the point OD = 1.0 on the x axis. This serum dilution value can be read graphically.

The sensitivity, specificity, positive and negative predictive values were calculated. “False positive” were titres of sera from H pylori negative patients that exceeded the mean titre of all sera from negative patients by more than 3 standard deviations. “False negative” were defined as the results of sera from patients known to be infected, with titres lying within this range of threefold standard deviation.

Results
The individual reciprocal antibody titres using the different antigen preparations in the H pylori positive and negative patients are shown in the figure. The ultracentrifuged cell sonicate, acid-glycine extract, and 120 kilodalton protein antigens resulted in two, one, and one false positive results, respectively, and six, 11, and six false negative results, respectively. In table 1 the outcome of the tests with the 15 sera giving discrepant results with the various antigens is presented. With the exception of serum 49, all sera giving false negative results with one or two antigens reacted with at least one of the other antigens. Consequently, using a mixture of the 120 kilodalton protein with an ultracentrifuged cell sonicate or acid-glycine extract, rendered all but one serum sample positive by ELISA. The combined use of these antigens thus increased the sensitivity of the test (significantly so in the case of the acid-glycine extract and 120 kilodalton protein; p = 0.01; McNemar test) and improved the negative predictive value without affecting the specificity (table 2).

Table 1 Comparison of ELISA results of sera from patients with histologically verified H pylori-associated gastritis and negative ELISA titres in at least one antigen preparation

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Serum No</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 2 3 11 22 32 35 44 49 50 53 61 66 72 76</td>
</tr>
<tr>
<td>UCS</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>AGE</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>P120</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>UCS + P120</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>AGE + P120</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
</tbody>
</table>

UCS = ultracentrifuged cell sonicate; AGE = acid glycine extract; P120 = 120 kilodalton protein.

Table 2 Specificity (%), sensitivity (%), and predictive values (%) of H pylori-IgG ELISAs with different antigen preparations

<table>
<thead>
<tr>
<th>Antigen</th>
<th>UCS</th>
<th>AGE</th>
<th>P120</th>
<th>UCS + P120</th>
<th>AGE + P120</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>84</td>
<td>70</td>
<td>84</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>Specificity</td>
<td>95</td>
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<td>98</td>
<td>100</td>
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<td>Positive predictive value</td>
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<td>96</td>
<td>97</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Negative predictive value</td>
<td>87</td>
<td>78</td>
<td>87</td>
<td>98</td>
<td>98</td>
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</tbody>
</table>

UCS = ultracentrifuged cell sonicate; AGE = acid glycine extract; P120 = 120 kilodalton protein.

Discussion
The nature of antigens commonly used for serodiagnosis of H pylori infection may be classified into three categories: (i) whole cell antigens and ultrasonicates of them; (ii) partially purified; and (iii) highly purified antigens.

Whole cell antigens and their sonicates possess the theoretical advantage of exposing a maximum number of surface antigens. This seems to be desirable in view of the extremely variable immune response to H pylori, but it also increases the risk of non-specific binding of immunoglobulins and of cross reaction with Campylobacter species. Indeed, there are reports of unacceptably high numbers of false positive results of ELISAs with a specificity of only 73–78% when a whole cell antigen is used; the sensitivity, however, ranged well above 90% when using this type of antigen.

Ultrasonicated cells seem to offer a somewhat better specificity when used as an antigen. In IgG or IgA ELISAs Perez-Perez et al found specificity values of 90%, and 80%, respectively, and of 95%, when combining the results of both tests. With a similarly prepared
Comparison of ELISA antigen preparations

antigen—that is, a shortly pelleted ultrasonicated, we have found a specificity of 98% (but a sensitivity of only 76%) when testing the sera of this study (unpublished data). These numbers are greatly influenced by the cut-off value chosen for "positive" and "negative". Loffeld et al. used two cut-off values in their test and found positive and negative predictive values of 100% for both. The results of nearly 20% of sera examined were, however, not in the interpretable range.

Among the partially purified antigens the most commonly used are an ultracentrifuged cell sonicate, an acid glycine extract, and a crude urease preparation. As can be seen from our results the two former types of preparation give an excellent specificity but insufficient sensitivity. The choice of the cut-off value, which in this study was fixed relatively high (mean titre of negative sera + 3SD), has an important role in determining the specificity and sensitivity. Goodwin et al. reported the sensitivity and specificity of their test as 81% and 97%, respectively, if they fixed the cut off at 300 ELISA units, 99% and 78% at 150 ELISA units. For an ELISA with an acid glycine extract antigen, Newell and Stacey tested more than 600 sera and found the sensitivity and specificity to be 92% and 89%, respectively. Compared with other types of preparation, a crude urease preparation does not seem to offer special advantages; in contrast, the proportion of false positive results is relatively high. This fact, however, was thought by Dent et al. to originate from a failure to show H pylori in bacteriological culture or histological examination rather than from a faulty serological test.

Generally, highly purified antigens such as purified urease, N-acetylmuraminylpentapeptide binding haemagglutinin, and the 120 kilodalton protein are very specific but have poor sensitivity. Yet, for the latter antigen the test sensitivity was not lower than that of both of the more complex antigens. One problem with the 120 kilodalton protein is that it is not demonstrable in all strains of H pylori.

The most important finding of this study is the fact that the three antigens studied, although giving only suboptimal sensitivity when used alone, produce optimal sensitivity and specificity when used in combination. Coating microtitre plates with the antigen mixture is simple and the results observed with the combination are those expected from the results of the antigens alone. Only one serum sample was found to be a false negative with each of the antigens and consequently also with their mixtures. Perhaps the patient was infected with a less common variant of H pylori so that his immune response remained undetected with antigens prepared from only one isolate.

In summary we conclude that the combination of a prepurified complex antigen of H pylori with its 120 kilodalton protein results in an antigen rendering the ELISA highly specific and very sensitive. This antigen combination thus seems well suited for accurate diagnosis of H pylori infection.