Western blotting is useful in the salivary diagnosis of *Helicobacter pylori* infection

L D Ballam, M A Mendall, M Asante, J Morris, D P Strachan, P H Whincup, D G Cook

Abstract

**Background**—The salivary diagnosis of *Helicobacter pylori* infection offers attractive possibilities for the epidemiological study of infection in children. Salivary enzyme linked immunosorbent assay (ELISA) is less reliable than serum ELISA, owing to variable transudation of immunoglobulin. In addition, children are more difficult to study because of lower specifc serum antibody concentrations to *H pylori*. The performance of salivary western blotting in comparison with serum western blotting and serum ELISA was investigated in school children.

**Subjects and methods**—Paired serum and saliva specimens were obtained from 665 school children aged 9–11 in 10 British towns. All saliva and serum specimens were first analysed by ELISA; subsequently, western blotting of both specimens was performed on 31 and 34 specimens, respectively, to establish the criteria for positivity for western blotting. The remaining 121 specimens were then tested blindly and saliva was compared with the serum.

**Results**—The sensitivity and specificity of salivary ELISA in the 665 specimens was 32 of 50 (64%) and 530 of 691 (87%), respectively, when compared with serum ELISA. The western blotting validation was performed on 28 subjects with positive serum and positive salivary ELISA, 28 saliva positives with negative serum, 16 saliva negatives with positive serum, and 50 doubly negative subjects. Compared with serum western blots, the sensitivity and specificity of salivary western blots was 38 of 47 (81%) and 68 of 75 (91%), respectively. Using serum ELISA as the gold standard, the sensitivity and specificity were 32 of 44 (73%) and 72 of 78 (92%), respectively, the specificity being significantly higher than salivary ELISA (p < 0.001).

**Conclusion**—Salivary western blotting for IgG is useful in the diagnosis of *H pylori* infection and is superior to ELISA. It also permits the identification of pathogenic strains.


Keywords: *Helicobacter pylori*, western blotting; enzyme linked immunosorbent assay; salivary specimens

*Helicobacter pylori* is a chronic bacterial infection of the human stomach that is associated with peptic ulcer and gastric cancer, as well as with possible extra-intestinal manifestations. The diagnosis of *H pylori* can be made on biopsies obtained at endoscopy, but these are unsuitable for epidemiological studies, which have relied on serology and the urea breath test. Salivary antibody testing is a potentially useful alternative for epidemiological studies, especially in children, because it is non-invasive and no sample preparation is required.

The specific antibody response to *H pylori* can be detected using a variety of methods, but ELISA and immunoblotting (western blotting) for specific IgG are the most reliable. ELISA is more convenient than western blotting, but specific anti-*H pylori* IgG values tend to be lower in children than adults, and most assays have reported a slightly lower sensitivity when compared with adults.

Although IgA is the predominant immunoglobulin present within saliva, poor sensitivity and specificity are obtained in comparison with IgG when assayed by ELISA. IgG gains access to saliva by transcapillary leakage, but values are approximately 1/1000 of those in serum. The low concentrations, together with variable transcapillary leakage, are the main reasons why salivary IgG determination is less reliable than serum determination. Immunoblotting is a sensitive and qualitative method for determining the presence of antibodies, and hence would be expected to overcome some of these difficulties. It also has the advantage of being able to detect antibodies to CagA and VacA, proteins that are markers of *H pylori* virulence.

Therefore, we investigated whether salivary western blotting was superior to salivary ELISA for the detection of IgG antibodies to *H pylori* compared with serum ELISA and serum western blotting. We also determined whether salivary western blotting had the ability to detect antibodies to VacA and CagA, as determined by serum western blotting.

**Subjects and methods**

Our report is based on the 8–11 year old examination of the “ten towns” children study. The general design of our study has been described elsewhere. Paired saliva and serum samples were available for 665 children from the last five towns screened; three with particularly low adult cardiovascular mortality (Leatherhead, Bath, and Tunbridge Wells) and two with particularly high cardiovascular mortality (Rhonda, Rochdale). ELISA antibody determinations were performed on both serum and saliva with the Launch Premier ELISA kit™ (Launch Premier, Longfield, Kent, UK). This has been shown previously to be 100% sensitive and 96% specific for serum in adults. Serum was tested according to the manufacturer’s instructions. Optimum performance for salivary ELISA was obtained when undiluted saliva was incubated with IgG conjugate.
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(ELISA) values (n = 129).

Figure 2 Frequency distribution of serum enzyme linked immunosorbent assay (ELISA) values (n = 129).

Figure 1 Frequency distribution of optical densities in 665 subjects who had salivary western blotting to diagnose *Helicobacter pylori* infection.

(A2290; Sigma, Poole, Dorset, UK) at a concentration of 1/1500.

The specimens selected for the validation study had an over representation of false positive saliva by ELISA to allow a better evaluation of specificity. All the seropositive specimens from the original 665 paired samples tested on ELISA with remaining saliva were included. In total, 122 paired saliva and serum samples were studied by western blotting. They comprised 28 subjects who were ELISA seropositive and salivary IgG positive, 16 subjects who were seropositive and saliva IgG negative, 28 subjects who were seronegative but saliva IgG positive (OD > 0.41), and 50 subjects who were seronegative and saliva IgG negative. A further seven samples were unreadable owing to gross contamination or insufficient saliva.

Criteria for a positive serum western blot were first established on 18 positive and 16 negative sera measured by ELISA, and for a positive salivary western blot on saliva specimens from 13 seropositive and 18 seronegative subjects by serum ELISA. When the criteria had been established the remainder of the samples were tested blindly.

Western blotting was performed as follows: 100 µg/ml of a crude lysate of a CagA VacA positive strain of *H pylori* was run on a 10–15% gradient gel and then blotted on to nitrocellulose for one hour at 20 V, 300 mAmPs. The *H pylori* were grown on selective plates at 37°C in a controlled atmosphere (5% oxygen, 10% carbon dioxide) for five days and then washed and stored in phosphate buffered saline (PBS) at −70°C. The nitrocellulose was blocked overnight at 4°C in blocking solution (15 g Marvel milk in 200 ml PBS). The nitrocellulose was incubated with 5 µl serum and 1 ml saliva and control serum samples for one hour and at the dilutions stated below in TTBS (300 ml PBS/Tween, 9 g Marvel milk). The blots were washed in PBS/Tween (one litre PBS/4 ml Tween 20) three times for 10 minutes. The IgG conjugate (HRP conjugated goat antihuman IgG; catalogue number A2290; Sigma) was diluted at the optimum dilution stated below in TTBS and incubated again for one hour. Washing was then performed four times for 15 minutes in PBS/Tween and the signal developed using the Amersham ECL (Amersham, Little Chalfont, Buckinghamshire, UK) detection system.

Conjugate, serum, and saliva concentrations were optimised using paired samples. The optimum dilution of saliva was 1/4 to minimise sample use and 1/500 for the IgG conjugate. The optimal concentrations for serum were an IgG sample dilution of 1/5000 and IgG conjugate concentration of 1/6000.

Some saliva specimens contained an excess of debris on visual inspection and were centrifuged for five minutes at 5000 × g before testing.

STATISTICAL METHODS

The sensitivity of saliva ELISA and saliva western blot test were compared by focusing on “true” positives as defined by serum ELISA and serum western blot, respectively. The sensitivity of the two tests was then compared using McNemar’s test for paired samples with continuity correction. The specificity of the two saliva tests was similarly compared by focusing on the “true” negatives defined by the gold standard.

We calculated $\chi^2$ statistics for comparing the agreement for saliva and serum western blot using EPI INFO version 6.

Results

**PERFORMANCE OF SALIVARY ELISA VERSUS SERUM ELISA**

Of the 665 sera and saliva samples tested by ELISA, 50 (8%) were seropositive (fig 1). The sensitivity and specificity of salivary ELISA against serum ELISA as gold standard was 32 of
50 (64%) and 530 of 619 (87%), respectively, at a cut-off point of 0.41 OD (optical density) units for saliva and 0.1 OD units for serum (fig 2), as suggested by the manufacturer. The cut-off point of 0.41 maximised specificity without compromising sensitivity too far (fig 3).

ESTABLISHING CRITERIA FOR POSITIVE AND NEGATIVE SALIVARY WESTERN BLOT

The criteria for positivity on serum/saliva western blotting were either the recognition of the CagA (120 kDa) band alone, which has previously been shown to be specific for the presence of H pylori infection, or a minimum of six bands from any other region of the blot, because several bands between 30 and 60 kDa were non-specific, as has been reported previously. Using these criteria, 17 of 18 (94%) positive sera were western blot seropositive and 16 of 16 (100%) negative sera were western blot seronegative (fig 4).

The same criteria were applied to the saliva western blot samples and 11 of 13 (84%) of the saliva specimens from seropositive subjects were western blot positive, and 18 of 18 (100%) of the saliva specimens from seronegative subjects were western blot negative (fig 5).

PERFORMANCE OF SALIVARY WESTERN BLOT COMPARED WITH SERUM WESTERN BLOT

A total of 16 samples had to be centrifuged before western blotting and of these seven still yielded uninterpretable blots and were excluded from the analysis. Using the serum western blot as the gold standard, the sensitivity and specificity of the salivary western blot was 38 of 47 (81%) and 68 of 75 (91%), respectively (table 1).

The sensitivity and specificity of saliva ELISA on these same specimens was 29 of 47 (62%) and 48 of 75 (64%), respectively. Saliva western blotting was significantly more sensitive (p = 0.039) and significantly more specific (p = 0.00005) than saliva ELISA.

The presence of bands to the cagA and vacA proteins on the salivary and serum western blots were compared, with good overall agreement between the two methods (κ = 0.75, p < 0.000001 for CagA; κ = 0.66, p < 0.000001 for VacA; table 2).

PERFORMANCE OF SALIVARY WESTERN BLOT AGAINST SERUM ELISA

The sensitivity and specificity of saliva western blot compared with serum ELISA was 32 of 44 (73%) and 65 of 78 (83%), respectively (table 3). The sensitivity of salivary western blotting was not significantly greater than salivary ELISA (p = 0.39), whereas specificity was significantly greater (p = 0.0071).

COMPARISON OF SERUM WESTERN BLOTTING AGAINST SERUM ELISA

There was good overall agreement between serum western blot and serum ELISA (κ = 0.84, p < 0.000001; table 4). Of the six ELISA negative, but western blot positive,
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Discussion
Our study demonstrates that western blotting has a significantly better specificity than ELISA for the detection of H pylori antibodies in the saliva of children and is comparable with serum ELISA. Therefore, salivary western blotting might be useful in the study of children, where high specificity is of particular importance, owing to the low prevalence of infection. In comparison with serum western blotting, salivary western blotting was more sensitive than salivary ELISA. It was also superior when both were compared with serum ELISA, although this failed to reach significance.

Our study might have underestimated the specificity of salivary western blotting significantly, because a stratified sample was used, in which saliva positive serum negative specimens on ELISA were over represented. Although western blotting of saliva was very specific, it had a slightly lower sensitivity than serum ELISA, using serum western blot as the gold standard. However, specificity is more important than sensitivity in studying low prevalence populations, such as children in the developed world.

The qualitative nature of the western blot does appear to overcome the problems of variable transudation of serum immunoglobulin into saliva, which might affect ELISA optical density. Western blotting has the additional advantage of providing more information from the saliva samples, such as seropositivity to CagA or VacA, which can be used to type the organisms with which the patient is infected. There was good agreement between bands detected on serum and saliva western blotting, supporting the use of saliva for this purpose.

Saliva has some disadvantages compared with serum. In a number of specimens there was excessive debris, which required centrifugation. Unfortunately, there were still seven samples that yielded uninterpretable blots and these were not included in the results. Another potential disadvantage is the sample volume required, although only 1 ml was used, which is not excessive.

Other problems that might impair the sensitivity of salivary western blotting are the freeze thawing of samples and the circumstances of sample collection. In our study, the saliva was three years old and had been subjected to a previous freeze–thaw cycle. The means of collection might also be important: theoretically, unstimulated saliva is better than stimulated saliva.

Saliva has several advantages over other methods of determining H pylori status. It is non-invasive, unlike serum testing, and it is much cheaper and more convenient than C13 urea breath testing. New tests to detect H pylori antigen in faeces hold promise, but faecal specimens are more difficult to obtain, particularly in children, and harder to work with in the laboratory. Preliminary reports of evaluation in children have given promising results.

Although western blotting is a reliable method and overcomes the inconvenience of a capture assay, such as ELISA, it is time consuming, but could and is being automated. However, because of the ease of collection and non-invasiveness, saliva remains attractive for the diagnosis of H pylori infection, and salivary western blotting is therefore a potentially useful method for epidemiology and paediatric diagnosis.

Table 2 Presence of cagA and vacA bands on serum and salivary western blots

<table>
<thead>
<tr>
<th></th>
<th>Serum WB positive</th>
<th>Serum WB negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA serum positive</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>cagA serum negative</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>vacA serum positive</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>vacA serum negative</td>
<td>92</td>
<td>4</td>
</tr>
</tbody>
</table>

$k_1 = 0.75$ $k_2 = 0.66$

Table 3 Performance of salivary western blot (WB) compared with serum enzyme linked immunosorbent assay (ELISA) by salivary ELISA status

<table>
<thead>
<tr>
<th>Salivary WB</th>
<th>Serum ELISA positive</th>
<th>Serum ELISA negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>44</td>
</tr>
</tbody>
</table>

$k_1 = 0.75$ $k_2 = 0.66$

Table 4 Agreement between serum enzyme linked immunosorbent assay (ELISA) and serum western blot (WB)

<table>
<thead>
<tr>
<th></th>
<th>Serum WB positive</th>
<th>Serum WB negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ELISA positive</td>
<td>41</td>
<td>3</td>
</tr>
<tr>
<td>Serum ELISA negative</td>
<td>6</td>
<td>72</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>75</td>
</tr>
</tbody>
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Rationalised virological electron microscope specimen testing policy

I read the two letters from McCaughey and Curry and their respective colleagues1 2 with astonishment. Their debate on a policy for electron microscopy (EM) use reminded me of those mediaeval ones about the number of angels that could dance on the head of a pin. Interesting debate, pity it missed the point. They failed, I think, to address three important aspects:

1. The nature of virus diagnosis. No one can pretend that virus laboratories can investigate every individual “viral” illness in the community, but they have an obligation to monitor what is prevalent in it. Although attractive to a cash strapped service, selecting specimens on the basis that some are more likely to yield positives than others misses the point of diagnosis—more than one virus can cause many “virus like” syndromes and what is detected does not parallel exactly the presence of symptoms. To discard specimens taken from those who are recovering because the yield may be low, or where the cause is apparently obvious, strikes me as arrogant. If someone has taken the trouble to send a specimen, it seems reasonable to look at it, if only in the hope that the sender might be encouraged to send others in the future especially if it turns out to be positive. It is a constant battle to get worthwhile virological specimens sent to the laboratory—chocking them off is not a plan. Moreover, I would further confirm the Irish view that solid stools may often yield positives and also that making the diagnosis by holding the specimen up to the light (metaphorically speaking) is very tempting. Trends in infection can only be given some credence if the specimen base remains more or less constant. Arbitrary and variable selection of what will be examined, and what will not, is not likely to be effective. ELISA for some diseases is becoming larger, although electronic microscopy (EM) to a much greater extent than that originally intended by either Professor Madeley’s comments are very welcome, but his letter opens up the debate about this issue.

2. The question of money. EM is unique among virological laboratory techniques in that the major costs of using it are not the equipment, staff are incurred in setting it up in the first place. Running costs, in comparison, are trivial, but savings are thought to be possible by allowing the operator to work only part time. Used full time, the technique becomes less expensive for each specimen the more it is used, but this use must be sensible, and humane to the operator. Microscopy, and EM in particular, can never really be a part time occupation—the operator needs to keep in constant practice and to be committed to it. The cream of his/her work should not be skimmed off by using other techniques (enzyme immunoassays, etc.)—to detect rotavirus or adenovirus—for example. Like everyone else, electron microscope scopists thrive on getting positive results and these reward immensely what can be a lonely working existence. Moreover, the laboratory gets several simultaneous tests for its money, results are produced more quickly than with other tests and, in most cases, with better sensitivity.

3. The need to retain EM as a non-centralised resource. With changes in travel and climate, “new” viruses can appear anywhere at any time. Part of characterising a putative new virus, whether truly novel or merely transferred to a new habitat, is to know what it looks like—other properties correlate surprisingly well with structure and having only a national, or even a regional, facility for this purpose is usually a recipe for serious delay. Specimens from the periphery do not carry the same urgency as the facility’s own work, especially if the latter has to meet internal performance targets. Passing perhaps the most interesting part to another laboratory is hardly likely to encourage the local virus hunters either.

Clamping EM into an “rational policy”, instead of encouraging its use wherever possible, will ultimately cause it to die a slow death from a downward spiral of discouragement. Put in rugby terms, use it or lose it. EM is the only truly “catch all” technique in diagnostic virology. If lost, it will be replaced (partially, even at best) with a battery of tests which, collectively, will cost more, take longer to do, and will frequently miss the dual or triple gut infections that are common in children.

The authors reply

Madeley’s perception of the continuing usefulness of diagnostic electron microscopy (EM) in virus laboratories is very different from ours. We believe that diagnostic EM is becoming largely redundant and will continue to be replaced by other methods. Increasingly, these alternatives are molecular. Such assays are preferable to EM on grounds of cost, sensitivity, and the inherent versatility of generic methodology. In our laboratory, we have replaced EM as our routine first line test for the diagnosis of primary and recurrent herpes simplex infections, herpetic zoster infections and a comparison with electron microscopy. J Med Virol [in press].


The authors reply

Professor Madeley’s comments are very welcome but his letter opens up the debate about electron microscopy (EM) to a much greater extent than that originally intended by either the rationalised EM policy paper1 2 or the recent correspondence relating to it.1 2 We strongly agree that EM should not be allowed to wither and die, but maintaining the relative abundance of EM units that existed into the late 1980s was untenable for two main reasons. First, the development of new investigative and diagnostic technologies (initially

In the current climate of adverse publicity and a UK manpower crisis in histopathology, the book’s aim to “project the pathologist and break some of the myths surrounding his profession” is most welcome. Dr. Anim appears well qualified to undertake such a task, having worked in Kuwait, Ghana, and London, and having experience in the four main pathology disciplines. Despite being a histopathologist, he succeeds in minimising the nomenclature fixation of most accounts and gives a considered overview of each branch of pathology, highlighting the often overlooked educational and management aspects of the job.

The book comprises a brief history of pathology and its subspecialities, analyses various training schemes and practices worldwide, and touches on future developments. It is more than just a factual account because the author puts forward opinions on the role of pathology in the undergraduate curriculum, the need for close clinical liaison, and the frustrations experienced by pathologists trained abroad who return to work in less developed countries.

The author acknowledges that some of his observations on training might be deficient owing to the speed of recent modifications which, not surprisingly, is the case in his coverage of the Royal College of Pathologists’ examination of the specialist training register and the concept of continuing medical education are notable omissions.

There are several appendices pertaining to training in the UK, USA, China, and the USSR, but these would benefit from the inclusion of contact addresses/web sites for organisations such as the RCPPath, ACP, IBMS, and IAP and their overseas equivalents. Although the information in the text seems up to date, the references are disappointing, with many citations of recent trends/advances dating from the mid-eighties.

The price is extremely reasonable, which encourages one to overlook the occasional typographical errors such as: “the part 2 is less formal and may consist of a dissertation”!

In my opinion, the author achieves his aim and this book will be of value to those called upon to explain the work of a pathologist to health professionals or lay people, and to those involved in giving careers advice.
that goes into the required details when discussing the diseases that are specific for the upper aerodigestive tract. The growth in knowledge and proliferation of diagnostic entities associated with the upper aerodigestive tract are reflected in the fact that the current fascicle counts 455 pages, whereas the head and neck fascicle from the previous series had 343. Another difference to the previous edition is the use of colour illustrations, which greatly enhances the quality of the book.

Of course, there are some issues on which there may be disagreement with the authors. The use of the term transitional carcinoma as a synonym for undifferentiated carcinoma is confusing because in the WHO classification this definition is used as a synonym for cervical cell carcinoma, the tumour originally described by Ringertz in his seminal monograph. Moreover, equating psammomatosi ossifying fibroma with juvenile ossifying fibroma is unjustified: these lesions differ in histology as well as in predilection site and age. Finally, the discussion of the bony lesions occurring in the jaw is meagre but I suppose they will be the subject of a separate fascicle, as was the case in the previous series.

I can heartily recommend this book for anyone who has diagnostic responsibilities for specimens from the upper aerodigestive tract.

PIETER SLOOTWEG

CD-ROM review


I am a lover of well written textbooks and, I suspect, like most histopathologists a relatively late adopter of new technologies such as the use of the digital image. I therefore faced the review of this CD-ROM atlas of surgical pathology with some trepidation; the reader should take this into account. This CD-ROM installs, or uses, Quick Time, Adobe Acrobat, and Folio Bound Views to present and navigate between what is stated to be over 4000 images taken from 1500 different lesion types. The images are almost all haematoxylin and eosin stained sections. The quality of photomicrography is good—illumination is even, the resolution is fine for someone used to navigating electronic indexes. However, they would need a slight over emphasis on high power images, the inability to select one’s own field, and the lack of resolution compared with real microscopy. On the positive side, however, was the requirement to make a commitment to a diagnosis on the basis of a given field. This is obviously something that could be used in the future for testing professional interpretation in a very standardised way.

All of this raises the question “who would derive most value from the program?”. I don’t think there is a lot of new information here for existing consultants. However, I can imagine the images being useful in teaching (I should note that I attempted to cut and copy some of the images out of the program, without knowing whether I was allowed to or not, but in any event, the program would not allow me to do this). Trainees might prefer this to a paper atlas because a large number of reasonably high quality images are contained here, and the indexing is fine for someone used to navigating electronic indexes. However, they would need to be at a level where they know the names of basic histological patterns. I would therefore envisage that this would be invaluable to SpRs as an adjunct to viewing slide collections and participating in bench work.

I would recommend that departments set up an electronic learning resource for their trainees add this to the collection of available CD-ROM programs and think that practical use would be seen at least by those trainees who enjoy operating this type of technology.

TIM STEPHENSON

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Notices

Muscle Disorders: Pathophysiology

Applications and Techniques in Veterinary Pathology

New Millennium Bugs

Cytopathology Update: Making Cervical Cytopathology Work

27 September 2000, 5 October 2000, 18 October 2000, and 7 December 2000, respectively

Four one day symposia held at the Royal College of Pathologists, 2 Carlton House Terrace, London, SW1Y 5AF, UK. The symposia are open to members of the college, to trainee pathologists, and to workers in other disciplines with an interest in the subject. The programme is approved for CPD.

Further details: Scientific Meetings Officer, Royal College of Pathologists, 2 Carlton House Terrace, London SW1Y 5AF, UK, tel: +44 020 7451 6740/6739; email: www.repath.org

Practical Adult Cardiovascular Pathology Course

6–8 November 2000

Royal Brompton Hospital, Imperial School of Medicine, and National Heart and Lung Institute

This “hands on” course approaches in detail the problems that face the diagnostic pathologist when dealing with cardiovascular pathology. Congenital heart disease will be highlighted. The approach to a cardiac necropsy and sudden death will be emphasised. Cardiac specimens will be made available for analysis and practical demonstrations as well as video demonstrations. A slide seminar is also included. The course is aimed at trainees studying for the MRCPATH and also senior pathologists who wish to update their knowledge of cardiac disease, both congenital and adult. Course fee £300.00 or £200.00 for juniors in training.

Details from: Short Course Office, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY; tel: +44(0)20 73518172; fax +44(0)20 73518246; email: shortcourse.NHLI@IC.AC.UK

XIII International Congress for Quantitative Diagnostic Pathology

October 2000

Adelaide, Australia

The web site for this meeting will be available at the following address from 12th May 2000: http://som.finders.edu.edu/fusa/AnatPath/congress/main1.htm

www.jclinpath.com

The authors would like to apologise for an error that occurred in this paper. It was stated throughout the paper that 665 children were tested but in fact the number was 669. In addition, in the first sentence of the results section of the abstract 530 of 691 (87%) should be 530 of 619 (86%). The authors stress that these errors do not change the message of the paper.