Cryptosporidium Antigen Detection in Human Feces by Reverse Passive Hemagglutination Assay

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Received 9 May 1994/Returned for modification 23 July 1994/Accepted 16 August 1994

A reverse passive hemagglutination (RPH) assay was developed for Cryptosporidium oocyst antigen with an antioocyst monoclonal antibody (MAb; MAb-C1) coupled to stabilized sheep erythrocytes. RPH was compared with microscopy of auramine-phenol-stained smears of 56 oocyst-positive fecal samples, each of which was tested blindly by RPH with two oocyst-negative samples received on the same day (a total of 112 controls). Thirty-nine additional fecal samples from human immunodeficiency virus type 1 antibody-positive patients with diarrhea (10 of which were positive in auramine-phenol-stained smears) were stored at −20°C before testing. Thirty specimens with a variety of other fecal pathogens (all negative for oocysts) were also tested. Of the 237 samples tested, 69 were positive by one or both methods: 65 by RPH and 66 by microscopy. The kappa coefficient of agreement between the methods was very high at 0.926. The sensitivity of RPH was 93.9%, the specificity was 98.2%, the positive predictive value was 95.4%, and the negative predictive value was 97.7%. Visible oocyst numbers and RPH titers were measured after storage of fecal samples and oocyst concentrates for 8 days at 4°C. Oocyst morphology was generally poor in specimens from the human immunodeficiency virus type 1 antibody-positive group, and it degenerated during the 8-day storage experiments. MAb-C1-reactive antigen eluted from oocysts to give progressively higher reciprocal titers during storage, and it was partially removed from the oocysts by concentration. RPH is a promising technique for the detection of Cryptosporidium antigen in human feces and may be useful when specimens are stored before testing. Studies of the sensitivity of Cryptosporidium immunooassays should take into account the possible release of antigen from oocysts.

Although long recognized as an animal pathogen, infection of humans with Cryptosporidium spp. has been reported frequently only during the past decade. This followed appreciation of its connection with intractable diarrhea in patients with AIDS (10) and of the public health implications of its frequent presence in surface waters (13). Cryptosporidiosis has a worldwide distribution. It is detected in about 16% of patients with AIDS and diarrhea in hospitals in the United States, and this proportion rises to about 50% in developing countries (10).

Laboratory diagnosis worldwide currently relies upon the well-established but time-consuming method of microscopy of Ziehl-Neelsen- or auramine-phenol-stained fecal smears (3). A low level of sensitivity is a problem with these methods, and it may be increased only by technically demanding concentration procedures (17). Immunofluorescent stains are increasingly used, but not all studies have shown their use to be straightforward or always to improve sensitivity (2, 8, 9, 11). Experience with several immunodiagnostic assays has been published (1, 4, 12, 14–16). However, few comparative studies of these methods have been performed outside the research laboratory setting, none has shown significantly improved sensitivity, and nonspecific reactions have sometimes been observed (4). Today, diagnostic microbiology laboratories in the United Kingdom screen the majority of fecal specimens from patients with community-acquired cases of diarrhea for Cryptosporidium oocysts; in our laboratory about 7,000 microscopic examinations of auramine-phenol-stained material were performed in 1993. Elsewhere, many laboratories restrict the examination to feces from human immunodeficiency virus type 1 (HIV-1) antibody-positive patients. A sensitive, specific, and cost-effective diagnostic method would be a major advance.

Reverse passive hemagglutination (RPH) involves the agglutination by specific antigen of glutaraldehyde-stabilized, protease-sensitized sheep erythrocytes that have been coupled to immunoglobulin by chromic chloride (6). The sensitivity of the method exceeds that of enzyme-linked immunosorbent assay (ELISA) in some systems, and the technique has been successfully used for antigen detection in several body fluids, including feces. Freeze-drying allows indefinite storage of reagents, and the reactions are stable, easily read, and controlled (7).

We compared RPH with semiquantitative microscopy of auramine-phenol-stained smears of feces from patients with community-acquired diarrhea, patients with AIDS and diarrhea, and patients with a variety of other fecal pathogens.

MATERIALS AND METHODS

At the Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge (CMPhL), stools were cultured for Salmonella, Shigella, and Campylobacter spp. by routine methods. Clostridium difficile cytotoxin and Clostridium perfringens enterotoxin were detected by Vero cell culture. Stools from patients with bloody diarrhea and hemolytic uremic syndrome and from children out of hospital were investigated for Escherichia coli O157. Fecal samples from patients recently returned from areas where vibrios and Aeromonas spp. are endemic were also cultured for pathogenic vibrios, and samples containing blood were also cultured for...
Aeromonas spp. A wet preparation and a formol saline concentration for parasites were performed on patients recently returned from outside the British Isles or those with bloody diarrhea or AIDS or when requested clinically.

Auramine-phenol staining for Cryptosporidium spp. was performed on all samples of feces from patients between the ages of 6 months and 44 years (5). A pea-sized portion was emulsified in 0.5 ml of quarter-strength nutrition’s solution, giving a 20% solution, and a 10-μl loopful was smeared in a well in a monosop slide (code O11; Hendley, Loughton, United Kingdom) and allowed to dry in air. A positive control was included on each slide. Staining was performed with auramine-phenol for 15 min, 3% acid-alcohol for 5 min, and potassium permanganate for 30 s, with washes after each step. After air drying, the slide was examined under a fluorescence microscope at ×250 magnification, and the number of cysts per high-power field was recorded. Fecal samples found to be positive for Cryptosporidium oocysts were each matched with two Cryptosporidium-negative samples from other patients that had arrived on the same day. Each set of three specimens was randomized and coded and submitted blind for RPH testing, usually on the day of arrival.

Fecal specimens from patients with AIDS and diarrhea were collected from inpatients at the Middlesex Hospital, London. These were immediately frozen at −20°C and were transported to CMPHL for routine fecal culture, parasite concentration and microscopy, and blind RPH testing of the results of routine investigations. As additional controls, fecal specimens from patients with diarrhea caused by other pathogens submitted to CMPHL and from patients with no causative diagnosis were also tested by RPH.

RPH technique. Anticryptosporidium oocyst monoclonal antibody (MAB; MAB-C1; batch 11), an immunoglobulin M (IgM) antibody of the kappa isotype, was generously donated as ascites by the Central Public Health Laboratory, Colindale, London. Ascitic fluid was clarified by centrifugation and was filtered through a 0.22-μm-pore-size Millipore filter, and then IgM was precipitated at 4°C with 20 volumes of 2% boric acid. After centrifuging at 10,000 × g for 15 min, the precipitate was dissolved in saline and was dialyzed overnight against physiological saline. A mouse kappa IgM (ABPC 22; no. M-7394; Sigma) was coupled to sheep erythrocytes as an immunoglobulin-erythrocyte control, and uncoupled erythrocytes were used as the erythrocyte control.

Antibody-coupled erythrocytes were prepared as follows (7). Sheep erythrocytes washed in phosphate-buffered saline (PBS) were incubated for 20 min at 37°C with α-chymotrypsin in acidified PBS-azide complement fixation test buffer (BR16; Oxoid, Basingstoke, United Kingdom). Stock chromic chloride coupling solution was made by dissolving CrCl3 · 6H2O (BDH; Poole, United Kingdom) to a 1% concentration in pyrogen-free physiological saline and adjusting the pH to 5.0 with 5 M sodium hydroxide. Purified immunoglobulin was then coupled at a concentration of 0.5 mg ml−1 to sheep erythrocytes in piperazine buffer with an optimal 1:180 dilution of chromic chloride coupling solution (6, 7). Coupling was confirmed by titration with sheep anti-mouse globulin (Serotec Ltd., Oxford, United Kingdom). Samples of 1 ml of coupled cells were stabilized with 100 μl of glutaraldehyde (30 μl in 5 ml of PBS; BD) and were then freeze-dried in PBS with 1.5% sucrose–1.0% bovine serum albumin (Calbiochem, La Jolla, Calif.) in an Edwards ASP230B freeze-dryer overnight. Before use, the cells were reconstituted with gentle mixing in PBS to their original volumes at 4°C for 24 h; shorter reconstitution times gave a weaker distinction between positive and negative results.

Fecal samples were suspended in PBS at 25% (wt/vol). The fecal suspensions were placed in a boiling water bath for 3 min, which we observed improved the quality of the agglutination reactions. Following sedimentation and cooling for 15 min at room temperature, the supernatants were diluted 1:2 and 1:20 in PBS containing 0.1% normal mouse serum (Serotec). Serial twofold dilutions of these supernatants were made along 96-well microtiter plates (SeroWell; Bithynia Sterilin, Stone, United Kingdom) with PBS containing 1% normal mouse serum, from 1:2 to 1:256 and from 1:20 to 1:1,280, respectively.

Plates were shaken to mix the contents of the wells, and the result of hemagglutination was read by the sedimentation pattern after 30 min at room temperature. A negative result for a well was indicated by a button of unagglutinated erythrocytes settling at the bottom of the well, whereas a positive result was indicated by a diffuse coating of agglutinated cells over the bottom of the well (7). An RPH result for a fecal specimen was considered positive (i) if the titer in the MAB-C1 dilution series that began at 1:20 was two or more dilutions higher than the highest titer in either of the control dilution series that began at 1:20 or, if the sample was negative by criteria (i), then (ii) if the titer in the MAB-C1 dilution series that began at 1:2 was three or more dilutions higher than the highest titer in either of the control dilution series that began at 1:2.

Effects of specimen storage and concentration on RPH titer and oocyst counts. Eight fecal samples that had been submitted to CMPHL for diagnostic purposes were chosen to study the effects of storage and concentration procedures. They were oocyst microscopy positive, large in volume, and gave high RPH reciprocal titers without nonspecific reactivities.

Twenty-five percent suspensions of each of the eight freshly received samples were made in PBS, and aliquots of each of these were stored at 4°C. Their Cryptosporidium antigen reciprocal titers and oocyst counts were determined on days 1, 3, 6, and 8. The RPH assays were performed in the same way as the diagnostic tests, with the exception that fecal suspensions were filtered through 0.22-μm-pore-size filters (Millipore) after removal from the boiling water bath. This step was included because preliminary experiments indicated that nonspecific reactivity increased with storage and that it could be removed by filtration without significantly altering the specific titer (data not shown). Quantitation of the oocysts was performed by use of an improved Neubauer counting chamber, with calculation of the number of oocysts per milliliter in the original fecal sample.

Oocyst concentrates were made by mixing equal volumes of the eight freshly received fecal samples and dithiothreitol (Mucolyse PL-701; Pro-Lab Diagnostics, Wirral, United Kingdom) in 10-ml centrifuge tubes and holding the mixtures at room temperature for 15 min. Ether (2 ml) was added to each tube, and the contents were mixed by shaking before centrifuging at 900 × g for 3 min. After the solid surface plug and supernatant were discarded, the pellet was resuspended and washed three times in PBS (pH 7.2), centrifuging at 900 × g for 10 min after each wash, and was then resuspended in 1 ml of PBS. One volume of each oocyst suspension was loaded to a discontinuous step density gradient consisting of equal volumes of 0.5, 1.0, 2.0, 4.0, and 6.0% Ficoll (400) in PBS containing 16% sodium diatrizoate. Each gradient was centrifuged at 900 × g for 60 min at room temperature. The oocysts were harvested from a clearly defined band at the former location of the 0.5% Ficoll layer. After washing three times in PBS, the oocysts were resuspended in 1 ml of PBS and their concentrations were determined by counting in an improved Neubauer chamber. Oocyst concentrates were then added to eight aliquots of a Cryptosporidium-negative sample of feces to
TABLE 1. Correlation between RPH assay* of Cryptosporidium antigen and microscopy of auramine-phenol-stained smears for oocysts in human stool specimens

<table>
<thead>
<tr>
<th>RPH result</th>
<th>No. of specimens with the following microscopy result:</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>62</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
</tr>
</tbody>
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* Sensitivity, 93.9%; specificity, 98.2%; positive predictive value, 95.4%; negative predictive value, 97.7%; kappa coefficient of agreement, 0.926.

give a concentration of about $10^5$ to $10^6$/ml. These mixtures were stored at 4°C. After 1, 3, 6, and 8 days the oocyst concentrations in each mixture were determined by light microscopy with quantitation in an improved Neubauer chamber, and antigen concentrations were determined by performing RPH assays.

RESULTS

In total, 237 specimens from patients were tested. These included 56 from CMPHL with positive microscopy results and the associated 112 controls with negative microscopy results. Thirty-nine stool samples came from episodes of diarrhea from HIV-1 antibody-positive patients (10 microscopy positive), all of which were clinically compatible with Cryptosporidium infections and negative for other pathogens. Thirty samples came from known Cryptosporidium-negative patients at CMPHL (10 with giardiasis, 8 with C. difficile toxin, 2 with E. coli O157, 1 each with Endolimax sp. and Iodamoeba sp., and 8 with diarrhea of unknown cause acquired in the United Kingdom).

Results from the dilution series starting at 1:20 usually gave more easily interpretable reactions, stronger hemagglutination patterns, and less residual nonspecific reactivity than tests performed in the dilution series that began at 1:2. Occasional prozone phenomena were observed with the supernatants tested in the 1:2 series, which were diluted out in the 1:20 series. In the 1:20 series, 62% of the tests showed no nonspecific reactions, 93% gave reactions of one or no wells, and no tests showed nonspecific reactivity of greater than two wells. Only 36% of the nonspecific reactions were predominantly against immunoglobulin-coupled erythrocytes (defined as a titer 2 or more dilutions greater against immunoglobulin-coupled erythrocytes than against erythrocytes alone). No samples reacted only or predominantly against uncoupled erythrocytes.

Table 1 summarizes the results obtained from all 237 samples from patients with diarrhea tested by microscopy and RPH, and Fig. 1 shows the RPH titers obtained from the 69 specimens that were positive by one or both methods. Sixty-five specimens were positive by RPH; 63 of these were positive in the MAb-C1 dilution series that began at 1:20 by criterion (i) above. The remaining two gave titers of 16 in the series that began at 1:2. All 30 negative control samples from patients with known causes of diarrhea and negative smears for Cryptosporidium oocysts were negative by RPH. Of the 66 specimens positive by microscopy, only 4 were negative by RPH, all of which were diagnostic samples submitted to CMPHL. Of the 171 specimens negative by microscopy, 3 were positive by RPH, all of which were from HIV-1 antibody-positive patients. Assuming microscopy of auramine-phenol-stained smears to be a reference technique, the sensitivity of RPH was therefore 93.9%; the specificity was 98.2%, the positive predictive value was 95.4%, and the negative predictive value was 97.7%. The kappa coefficient of agreement (5) between the methods was very high at 0.926.

Table 2 illustrates the results obtained from the 168 clinical diagnostic specimens referred to CMPHL combined with those obtained from the 30 negative control samples. The sensitivity of RPH for this group of specimens was 92.9%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 97.3%. The kappa coefficient of agreement between the methods was very high at 0.949.

Table 3 shows the results obtained with the 39 specimens from HIV-1 antibody-positive patients. For this group of specimens, the sensitivity was 100%, the specificity was 89.7%, the positive predictive value was 76.9%, and the negative predictive value was 100%. The kappa coefficient of agreement between the methods was high at 0.816.

One specimen from an HIV-1 antibody-positive patient that was positive by RPH (reciprocal titer, 160) but that initially screened negative by microscopy was found to contain very scanty but characteristic oocysts (less than one per high-power field) on review of the film by senior staff. This sample was scored as RPH positive and microscopic negative. Two other specimens from HIV-1 antibody-positive patients with diarrhea were positive by RPH (reciprocal titers, 20 and 160) but negative by microscopy even on repeat examination.

All four samples in which oocysts were seen by microscopy but which gave negative RPH results were from specimens submitted to CMPHL for routine diagnostic purposes. None of

FIG. 1. RPH reciprocal titers obtained with the 69 stool specimens that were positive by microscopy or RPH. CMPHL (open bars), samples referred to CMPHL for diagnostic purposes; HIV+ (shaded bars), samples from HIV-1 antibody-positive patients.

TABLE 2. Correlation between RPH assay* of Cryptosporidium antigen and microscopy of auramine-phenol-stained smears for oocysts in stool specimens submitted to CMPHL for diagnostic reasons

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<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>52</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
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* Sensitivity, 92.9%; specificity, 100%; positive predictive value, 100%; negative predictive value, 97.3%; kappa coefficient of agreement, 0.949.
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

*Sensitivity, 100%; specificity, 89.7%; positive predictive value, 76.9%; negative predictive value, 100%; kappa coefficient of agreement, 0.816.

the samples gave high reciprocal titers in the control dilution series that might have masked a true-positive reaction, and three of the four samples had only low numbers of cysts per high-power field. Thirty-five characteristic oocysts per high-power field were seen in the fourth stool sample, but RPH titers were identical on repeat testing. Unfortunately, this was a very low volume specimen, and no additional samples could be obtained from the patient, so further investigations were not performed.

Figure 2 shows the effects of storage on the mean oocyst concentrations (oocysts per milliliter) and the geometric mean reciprocal RPH titers seen in the eight fecal samples studied after 1, 3, 6, and 8 days of storage. Mean oocyst counts in the eight clinical specimens of feces fell progressively from 1.05 × 10⁶ (standard deviation [SD], 1.69 × 10⁶) to 2.53 × 10⁵ (SD, 3.56 × 10⁵) per ml, and in the fecal sample with added oocyst concentrates they fell from 5.80 × 10⁵ (SD, 1.11 × 10⁶) to 5.48 × 10⁵ (SD, 8.23 × 10⁵) per ml, with many oocysts appearing degenerate at 6 and 8 days. Geometric mean reciprocal RPH titers rose progressively from 234 (range, 8 to 2,048) to 861 (range, 8 to 32,768) in the stored clinical specimens of feces, and fell progressively from 144 (range, 2 to 1024) to 2.5 (range, <2 to 8) in the samples to which oocyst concentrates were added.

FIG. 2. Effects of storage at 4°C on oocyst numbers and RPH reciprocal titers. Each measurement is the mean of eight samples. Bars, oocyst numbers per milliliter; lines, geometric mean RPH reciprocal titers; open bars and triangles, eight fecal specimens from patients with natural infections; shaded bars and squares, oocyst-negative fecal specimen to which oocyst concentrates from eight patients with natural infections were added.

**DISCUSSION**

In the preliminary assessment described here, RPH appeared to be a rapid and specific method for detecting Cryptosporidium antigen in human feces, with a sensitivity equivalent to that of the most commonly used diagnostic laboratory test, fluorescence microscopy with auramine-phenol staining. The kappa coefficient of agreement between RPH and microscopy was very high (0.926); this coefficient is the most appropriate measure, because no method for the routine detection of Cryptosporidium oocysts can currently be considered a “gold standard.” Considering the results from all stool specimens from patients with diarrhea, our experience of RPH compared with stool microscopy was similar to that of ELISA in the hands of Newman et al. (12) (specificity, 93.9 versus 96.4%; positive predictive value, 95.4 versus 97.6%). RPH, however, was more sensitive (93.9 versus 83.3%) and gave a higher negative predictive value (97.7 versus 77.1%). Before firm conclusions are drawn, these methods should be compared with the same samples of feces, but RPH appears to be a screening test that is at least as promising as ELISA. RPH should also be compared with immunofluorescence microscopic methods. It is possible that bias toward the increased sensitivity of RPH was introduced into the present study because one of the three fecal samples submitted for testing was known to be positive. However, preliminary experiments showed that storage of fecal samples altered RPH titers, and only one positive sample was submitted to CMPHL per week; hence, practicability dictated that the study be designed in this way.

Diagnostic assays for Cryptosporidium antigens may have different sensitivities and specificities for samples from HIV-1 antibody-positive and healthy patient groups because of the differences in the prevalence of Cryptosporidium spp. and other causes of diarrhea between the groups. Oocyst morphology in most specimens from the HIV-1 antibody-positive patient group was poor, and inexperienced staff found it difficult to assess this group of smears. One sample judged to be microscopy negative routinely was found to contain very scanty typical oocysts on careful review by senior staff. We suspected that the negative microscopy results in this and the other two specimens from HIV-1 antibody-positive patients that also gave positive RPH reactions may have been artifacts as a result of oocyst degeneration during the storage period. There was no evidence of cross-reactivity of these samples with either control reagent, and we found that MAb-Cl did not react with fecal specimens containing Giardia cysts, C. difficile toxin, E. coli O157, Endolimax sp., or Iodamoeba sp. Others have also found that MAb-Cl does not react with Campylobacter jejuni, Salmonella enteritidis, or rotavirus (4). We therefore performed the 8-day storage experiments to investigate the effects of time and concentration procedures on oocyst morphology and antigen release. Chapman et al. (4) used an enzyme immunoassay with MAB-C1 and found similar titers in fecal suspensions and supernatants, suggesting that the antigen may be soluble or finely particulate. We found the antigen to elute from fresh oocysts, reaching progressively higher titers in the soluble phase, while the oocyst structure deteriorated during the same period. RPH with MAB-C1 therefore may be especially useful for the detection of Cryptosporidium antigen in specimens that have been stored or delayed in transit. Much detectable and elutable antigen was removed if oocysts were subjected to a vigorous concentration procedure, and the initially low RPH titers fell progressively during the 8-day study period. Presumably, this was caused by degradation of residual soluble or particulate antigen, with little antigen remaining on
the oocysts and being eluted. Comparison of the sensitivities of microscopic and RPH-based methods is therefore complicated by the progressive elution of antigen from and the loss of the characteristic morphologies of fresh oocysts in clinical specimens. Similar assessments of the roles of free and oocyst-bound antigen and of the way in which they change with storage have not been reported for other Cryptosporidium immunoassays with MAb-C1 or other MAb's, but measurements of the sensitivities of other methods should take this observation into account.

None of the specimens positive in auramine-phenol-stained smears but negative by RPH showed signs of nonspecific reactivity that might have masked significant reactions. Three of the four specimens contained only low numbers of morphologically typical oocysts and might have been at the limits of sensitivity of the RPH assay. One sample contained 35 characteristic oocysts per high-power field, but was repeatedly negative by RPH. These oocysts might have belonged to a Cryptosporidium species that did not react with MAb-C1, but further investigations were not possible because of a limited sample volume.

Two RPH tests were performed on each microtiter tray, with one tray for each of the dilution series beginning at a dilution 1:2 and one beginning at a dilution of 1:20. A diagnostic system based on RPH could involve a more limited series of dilutions and use a single immunoglobulin-coupled erythrocyte control; hence, more tests could be accommodated per plate. Alternatively, a pair of screening dilutions for test and control specimens could be used, with further dilutions set up only if nonspecific agglutination was seen. Testing of additional samples will be necessary to resolve this issue. After reconstitution of the reagents, RPH results are available within 40 min, which includes only about 15 min of hands-on time. This is considerably faster than microscopy if more than a few tests are to be performed, and quantitative results are obtained. As with the currently available ELISA-based methods (12), batch testing of large numbers of samples by RPH is possible, and RPH needs little capital expenditure because the results are assessed by eye and expensive reading equipment is not required.

We found RPH with MAb-C1 to be a promising technique for the detection of Cryptosporidium antigen in human feces, and it may be especially useful when specimens are stored before testing and when batch testing of large numbers of samples is required. Studies of the sensitivity of Cryptosporidium immunoassays should take into account the possible gradual release of antigen from oocysts during storage of fecal specimens.

ACKNOWLEDGMENTS

We are grateful to R. R. A. Coombs (emeritus professor, University of Cambridge, Cambridge, United Kingdom) and J. McLauchlin (Central Public Health Laboratory, Colindale, London) for many helpful discussions and to the Addenbrooke’s Hospital Trust Funds Committee, the East Anglian Regional Health Authority Research Committee, and Serotec Ltd. for support.

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