

An evaluation of the staphylococcal co-agglutination test for the detection of group A rotavirus in human faeces

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Abstract

The group A rotavirus staphylococcal co-agglutination test was evaluated and its sensitivity and specificity compared with an in-house enzyme-linked immunosorbent assay (ELISA) and a commercial latex agglutination test (Rotalex). In addition, the storage stability of the staphylococcal reagents was ascertained. Examination of 136 clarified suspensions of diarrhoeal faeces by the staphylococcal co-agglutination test revealed a high proportion of false positives (26%) and uninterpretable results (34%) due to non-specific agglutination. Non-specific agglutination could be removed effectively by prior absorption of the clarified faecal specimens with unsensitized staphylococci. The staphylococcal co-agglutination test was less sensitive and specific than the in-house enzyme-linked immunosorbent assay but was comparable to the Rotalex slide latex agglutination test. The staphylococcal reagents have a shelf life of at least 29 weeks.

Key words: Diagnosis, human rotavirus infection, co-agglutination, latex agglutination, ELISA.

INTRODUCTION

Group A rotaviruses are the main causes of acute diarrhoea particularly in infants and young children throughout the world.¹ Rapid and reliable laboratory diagnosis of human rotavirus infection is important for patient management especially in the control of hospital infection.

A variety of techniques such as direct electron microscopic visualization, various immunoassays (such as: enzyme-linked immunoassay, immunofluorescence, radioimmunoassay), and polyacrylamide gel electrophoresis have been reported for the diagnosis of rotavirus-associated gastroenteritis.^{2,3,4,5} However, the slide latex agglutination method^{5,6,7} with visual detection is a suitable laboratory test for routine diagnostic purposes as it is rapid, simple to perform and does not require any sophisticated or expensive equipment. Furthermore, as the test is performed individually it is convenient when small numbers of samples are tested. Although the latex particle is currently the ubiquitous solid phase immunoabsorbent used in slide agglutination tests to detect a wide range of antigens⁸, *Staphylococcus aureus* rich in protein A has been used before for the direct detection of a number of microbial antigens.^{9,10,11,12} However, its application has never been widespread and has since been surpassed by the latex par-

ticle. In view of this, it would seem a retrograde step and an anachronism in present-day technology to reconsider the use of staphylococci as the solid phase immunoabsorbent in a slide agglutination test for rotavirus detection. However, the consideration is not without merit in certain countries. In poor countries commercial laboratory reagents are expensive and the financial burden is exacerbated by the large number of diarrhoeal cases.¹³ The ability to produce cheaply reagents for a simple in-house slide agglutination test for rotavirus detection would make the diagnosis of rotavirus-associated diarrhoea within the reach of more diagnostic laboratories in poor countries.

Although an in-house slide agglutination test can be prepared using latex particles as the solid phase immunoabsorbent, protein A-containing *Staphylococcus aureus* bacterium has several advantages. Unlike the latex particle, *Staphylococcus aureus* - an easily cultured bacterium - would provide an unexhaustable source of solid phase immunoabsorbent. The attachment of antibodies to staphylococci is simple, rapid and does not involve any chemical coupling. Although antibodies can also be attached to latex particles by passive adsorption, several factors would have to be determined to achieve optimal coupling and proper orientation of the antibody molecules. Furthermore, chemical coupling is

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essential for more defined orientation of the bound antibodies and longer shelf life.⁸ Thus the staphylococcal co-agglutination (SCA) test has the potential to be a highly cost effective low budget in-house slide agglutination test for rotavirus detection. However, the issues that need to be considered are the performance and stability of the reagents. In an earlier study, the rotavirus SCA test was reported to be as sensitive as a commercial ELISA (Rotazyme) and more sensitive than a commercial latex agglutination test (Rotalex).¹² However, the small sample size of 10 rotavirus-positive and 10 rotavirus-negative specimens made such conclusions equivocal. Furthermore, stability of the reagents on storage was not reported.

The objectives of this study were to evaluate the performance of the SCA test for the detection of rotavirus in human faeces and to compare its sensitivity and specificity with an in-house ELISA and the commercial Rotalex latex agglutination test, as well as to determine the storage stability of staphylococcal reagents.

MATERIALS AND METHODS

Preparation of faecal suspensions

Diarrhoeic faeces were collected from children hospitalized with diarrhoea at the Kuala Lumpur General Hospital. Non-diarrhoeic faeces were collected from children in the community. Faeces for the co-agglutination test and latex agglutination were prepared by making a 10-20% faecal suspension in phosphate buffered saline (PBS) and clarified by centrifugation at 1000g for 15 minutes at room temperature. Faecal suspension for ELISA was prepared in the same manner but with PBS containing 0.05% Tween 20.

Absorption of clarified faecal suspensions with staphylococci was carried out by mixing 50 μ l of the clarified faecal suspension with an equal volume of a 50% (v/v) staphylococcal suspension. The mixtures were either allowed to stand at room temperature for 15 minutes and shaken occasionally, or were rotated on a rotator. The bacterial cells were then pelleted by centrifugation and the supernatant examined for rotavirus by the co-agglutination test.

Preparation of hyperimmune anti-rotavirus sera

The cell culture-adapted SA11 strain of simian rotavirus and pooled human rotaviruses from diarrhoeic faecal specimens collected from infants and children admitted to the Kuala Lumpur

General Hospital were used to raise hyperimmune sera used in this study. Both rotavirus preparations were concentrated by precipitation with polyethylene glycol and partially purified by centrifugation through a 45% glucose layer onto a caesium chloride cushion. The pooled human rotavirus preparation was further purified by centrifugation through a 56% caesium chloride gradient.

Hyperimmune rabbit anti-SA11 rotavirus sera and hyperimmune guinea pig anti-human rotavirus sera were prepared by intramuscular (flanks) and intradermal (back) injections with the respective virus preparations mixed with an equal part of complete Freund's adjuvant during the first inoculation. Incomplete Freund's adjuvant was used in the two subsequent injections. Each inoculation was one month apart. Sera were collected 10 days after the third inoculation. The hyperimmune rabbit and guinea pig rotaviral sera were determined to have end-point titration of 1:40,000 and 1:20,000 respectively as measured by a competitive ELISA.

Preparation of staphylococcal reagents

Staphylococcus aureus Cowan I strain (NCTC 8530) were grown overnight on nutrient agar plates. The bacteria were scrapped off the plates, washed twice with phosphate buffered saline (PBS) and treated for 3 hours with 0.4% formaldehyde in PBS at room temperature. After 2 washings the bacteria were suspended to about 50% (v/v) in PBS and heat-treated at 80°C for 30 minutes in a water bath. The bacteria were then washed twice and suspended to about 80% (v/v) in sterile PBS containing 0.1% sodium azide. The suspension was kept at 4°C until use.

In the preparation of sensitized staphylococci (test reagent), the bacteria concentration was first adjusted with PBS to give an absorbance reading of 1.9 at a wavelength of 450 nm with a photometer (Spectronic 20, Bausch & Lomb, England) which was equivalent to 0.75% (v/v) packed cells pelleted at 4000g for 10 minutes. Anti-SA11 rotavirus serum was added to the suspension to a final dilution of 1:500 and incubated at 37°C for 15 minutes. The antibody-coated staphylococci were washed twice and suspended to the initial volume in PBS containing 0.1% sodium azide. A similar suspension of uncoated staphylococci was used as the control reagent.

The staphylococcal co-agglutination (SCA) test

Two separate drops (15 μ l each) of clarified

faecal suspension were mixed with one drop (15 μ l) of each of the test and control reagents on a microscopic slide. Each mixture was spread to an area of about 1.5 cm diameter. The slide was tilted continuously by hand for 2 minutes and the development of agglutination observed with the naked eye against a dark background. Specimens which formed granular agglutinates with test reagent and not with control reagent were considered rotavirus-positive. Those which agglutinated both test and control reagents were considered to have given uninterpretable results.

The in-house enzyme-linked immunosorbent assay (ELISA)

Faecal samples were examined by an in-house indirect double antibody sandwich ELISA based on the WHO ELISA kit for the detection of group A rotaviruses.¹⁴ Briefly, in the screening test 96-well flat-bottomed Immulon 2 plates (Dynatech Laboratories, Inc., Virginia, USA) were coated with rabbit anti-SA11 serum. Captured rotavirus antigens were reacted with guinea pig anti-human rotavirus serum. The attached guinea pig antibodies were in turn reacted with a goat anti-guinea pig antibody preparation conjugated to alkaline phosphatase (American Qualex, La Mirada, USA) and the attached enzyme-antibody conjugate detected by enzymatic reaction on the substrate p-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets, Sigma, St. Louis, USA). The results of all assays were measured by spectrophotometry. All faecal samples positive by the screening test were

tested by the confirmatory test which was a blocking test based on the competitive binding of bound rabbit anti-rotavirus antibodies adsorbed onto wells and added free rabbit anti-rotavirus antibodies for rotaviral antigen. Samples that were positive by both the screening and confirmatory tests were considered rotavirus-positive. The sensitivity and specificity of the in-house assay were similar to the WHO ELISA and a commercial ELISA (Dakopatts, Copenhagen, Denmark) (unpublished data). The in-house ELISA-confirmed positive and negative results were used as references for comparison with other tests.

The latex agglutination test

Rotavirus was detected by the Rotalex test (Orion Diagnostica, Espoo, Finland) which was performed according to the manufacturer's instructions except that the reagents volume used was fixed at 15 μ l instead of a drop from the container.

RESULTS

Examination of untreated faecal specimens by the SCA test

Table 1 shows that all of 100 rotavirus ELISA-negative normal faecal samples did not agglutinate either the test or the control SCA reagents. Examination of 43 rotavirus ELISA-positive diarrhoeal faecal samples revealed that 18 (42%) agglutinated both control and test reagents while 2 others did not show agglutination with both test and control reagents. When 93 rotavirus

TABLE 1: Examination of untreated normal and diarrhoeal faeces by the staphylococcal co-agglutination test

Faecal specimens			Results of co-agglutination test			
Type	Rotavirus ^a status	No. tested	True positives and negatives	False positives	False negatives	Uninterpretable ^b results
Normal	Neg	100	100(100)	0	NA	0
Diarrhoeal	Neg	93	30(32)	35(38)	NA	28(30)
	Pos	43	23(53)	NA	2(5)	18(42)
		136	53(39)	35(26)	2(1)	46(34)

() = %

a Based on the results of an in-house ELISA

b Agglutinated both control and test reagents

NA = Not applicable

negative diarrhoeal faeces were examined, 35 (38%) agglutinated the test reagent but not the control reagent and 28 (30%) agglutinated both test and control reagents.

Examination of faecal specimens absorbed with uncoated staphylococci by the SCA test

In order to remove non-specific agglutination, 18 of the faecal samples (7 rotavirus-negative specimens, 11 rotavirus-positive samples) that showed non-specific agglutination by the SCA test and control reagents were absorbed with unsensitized staphylococci as described. Test on the treated specimens showed that non-specific agglutination was eliminated from all the specimens; all rotavirus-negative samples retested negative and all rotavirus positive samples retested positive.

Comparison of results of the SCA test on absorbed faecal specimens with results from ELISA and latex agglutination on the same untreated specimens

A different batch of single faecal samples from 105 diarrhoeic children was examined by the SCA test after prior absorption with unsensitized staphylococci, and by an in-house ELISA. The results in Table 2 show that overall agreement among the 2 techniques was found in 96 samples (91%). Seven samples (7%) were either false positives or false negatives and 2 (2%) were uninterpretable as agglutination was observed with both test and control reagents. Based only on the total number of specimens that gave interpretable results, the sensitivity and specificity of the SCA test compared to ELISA were 88% (28/32 positives) and 96% (68/71 negatives), respectively.

A total of 102 of the same samples were also examined by the commercial Rotalex latex agglutination (LA) test. The results in Table 2 show that there was a 91% agreement between the SCA test and LA. The sensitivity and specificity of the SCA test compared to LA were 93% and 94%, respectively. However, of the 4 specimens tested negative by latex agglutination and positive by the SCA test, 2 were in fact ELISA positive. Conversely, 1 of the 2 specimens tested negative by latex agglutination and positive by SCA test was tested negative by ELISA. When sensitivity and specificity were recalculated based only on those latex agglutination results that were similar to those of ELISA, the sensitivity and specificity of the SCA test were 96%

TABLE 2: Comparison of the staphylococcal co-agglutination (SCA) test with an in-house ELISA and a commercial slide latex agglutination test

Pattern of results	No. (%) associated with a particular pattern
SCA ^a /ELISA+	28 (26)
SCA-/ELISA+	4 (4)
SCA-/ELISA-	68 (65)
SCA+/ELISA-	3 (3)
SCA uninterpretable results/ ELISA + or -	2 (2)
	<hr/> 105 (100)
SCA ^a /LA+	25 (25)
SCA-/LA+	2 ^b (2)
SCA-/LA-	68 (66)
SCA+/LA-	4 ^c (4)
SCA uninterpretable/ LA + or -	2 (2)
SCA + or -/LA uninterpretable	1 (1)
	<hr/> 102(100)

a Specimens were absorbed with unsensitized staphylococci before testing using the SCA test

b 1 specimen was ELISA-negative

c 2 specimens were ELISA-positive

and 97% respectively of the slide latex agglutination test.

The effect of storage on the performance of the staphylococci reagents in the SCA test

Ten rotavirus-positive and 10 rotavirus-negative faecal specimens were examined by a batch of SCA reagents kept for 0, 2, 4, 8, 10, 15, 17, 19, 23 and 29 weeks after preparation. The results revealed that during the whole 29-week period all rotavirus-positive specimens retested positive and all rotavirus-negative specimens retested negative. The intensity of the agglutination at 2 minutes was graded 3+, 2+ and 1+ for strong, moderate and weak agglutinations, respectively. All specimens examined with newly prepared reagents were graded 3+ and this was maintained with all reagents stored over the 29-week period.

DISCUSSION

In this study a high proportion of untreated faecal samples examined with the staphylococcal co-agglutination test gave uninterpretable results (34%) due to agglutination of both test and control reagents. In addition, a high proportion of false-positives (26%) was observed. In an earlier study by Skaug et al.¹² it was reported that 52 per cent of the diarrhoeal faecal samples examined directly agglutinated the control reagent. However, it was not reported whether false positives also occurred. It is clear that the SCA test is not suitable for the examination of untreated faecal suspensions. Absorption of clarified faecal specimens proved effective in removing non-specific agglutination in this study and an earlier report.¹²

Latex is currently the solid phase immuno-adsorbent used in the slide agglutination test. Comparison of latex agglutination results to ELISA results which were read photometrically and which included a confirmatory test revealed that the sensitivity and specificity of latex agglutination was lower, with sensitivity figures of 80% and 84% and specificity figures of 95% and 100% those of ELISA.^{5,15} The sensitivity and specificity of the SCA test when compared with the in-house ELISA were 88% and 93%, respectively. It was therefore not unexpected that when the SCA test and the Rotalex latex agglutination test were compared directly the sensitivities and specificities of the 2 tests were essentially the same when latex agglutination results compatible with those of ELISA were considered. Thus the performance of the SCA test was of the same standard as latex agglutination - a test which may inspire more confidence and which may be considered technologically more advanced.

The SCA reagents were shown to have a long shelf-life: the reagents were still reactive after 7 months of storage although the coupling was not chemically mediated. The long shelf life reduces the necessity for frequent preparations as it allows a large batch to be prepared and used over a long period of time.

Thus, when the overall performance of the SCA was considered, the features that supported its use as a routine diagnostic laboratory method in rotavirus detection were the ease of preparation of reagents, long shelf-life of the reagents, the use of small amounts of test and control reagents for each test and, like all slide agglutination tests, it was quick and simple to perform. Furthermore, the level of sensitivity and specificity

were comparable to a commercial latex agglutination test (Rotalex) and, although not as high as ELISA, it would be acceptable in a situation where no alternative test is available. However, unlike the latex agglutination test, the need to treat the faecal specimen prior to testing introduces an additional step to the SCA test. Absorption treatment not only increased the time of the test but, more importantly, required more production of staphylococci as a high concentration of cells is needed for absorption. Thus it may be concluded that the SCA test can be recommended as a routine diagnostic laboratory method in rotavirus detection in situations where a more sensitive alternative is not available and where few samples are to be tested. However, this would exclude it from poor countries with a high incidence of diarrhoea where it was originally targeted for use.

It is interesting to note that non-diarrhoeal faeces did not give rise to non-specific agglutination. Although normal and diarrhoeal faeces are obviously different, the reason for the appearance of non-specific agglutination in diarrhoeal faeces and not in normal faeces is not known.

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