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Development of a slide latex agglutination test for rotavirus antigen detection

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Abstract

The aim of this study was to optimize the conditions for the passive adsorption of polyclonal antibody onto plain surface polystyrene latex particles and its performance in a slide latex agglutination test for rotavirus antigen detection. Cleaning of latex particles by washing through repetitive centrifuging, decanting and resuspending in distilled water was adequate in removing surfactants from the particles' surfaces to enable coating. A study of antibody concentration, incubation temperature and buffer pH revealed that optimum coating was achieved with a 3-fold excess of antibody to the calculated total particle surface capacity for the antibody in a glycine-saline buffer of pH 9.2 at 40°C for 4 hours. The ionic strength and pH of the latex suspending buffer and the sample buffer were critical factors determining the sensitivity of the test and the appearance of non-specific agglutination. Ultrasonication, addition of glycerol and Tween 20, either individually or in combination, were able to suppress non-specific agglutination in some batches of latex reagents. Polyethylene glycol 6000 enhanced the quality of agglutination as well as reduced the time of its appearance, especially in reagents that produced poor agglutination.

Key words: rotavims, slide latex agglutination test, assay development.

INTRODUCTION

Group A rotavirus is an important cause of acute diarrhoea in infants and young children in Malaysia' and other parts of the world.' Rapid and reliable laboratory diagnosis of human rotavirus infection is important as it could affect patient management.'

The slide latex agglutination method[4] is a suitable laboratory test for routine diagnosis of rotavims-associated diarrhoea as it is rapid, simple to perform and inexpensive. Furthermore, as the test is performed individually it is convenient when small numbers of samples are tested.

Rotavirus detection by commercial slide latex agglutination test is relatively cheap when compared to other equipment-based tests. However, the test kit is still expensive in developing countries which have the greatest need of these reagents because of the high incidence of diarrhoea1 cases. Provided anti-rotavirus antibody preparations are available, substantial cost reduction in the use of the slide latex agglutination test can be achieved by developing the test locally as commercial supply of microspheres and method of coating them with ligands are available.

A number of variables affects the coating of polystyrene particles by ligands. Certain additives also influence the performance of the coated particles in the slide agglutination test.[5,6] Therefore, it is important to determine the optimum conditions for the performance of the test so as to obtain the best sensitivity and specificity. This report presents the results of a study on the effect of a number of factors on the passive adsorption of polyclonal antibodies onto polystyrene latex particles, and the performance of these particle reagents in the slide agglutination test. This study was the first stage in the development of a rotavirus slide latex agglutination test that reacts rapidly and has good stability, specificity and sensitivity.

MATERIALS AND METHODS

Antibody preparations

The preparation of guinea-pig hyperimmune sera against pooled human group A rotavirus from local children has been described previously.' Goat anti-guinea-pig hyperimmune serum used in coating latex particles to produce control latex reagent was raised by the same procedure. The gamma globulin fractions of the hyperimmune sera were isolated by fractionation with saturated ammonium sulfate ($[NH_4]_2SO_4$). The fractions, referred to as 'immunogloulin fractions' were dialyzed against phosphate bufferred saline until all $[NH_4]_2SO_4$ had been removed. The contents

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of the bag was centrifuged at 1000 *g* to separate any precipitate that has been formed and the clear supernatant filtered through a 0.2 p m filter and stored in aliquots at –20°C. The protein concentration of the immunoglobulin fractions as determined by the Coomassie Brillant Blue G250 method[5] ranged from 11 to 14mg/ml.

Latex particles

Plain surface polystyrene latex beads with diameter of 0.825 p m [Sigma Chemical Company, USA] were used. The particles which consisted of 10% solids content in an aqueous suspension were cleaned by washing in 5 cycles of centrifuging [5500 g, 15 min], decanting and resuspending in distilled water by vortexing and a brief ultrasonication. The washed particles were resuspended in distilled water to the original volume and stored at 4°C for further use.

Passive adsorption of antibodies onto latex particles

A 25 μl volume [2.5 mg solids] of washed latex beads was added to 300 μl of glycine-saline buffer [0.02M glycine, 0.03M NaCl, pH 9.2]. The suspension was sonicated using a ultrasonic probe [model UP-150, Sonicor Instrument Corporation, Copiaque, N.Y.] by rapidly turning the relative output to a reading of 0.6. Immunoglobulin fraction was added to the latex suspension at an amount 3 times in excess of the calculated surface saturation capacity of the total latex surface area for IgG. The mixture was vortexed for about 1 minute, taking care to avoid any frothing, and then allowed to incubate with gentle stirring at 40 C for 4 h. Excess [unbound] protein was removed by washing twice with 2 ml of glycine-saline buffer and the coated beads resuspended in 0.3 ml of the same buffer. Sodium azide at a final concentration of 0.05% was added and the reagent stored at 4°C. Modifications to this procedure as to antibody concentrations, incubation temperature and coating buffer pH were carried out and were stated in the appropriate sections of the text. Test latex reagent was a suspension of particles coated with a guinea-pig anti-rotavirus immunoglobulin fraction while control latex reagent consisted of particles coated with a goat anti-guinea-pig immunoglobulin fraction.

Slide latex agglutination test

A 10μl volume of sample was placed on a microscope slide and mixed with an equal volume of latex reagent. The mixture spread to a circular area of about 1.5 cm diameter. The slide was then rocked gently and the appearance of agglutination observed with the unaided eye against a dark background. The level of agglutination achieved was scored by taking a value of 4+ to represent high level agglutination that caused the suspending fluid to become clear due to the extensive clumping of the latex particles. Other levels of agglutination were expressed as values relative to this level. Equivocal agglutination was denoted by '±'.

RESULTS

Effect of coating antibody concentration, incubation temperature and coating buffer pH on the agglutination performance of coated latex particles

Washed beads were coated with guinea-pig anti-human rotavirus immunoglobulin fractions at concentrations 3, 6 and 12 times in excess of the calculated total surface area of the latex particles. The efficiency of coating was determined indirectly by the performance of the coated beads in a slide agglutination test against a rotavirus-positive sample. The results in Table 1 show that the performance of the beads [time of first appearance of unequivocal agglutination and level of agglutination achieved by 3 minutes following mixture of latex particles and sample] was similar for the 3 antibody concentrations used.

The coating of latex particles with 3 times the excess of antibody was studied at 3°C, 24°C and 42°C. The agglutination performance of particles coated at the 3 temperatures was similar when reacted with a high concentration rotavirus-positive sample. However, when tested against samples with lower concentrations of rotavirus, improved performance was observed with particles coated at higher temperature.

Latex particles were coated with 3 times the excess of antibody at 42°C in glycine-saline buffer pH of 7.8 [~isoelectric point] and 9.2. The performance of the 2 batches of latex reagents was the same when reacted with a high concentration rotavirus-positive sample. However, particles coated at a pH of 9.2 performed better when reacted with samples containing lower concentration of rotavirus antigens.

TABLE **1:** Effect of coating antibody concentration, coating temperature and buffer pH on the agglutination performance of latex beads **adsorbed** with anti-rotavirus antibodies

Variable	Agglutination of coated latex particles			
	R+	R+a	R+a[1:1]	R–
Antibody [pg][a]/ coating capacity of latex particles	b c			
560/12x	30s/4+			0
280/6x	30s/4+			0
140/3x	30s/4+			0
coating temperature/ incubation period				
3°C/24h	60s/3+	120s/3+	3.5m/2+	0
24°C/16h	40s/4+	75s/4+	90s/3+	0
42°C/4h	50s/4+	40s/4+	50s/4+	0
pH				
7.8	25s/3+	120s/2½+	3m/1½+	0
9.2	25s/4+	35s/4+	55s/3+	0

R+ = rotavirus-positive faecal sample
R+a & R+a[1:1] = Undiluted and diluted Rotalex positive control [Orion Diagnostica, Espoo, Finland]
R– = rotavirus-negative faecal sample.

[a]Added to 2.4mg of latex particles for 4h at 40°C.

[b]Time agglutination first detected following mixing of latex reagent with sample.

[c]Maximum level of agglutination achieved by the end of 2 min.

4+ = strong agglutination; ± = equivocal agglutination; 0 = no agglutination.

Effect of ionic strength on the performace of antibody-coated latex particles

Clarified suspensions of a rotavirus-negative faecal sample and a rotavirus-positive faecal sample were prepared in distilled water containing 0, 0.06M, 0.15M, 0.27M, 0.57M NaCl. Samples from the 3 series were reacted against 3 different batches of anti-rotavirus antibody-coated latex particles [test latex reagents] prepared in buffer containing 0.03M NaCl under optimum conditions as determined previously. The results in Table 2 show that test latex reagent batch b2/w1 was stable in the clarified rotavirus-negative faecal suspensions and the corresponding NaCl solutions used in their preparations up to the maximum NaCl concentration tested [0.6M]. However, deterioration of specific agglutination occurred at 0.6M NaCl: agglutination took twice as long to appear compared with a lower NaCl concentration of 0.3M and the level of agglutination achieved was 2-fold lower. In the case of test latex reagent b2/w2A, NaCl concentrations of 0.09M and higher in the latex reagent-sample admixtures caused flocculation, or non-specific agglutination [NSA] to occur in the rotavirus-negative faecal samples although the latex reagent was stable in the corresponding NaCl solutions. In contrast, test latex reagent b2/w2B produced NSA in both the rotavirus-negative samples and the corresponding NaCl solutions when the NaCl concentration in the latex reagent-sample admixture was 0.09M and higher.

Effect of pH on the performace of antibody-coated latex particles

Table 3 shows that the test latex reagent was stable in a 0.017M NaCl solution [sample medium] and also in a clarified rotavirus negative faecal suspension prepared from it. However, a sample medium consisting of a 0.34M NaCl solution caused flocculation of the test latex reagent. Similarly, a rotavirus-negative faecal sample prepared in it also produced NSA. The pH values of the admixtures of latex reagent and the 2 sample media were both 8.7. When NaOH was used to increase the pH of the sample medium containing 0.34M NaCl-and hence indirectly the

TABLE 2: Effect of ionic strength on agglutination of antibody-coated latex reagents

NaCl concentration" in latex-sample admixture [M]	Agglutination of anti-rotavirus antibody coated latex particles of batch no.								
	b2/w1			b2/w2A			b2/w2B		
	N	R–	R+	N	R–	R+	N	R–	R+
	b								
0.03	0	0	30s/4+	0	0	20s/4+	0	0	20s/4+
0.09	0	0	30s/4+	0	3m/1+	15s/4+	90s/2+	90s/3+	12s/4+
0.18	0	0	30s/4+	0	2m/3+	20s/4+	90s/4+	40s/4+	15s/4+
0.30	0	0	30s/4+	0	95s/3+	30s/4+	40s/4+	ND	15s/4+
0.60	0	0	60s/2+	0	2m/3+	60s/4+	50s/4+	ND	15s/4+

N = distilled water containing 0, 0.06M, 0.15M, 0.27M, 0.57M of NaCl

R– & R+ = rotavirus-negative and –positive faecal specimens prepared in the corresponding series of distilled water with varying amount of NaCl.

"Final concentration of NaCl in the latex-sample admixture. [Latex reagents contained 0.03M NaCl]

[b]Results at the end of 3 min after mixing of reactants.

ND = not done

pH of the admixture-NSA was inhibited at a admixture pH of 9.5 without detrimental effect on agglutination of the test reagent by a rotavirus-positive sample. At an admixture pH of 10.5, agglutination was slower and at 11.7, no agglutination of latex particles by the rotavirus-positive sample was observed.

Effect of sonication and the addition of Tween 20 and glycerol on the agglutination performance of antibody-coated latex particles

The efficiency of different methods in preventing NSA was tested on 3 different batches of test latex reagents which caused NSA. Test latex reagent batch no. 1 had slow-developing, low-

TABLE 3: Effect of pH on agglutination of anti-rotavirus antibody-coated latex particles

Sample medium [NaCl in distilled water]		pH of admixture of latex reagent buffer[a] and sample medium with different NaOH concentrations added	Agglutination of coated latex particles in:		
NaOH [M] added	pH		sample medium	R– sample	R+ sample
sample medium: 0.017M NaCl solution					
0	7.5	8.7	0	0	15s/4+
sample medium: 0.34M NaCl solution					
0	7.5	8.7	45s/3+	30s/3+	15s/4+
0.003	10.7	9.2	120s/1+	75s/2+	ND
0.006	11.0	9.2	105s/1+	60s/2+	ND
0.013	11.5	9.5	0	0	10s/4+
0.05	12.0	10.5	0	0	25s/4+
0.10	12.4	11.7	0	0	0

Rotavirus negative faecal samples [R–] and rotavirus-positive faecal samples [R+] were prepared in 2 sample media: one consisting of a 0.017M NaCl solution and another consisting of a 0.34M NaCl solution containing 0 to varying concentrations of NaOH.

"The pH of the latex reagent buffer which contained 0.03M NaCl was 9.2.

TABLE 4: Effect of sonication and addition of glycerol and Tween 20 on the agglutination of antibody-coated latex particles

Treatment	Agglutination of different batches of coated latex particles							
	Test reagent 1		Test reagent 2		Test reagent 3		Control reagent	
	R–	R+	R–	R+	R–	R+	R–	R+
Nil	3m/1+[a]	50s/4+	90s/4+	30s/4+	90s/1+	35s/4+	25s/4+	25s/4+
Sonication[b]	0	60s/4+	90s/4+	30s/4+				
Glycerol	0	60s/4+[c]	60s/2+	30s/4+[d]				
Tween 20 [0.025%]	0	60s/4+	60s/3+	30s/4+				
Combination[e] of treatments	0	75s/4+	120s/± 140s/3+	60s/4+	0	50s/4+	0	0

R– = rotavirus-negative faecal sample; R+ = rotavirus-positive faecal sample.

[a]The reaction time of latex reagent and sample was 3 min.

[b]The ultrasonic probe was turned on rapidly until it reached 0.6 relative output.

[c]Final concentration of 2.5%.

[d]Final concentration of 5.0%.

[e]Sonication, 5.0% glycerol, 0.05% Tween 20

TABLE 5: Effect of polyethylene glycol 6000 on agglutination of antibody-coated latex beads

PEG 6000[a]	Agglutination of different batches of coated latex particles							
	b1/w5		b1/w7		b2/w1		b2/w1B	
	R–	R+	R–	R+	R–	R+	R–	R+
0	0[b]	140s/3+[c,d]	0	210s/2+	0	30s/4+	0	0
1%	0	90s/4+	0	105s/3+	0	25s/4+	0	0
2%	0	60s/4+	0	120s/4+	0	20s/4+	0	0
4%	30s/3+	15s/4+	90s/2+	60s/4+	0	10s/4+	0	0

Test latex reagent b1/w5, b1/w7, b2/w1 were coated with anti-rotavirus antibodies.

Control latex reagent b2/w1B was coated with goat anti-guinea pig antibody.

Figures in the 1st and 2nd columns under each batch of reagent were results of the reaction of latex particles with a rotavirus-negative [R–] and –positive sample [R+], respectively.

[a]Final concentration in admixture of latex and sample.

[b]Results by the the end of 4 minutes.

[c]Time of first appearance of agglutination.

[d]Level of agglutination by the end of 4 min.

level NSA; reagent batch no. 3 produced low-level NSA at a faster rate; reagent batch no. 2 had high levels of fast-developing NSA. Included was a control latex reagent in which high levels of NSA appeared quickly.

The results in Table 4 shows that a brief period of ultrasonication, addition of glycerol and addition of polyethylenesorbitan monolaurate [Tween 20] removed NSA in test latex reagent batch no. 1. In test latex reagent batch no. 2, these 3 methods individually were unable to eliminate NSA although a combination of all 3 reduced and delayed it. In contrast, combined treatment was able to prevent NSA in test latex reagents batch no.1 and batch no. 3 and the control latex reagent.

Effect of polyethylene glycol 6000 on the agglutination performace of antibody-coated latex particles

Four batches of latex reagents prepared under similar optimising conditions were reacted with a rotavirus-negative faecal sample and a rotavirus-positive faecal sample in different concentrations of polyethylene glycol [PEG] 6000. The results in Table 5 show that the addition of PEG to a final concentration of 1% and 2% in the admixture of the rotavirus-positive sample and test reagents b1/w5 and b1/w7 reduced the time agglutination first appeared and increased the agglutination levels by 1+ to 2+. Conversely, there was no agglutination with the rotavirus-negative sample. However, raising the final PEG concentration to 4% produced agglutination with the rotavirus-negative sample. The effect of PEG was not as pronounced with test latex reagent b2/w1 which caused relatively fast agglutination. The addition of up to 4% PEG did not cause NSA in that batch. The control latex reagent b2/w1B did not produce agglutination with either the rotavirus-negative sample or the rotavirus-positive sample in the presence of up to 4.0% PEG.

DISCUSSION

Attachment of antibody [and other ligands] to latex particles can be accomplished by passive adsorption or covalent coupling.[1] However, the simplicity of passive adsorption made it the first choice for the development of latex reagent in this study. Gamma globulin fractions of polyclonal hyperimmune sera were used as coating antibody as passive adsorption of polyclonal whole IgG molecules to the surface of polystyrene particles occurs readily and hence sufficient active antibody coating can be achieved to allow usage of the particle reagents produced for diagnostic purposes. Furthermore, greater crosslinking can be expected between bound antibodies on the particle's surface and the antigen if polyclonal antibody rather than pure monoclonal antibody is used because in a polyclonal antibody preparation a variety of specific antibodies are directed at different epitopes of the antigen.

Commercial latex particles are usually supplied in a medium containing detergents. In addition, salts and soluble polymers may be present as impurities. Removal of impurities and surfactants is necessary as they prevent the attachment of ligands. Particle clean-up methods include diafiltration,[1] dialysis[1] and centrifugation/washing.[11] Maximum adsorption which needs totally clean particles are not required in the slide agglutination test. Therefore, in this study the use of repeated washing in distilled water and dispersal by brief ultrasonication after centrifuging was found to be adequate for particle clean-up even though it was reported that this method might not eliminate all removable surfactant groups.[5]

Surface coverage of a suspension of latex particles of a particular diameter for a specific ligand depends on a number of variables including coating antibody concentration, pH, buffer type and concentration.[6]

The adsorption of antibody that allows optimum agglutination is not neccesarily associated with large amounts of antibody adsorbed onto the particles as at very high concentrations of antibody the antigen might bind with antibody molecules on the same particle rather than crosslinking particles thus leading to decreased sensitivity. Therefore, in visible agglutination tests, coating with the right amount of antibody is critical in order that the agglutinating antigen will bridge across particles rather than bind solely to antibody molecules on one particle. Conversely, if too few antibody molecules are adsorbed, agglutination is also less likely to occur. The general recommendation is to use a 3 to 10 times excess of coating protein to the calculated total binding surface.[1] In this study it was found that particles coated with 3 times excess antibody performed just as well as those coated with higher excess of antibody. Incubation at a higher temperature produced coated particles that performed better based on the observation of more rapid agglutination and higher level of agglutination when reacted with samples containing lower concentrations of

rotavirus antigen.

The pH of the coating buffer also affect the number of antibody molecules adsorbed as it determines the compactness of the molecule and therefore the number that can be accommodated on the particle. At the isoelectric point of IgG [pH 7.8], the molecule is most compact and hence maximum molecules can be adsorbed. However, coating for optimum reaction was at a pH away from the isoelectric point of the antibody. This was evident as particles coated at pH 9.2 performed better when reacted with samples that had lower concentrations of rotavirus. This observation reaffirmed the view that in the slide agglutination test optimum coating for maximum sensitivity of detection and rapid reaction does not necessarily equate with maximum number of bound antibodies.

The antibody-coated particles prepared in this study were stable only in buffer of very low ionic strength. Although the stability of these particles could be maintained in the slide agglutination test by using latex reagent buffer and sample buffer of low ionic strength, the amount of electrolytes present in some faeces, especially those from patients with diarrhoea, might increase the total ionic strength of the admixture to a level that caused NSA. However, the stability of the latex particles can be altered not only by decreasing the ionic strength of the system but also by increasing it's pH.[12,13] The latter situation can therefore be used to maintain the stability of the latex particles even though ionic strength is increased by electrolytes in faeces. In this study, raising the pH of the admixture to 9.5 preserved the stability of the antibody-coated particles in the presence of high ionic strength without affecting the performance of the test. An additional advantage of maintaining a high pH is that antibodies are fully charged and this causes the arms of the antibodies to be completely extended,[14] thus allowing more effective contact and interaction with antigens.

Sonication and the addition of glycerol and Tween 20, either individually or in combination, were able to remove NSA from some batches of antibody-coated particles. Ultrasonication most likely dispersed clumped particles which might flocculate more readily than monodisperse particles. Glycerol, because of its high specific density, most probably prevented flocculation by keeping the non-crosslinked particles apart. Exposed hydrophobic polystyrene areas not covered by ligand can cause NSA due to interaction with sample proteins. This can be prevented by the use of surfactants which will be adsorbed onto the exposed area.[15] Blocking these areas with Tween 20, a non-ionic surfactant, was successful in overcoming NSA in only some batches of reagents, especially those with low levels of NSA.

PEG enhances agglutination by lowering the solubility of the antigen-antibody complexes thus allowing more rapid clumping." In this study, the enhancing effect of PEG was observed to be greater in latex reagents that agglutinated poorly. In this situation, not only was agglutination more rapid but also enhanced. The highest concentration of PEG that could be added without causing NSA was dependent on the particular batch of latex reagent.

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