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Evaluation of Seven Immunoassays for Detection of Rotavirus in Pediatric Stool Samples

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The performance of seven commercially manufactured rotavirus assays was evaluated with 144 pediatric stool specimens and compared with electron microscopy (EM) findings. The four enzyme-linked immunosorbent assays used were Rotazyme II, Pathfinder, IDL rotavirus immunoassay, and Enzygnost (Behring) rotavirus assay. The three latex tests were Meritec rotavirus detection test, Virogen Rotatest, and Bartels rotavirus latex test. Test outcomes were compared with EM on the basis of sensitivity, specificity, positive-negative predictive value, and the kappa statistic. Relative to EM, Meritec had the highest specificity (97%), followed by Virogen (95%), IDL (91%), Pathfinder (85%), Behring (81%), Bartels (72%), and Rotazyme (71%). The sensitivities were as follows: Rotazyme (92%), Pathfinder (89%), Bartels (86%), Virogen (86%), Behring (82%), Meritec (71%), and IDL (75%). Patient age and sex did not influence test results. Owing to the absence of a true standard, the tests were also compared with each other on the basis of the kappa statistic, the frequency of positive test results, and the frequency of samples in which a test differed from all other tests. Using these measures, the assays could be classified into three groups with progressively decreasing utility: group 1 (Virogen, Meritec, IDL, and EM), group 2 (Pathfinder and Behring), and group 3 (Rotazyme and Bartels). Laboratory criteria were also compared. Latex tests were faster and required less equipment than enzyme-linked immunosorbent assays. The Virogen latex assay showed the best overall performance, which made it our choice for rapid and accurate rotavirus diagnosis. However, in children who have gastrointestinal symptoms with negative rotavirus test results, EM will be useful until such time as immunological tests for other enteric viruses are available.

Rotavirus is a major cause of gastroenteritis in children (9, 17) and is frequently reported as causing nosocomial outbreaks of diarrhea (17). Access to rapid and accurate diagnostic service for the detection of rotavirus at a pediatric hospital is important not only for diagnosis of gastroenteritis, but also to prevent nosocomial spread of the disease. Several factors such as specificity, sensitivity, rapidity, and simplicity have to be taken into account when choosing an appropriate test. The requirement of special equipment and technical skills also has to be considered.

Laboratories with a special interest in rotavirus infection may have methods such as immunoelectron microscopy (10, 25) and polyacrylamide gel electrophoresis (14, 27) available for rotavirus detection. Immunoelectron microscopy is reported to be more sensitive than electron microscopy (EM) (25). Polyacrylamide gel electrophoresis is as sensitive as EM and also provides epidemiological information (14, 27). However, EM has traditionally been used as a "gold standard" in evaluations of rotavirus detection assays (6, 8, 11, 13, 15, 19).

Classical EM is highly specific and rapid but is not suitable for testing large numbers of specimens. It requires an electron microscope and a skillful operator, which may make the method unsuitable for small laboratories. Various immunoassays such as latex agglutination tests and enzyme-linked immunosorbent assays (ELISAs) are commonly used as an alternative to EM in diagnosis of rotavirus infection (12, 28, 30, 31). ELISAs have the advantage of giving numerical results which can be objectively interpreted. They lend themselves to testing on a large scale but are usually not cost effective for testing small numbers of specimens. Latex tests, on the other hand, are rapid and do not require expensive laboratory equipment. However, until recently, the sensitivities of rotavirus latex tests have been unacceptably low, and it has been suggested that a latex test must be used within a week of onset of disease to detect the presence of rotavirus antigen (23, 24). Several ELISAs and latex tests for rotavirus detection are now commercially available, and this study was intended to identify which assays are suitable for a pediatric clinical virus laboratory. We evaluated the overall performance of four commercially available ELISAs and three latex tests for detection of rotavirus in stool specimens. The potential problems with adopting EM as a standard were addressed by various statistical methods.

MATERIALS AND METHODS

Clinical specimens. A total of 144 stool specimens were examined for the presence of rotavirus antigen by four ELISAs and three latex agglutination tests. A total of 113 consecutive stool specimens received at the virus laboratory at British Columbia's Children's Hospital were tested. In addition, 31 specimens (kindly supplied by P. Middleton, The Hospital for Sick Children, Toronto, Ontario, Canada) were included in our study. EM was performed on all specimens. The age of the patients varied from 2 weeks to 18 years. Forty patients were <6 months; 29 were 6 months to 1 year; 44 were 1 to 2 years; 25 were 2 to 10 years; and 6 were >10 years. All specimens were coded, and technologists conducted tests without knowledge of the EM results. Since we wanted to obtain conclusions that would be directly applicable in the routine laboratory setting, the instructions of the manufacturer for use of the test were followed, consecutively incoming specimens were tested, and there were no other special selection criteria.

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TABLE 1. Technical data and reagents used for latex tests and ELISAs

Data and reagents	Meritec	Virogen	Bartels	Rotazyme	Pathfinder	IDL	Behring
Monoclonal antibody	No	No	No				
Monoclonal 1st antibody				No	No	No	No
Monoclonal 2nd antibody				No	Yes	No	No
Negative control antibody	Yes	Yes	Yes	No	No	No	No
Blocking antibody	No	No	No	No	Yes	No	No
Antigen source	SA-11	Bovine	SA-11	SA-11	SA-11	SA-11	Bovine
Negative control antigen	No	No	Yes	No	No	No	No
Positive control antigen	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Negative control latex/wells (nonimmune rabbit serum)	Yes	Yes	Yes	No	No	Yes	No
Equipment required	None	None	None	Yes	Yes	Yes	Yes
Testing time/specimen (min)	20	20	20	170	95	200	205
Wells				Yes	Yes	No	No
Test tube				Yes	Yes	No	No
Beads				Yes	No	No	No
Antibody-peroxidase				Yes	Yes	No	No
Antibody-alkaline phosphatase				No	No	No	Yes
Antibody-biotin				No	No	Yes	No
Avidin-peroxidase				No	No	Yes	No

The specimens were stored at −70°C until tested, and samples for the tests were collected randomly from each stool specimen. The number of specimens tested with each test were as follows: Pathfinder, 144; Rotazyme, 144; Behring ELISA, 131; IDL, 89; Virogen, 84; Bartels latex test, 101; Meritec latex test, 96; and EM, 144.

Latex assays. Technical details and reagents for latex assays are outlined in Table 1. One latex test (Meritec rotavirus latex detection procedure; Meridian Diagnostics, Inc., Cincinnati, Ohio) is distributed in both the United States and Canada. One latex test (Virogen Rotatest; Wampole Laboratories, Div. Carter Wallace, Inc., Cranbury, N.J.) is distributed only in the United States, but was provided to our laboratory for evaluation. The third latex test was only available for evaluation (Bartels Immunodiagnostic Supplies, Inc., Bellevue, Wash.).

ELISAs. Technical details and reagents for ELISAs are outlined in Table 1. The ELISAs used were all commercially available (Rotazyme II, Abbott Laboratories, North Chicago, Ill.; Pathfinder, Kallestad Laboratories, Inc., Austin, Tex.; IDL rotavirus immunoassay, International Laboratories, Inc., Chesterfield, Mo.; Enzygnost rotavirus assay, Behring Institute, Behringwerke, Marburg, Federal Republic of Germany). All ELISA results were determined by spectrophotometry.

EM. EM specimens were prepared by the agar diffusion method (1, 10). Briefly, a Formvar-coated 300-mesh copper grid (I.B. EM Services Inc.) was placed in a well of a microtiter plate containing 1% Noble agar (Difco Laboratories, Detroit, Mich.) A 10% (wt/vol) aqueous stool suspension was added and incubated for 30 min at room temperature. The specimen was negatively stained with 2% phosphotungstic acid. Each grid was examined in a Phillips 400 T microscope for 15 min for the presence of virus particles.

Criteria for positivity. The guidelines of the manufacturers for positivity were followed in all assays.

Statistical methodology. Diagnostic test results were analyzed in two ways: with EM as a standard; and on a comparative basis with each other. In the first case, results of diagnostic tests were compared with those of EM by using sensitivity, specificity, positive predictive value, negative predictive value, and the kappa statistic. In the absence of a standard, the kappa statistic, the percent positive test results and the proportion of samples in which the test result differed from all others were used for comparison. These comparative measures are defined below. Figure 1 presents some basic quantities used in calculating these measures.

Sensitivity is the proportion of samples in which the test is positive when EM is positive. With EM as test 2 in Fig. 1, sensitivity = $a/(a + c)$.

The specificity shows the proportion of samples in which the test is negative when EM is negative. With EM as test 2 in Fig. 1, specificity = $d/(b + d)$.

The positive predictive value is the proportion of samples in which EM is positive when the test is positive. With EM as test 2 in Fig. 1, positive predictive value = $a/(a + b)$.

The negative predictive value is the proportion of samples in which EM is negative when the test is negative. With EM as test 2 in Fig. 1, negative predictive value = $d/(c + d)$.

The kappa statistic (8) is an overall measure of agreement between two tests and is useful for measuring agreement in the absence of a standard. It compares the observed proportion of samples in which the tests agree with the proportion that would be expected to agree by chance. It is normalized to have values between 1 and $-p/(1 - p)$, where p is the proportion of samples in which agreement would occur by chance. Using the notation in Fig. 1, the kappa statistic is defined by: kappa = $(a + d - p)/(1 - p)$, where $p = (a + b)(a + c) + (c + d)(b + d)$. Landis and Koch (21) provide the following benchmarks for interpreting kappa: kappa less than 0.0 corresponds to "poor" agreement; kappa between 0.0 and 0.20 is "slight" agreement; kappa between 0.21 and 0.40 is "fair" agreement; kappa between 0.41 and 0.60 is "moderate" agreement; kappa between 0.61 and 0.80 is

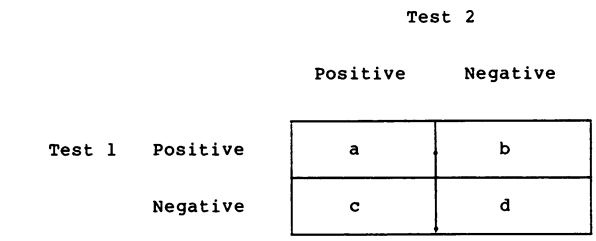

FIG. 1. A two-way table that serves as the basis for defining agreement measures.

TABLE 2. Evaluation of the performance of rotavirus kits relative to EM

Test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Kappa
Pathfinder	89	85	75	93	0.56
Rotazyme	92	71	62	93	0.55
Behring	82	81	68	92	0.59
IDL	75	91	81	85	0.68
Virogen	86	95	88	93	0.80
Meritec	71	97	92	87	0.71
Bartels	86	72	54	90	0.50

"substantial" agreement; and kappa between 0.81 and 1.00 is "almost perfect" agreement. Hypothesis tests of whether kappa is significantly greater than 0 are based on standard errors derived from approximations to its variance (2).

The proportion of positive test results is used to obtain an overall assessment of test performance. Statistical inference is based on the methods of Landis and Koch (21).

Age and sex effects on comparisons. Samples were classified into two groups on the basis of age: those from subjects less than 1 year old, and those from subjects exceeding 1 year of age. The effect of age and sex on outcome were examined by the Breslow-Day test for homogeneity of cross-product ratio across strata (4). A test based on the cross-product ratio instead of the kappa statistic was used because of its availability in existing statistical programs (SAS PROC FREQ).

Calculations were done using PROC CATMOD and PROC/FREQ in the SAS statistical program (29).

RESULTS

Table 2 summarizes results of analysis which used EM as a standard. Based on the kappa statistic, Virogen, IDL, and Meritec were in substantial agreement with EM, while the remaining tests were less so. Of these, Virogen had the highest sensitivity and was only slightly less specific than Meritec. It also had the greatest negative predictive value.

The frequency of positive test results was significantly

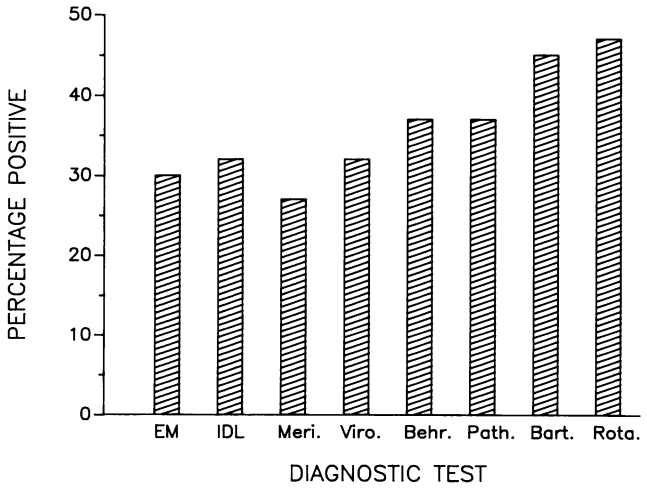

FIG. 2. Percentage of samples in which test results were positive. The numbers of samples in which each test was performed appear in the text.

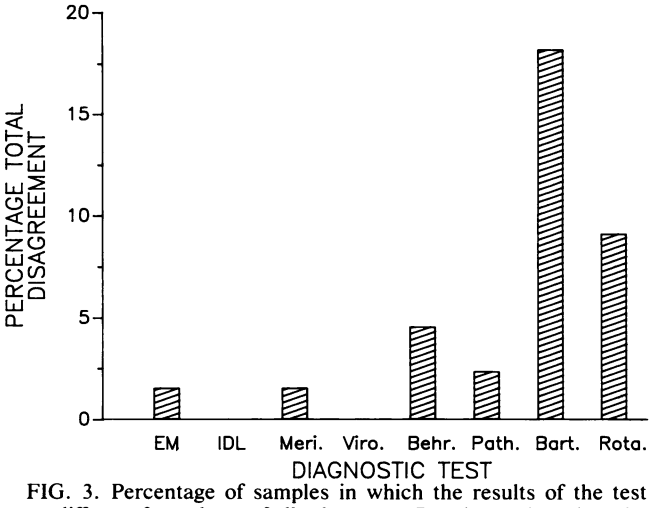

FIG. 3. Percentage of samples in which the results of the test were different from those of all other tests. Results are based on the 66 samples to which all tests were applied.

different between tests ($P < 0.001$). Figure 2 suggests that the tests can be divided into three groups on this basis as follows: group 1, Virogen (27% positive), Meritec (27%), IDL (31%), and EM (30%); group 2, Pathfinder (37%) and Behring (37%); and group 3, Bartels (45%) and Rotazyme (47%). Figure 3, which is based on the 66 samples in which all tests were performed, shows that on the basis of agreement with all other tests, the Bartels and Rotazyme assays were distinctly different from the remaining tests, with Bartels differing from all other tests in 17% of the complete samples and Rotazyme differing from all other tests in 9% of the complete samples.

The kappa statistic for each pair of tests is given in Table 3. It shows almost perfect agreement between the group 1 tests (Meritec, IDL, and Virogen), substantial agreement between these tests and the group 2 tests (Pathfinder and Behring), and moderate agreement between these tests and the group 3 tests (Bartels and Rotazyme). The group 2 tests were in moderate agreement with each other and in fair to moderate agreement with the group 3 tests. The group 3 tests were only in fair agreement with each other. Overall, the Bartels latex assay appeared to be most different from all remaining tests.

Age and sex did not have a significant effect on the comparative performance of diagnostic tests ($P < 0.12$ in all test pairs for each factor). The practical features of each test were also evaluated. The three latex tests require 20 min for

TABLE 3. Kappa statistic for intertest agreement between seven diagnostic kits for rotavirus detection[a]

Test	Kappa statistic						
	Bartels	Behring	IDL	Meritec	Pathfinder	Rotazyme	Virogen
Bartels	1.0	0.37	0.54	0.42	0.42	0.40	0.46
Behring		1.0	0.76	0.70	0.61	0.47	0.71
IDL			1.0	0.81	0.78	0.60	0.93
Meritec				1.0	0.75	0.56	0.83
Pathfinder					1.0	0.51	0.75
Rotazyme						1.0	0.61
Virogen							1.0

[a] Based on a number of samples in which both assays were used (n exceeded 66 in all cases).

analysis per specimen including controls. The ELISAs required around 3 h per specimen with the exception of Pathfinder, which only required 1.5 h (Table 1). The latex tests were simpler to perform and are suitable for processing urgent specimens. The latex tests required no specific equipment while the ELISAs did (Table 1). All the manufacturers of the ELISAs suggest that visual reading of results is possible, but the use of a spectrophotometer provides objective data. The Rotazyme test, with its tubes and antibody-coated beads, requires a special washer and ELISA reader. The Behring and IDL tests use 96-well plates, which can be washed manually or by any plate ELISA washer. The absorbance values may be registered by any ELISA reader for 96-well plates. Pathfinder recommends manual washing, and the test is performed in tubes. No special reader is available, which makes necessary either a reader suited for test tubes or transfer of the end product to a 96-well plate if using an ELISA plate reader.

For 40 of 144 specimens, absorbance values in the Rotazyme test were just above the equivocal reading (absorbance $\approx$ 0.200) defined in the package insert. The test is time consuming, and special equipment is required.

Behring Enzygnost and IDL are classical ELISAs performed in 96-well plates. There were no particular practical problems with these tests, but 3 h were required for analysis (Table 1).

The Pathfinder test was technically more attractive to perform than the other three ELISAs. This was partly due to the fact that the specimens and second antibody are added at the same time, which shortens the testing time. However, there were problems connected with this test. In a pilot study, false-positive readings (absorbance values of $\geq$0.600) occurred in 16 of 30 negative control tubes. A blocking antibody was supplied to check positive results. The test protocol recommended a dilution of blocking antibody which was insufficient to block all antigen when the antigen load in the specimen was heavy.

EM detected rotavirus in 44 of 144 specimens, enterovirus in a further 6 specimens, and adenovirus in 1 specimen.

DISCUSSION

The highest sensitivities of ELISAs were observed with Pathfinder (89%) and Rotazyme (92%), which had relatively low specificities of 85 and 71%, respectively. Two latex tests, Virogen and Meritec, showed the highest specificity (95 and 97%, respectively), but had lower sensitivity than either Rotazyme or Pathfinder (Table 2). The low specificity in the ELISAs must be interpreted with caution, since the specificity is calculated on the basis of EM as the standard. It is possible that an ELISA may have a higher sensitivity than EM and that the calculated specificities may be too low.

A discrepancy between EM and other tests performed was apparent in 10 of 144 specimens. Four of those were negative by EM but positive by the majority of the other tests. One explanation for this could be nonspecific binding of rotavirus antibody to bacterial or staphylococcal protein A (3, 18). This explanation would most likely not be valid if the second antibody was monoclonal as in the Pathfinder test. Another explanation could be that nonspecific factors in stools interfere with the enzyme reaction in the ELISA and give false-positive results. This would not explain positive reactions with the latex tests. If only disrupted virus particles were present in the specimen, they would be detected by the immunological tests, but would be difficult to find by EM. The other possibility is that the immunological tests are more sensitive than EM.

If, on the other hand, EM is positive and the majority of other tests are negative (6 of 144 specimens in our study), this could be explained by inhibitors in fecal specimens interfering with the first binding in the immunological step or by common antigenic determinants on observed virus particles being blocked by nonspecific fecal antibodies. An alternative explanation is that these viruses were non-group A rotaviruses or of different serotypes (16, 26).

On the basis of statistical measures, we concluded that the assays fell into three groups: group 1, Virogen, Meritec, IDL, and EM; group 2, Pathfinder and Behring; group 3, Bartels and Rotazyme. The Bartels test was consistently different from all the remaining tests. Two latex assays, Meritec and Virogen, are included in the group 1 tests. Our results indicate that the sensitivity of Virogen is substantially higher than that of the Meritec test (Table 2). This is surprising since it is reported that the Virogen latex test does not recognize rotavirus serotype 2, in contrast to Meritec, which recognizes all four serotypes (J. J. Mathewson, H. L. Du Pont, and S. L. Secor, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 100, 1986). It appears as though Virogen is an appropriate test in our geographical area, unless the prevalence of serotypes change.

The statistically derived grouping agrees with practical aspects of the tests. All tests in group 1 (except EM) include a negative control consisting of nonimmune rabbit serum (Table 1). The two ELISAs in group 2 had no negative serum control and recommended the use of diluent as a negative control (or a known negative stool specimen from the user's laboratory). The group 2 tests, Pathfinder and Behring, gave more positive results than group 1 (Fig. 3), but in the absence of a negative serum control, we cannot exclude the possibility that some of the positive results were false. The Pathfinder test gave positive results in 16 of 30 negative controls. We do not have an explanation for this observation, although considerable effort was made to explain this anomaly, including investigations into the efficiency of washing and control of water purity.

If false-positive results occur in ELISAs, a blocking antibody may be used to confirm positivity. Such an antibody was provided by Kallestad for their Pathfinder test. The procedure is suitable if there is enough antibody to block all antigen present, but it is perhaps not practical in a routine clinical setting since it delays provision of an answer to the clinician.

The group 3 tests, Rotazyme and Bartels latex test, were different from all other tests in the study. It has been reported that stools from neonates may give false-positive reactions with the Rotazyme test (7, 20). Our observations confirm the tendency of false-positive reactions with Rotazyme, but there was no indication with our material that this finding was correlated to either age or sex. The other test in group 3, the Bartels latex test, had a low specificity (72%) relative to EM (Table 2) and gave different results from those of all other tests in 17% of instances (Fig. 3).

The ideal rotavirus test for a virus laboratory at a pediatric hospital should be accurate, rapid, and simple to perform and should not require expensive equipment. Our opinion is that ELISAs require more equipment and time than the latex tests. It would not be cost effective to test a few or single specimens with an ELISA which is suited to batch testing and would be associated with delays in reporting results. In addition, the ELISAs in our study had lower specificity than two of the latex tests (Virogen and Meritec), which introduces uncertainty into interpretation of positive results with those assays. If the frequency of suspected false-positive results is

high, the interpretational problems with all positive specimens become significant. This is especially relevant when testing pediatric specimens, since some children may display asymptomatic shedding of rotavirus (5). A test with low sensitivity is not desirable, but if a choice between low specificity or low sensitivity has to be made, the low sensitivity may be more easily explained to the clinician.

If the rotavirus test is repeatedly negative but signs and symptoms still suggest an enteric viral infection, EM should be performed. In our study, EM detected viral pathogens other than rotavirus in seven specimens. Immunoelectron microscopy was not performed and may possibly have detected additional viral enteric pathogens (22).

In conclusion, a latex test with high specificity and acceptable sensitivity such as the Virogen test would be preferable to time-consuming ELISAs with a lower specificity. A latex test with both a high specificity and improved sensitivity remains ideal. If an ELISA is preferred, the IDL ELISA is the recommended test. At present, access to EM is invaluable in the examination of stool specimens from pediatric patients with gastrointestinal symptoms and negative immunological rotavirus test results.

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