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EVALUATION OF TECHNIQUES FOR THE DIAGNOSIS OF ROTAVIRUS GASTROENTERITIS IN PAEDIATRIC PATIENTS

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ABSTRACT

Rotavirus is the major etiology of acute gastroenteritis in infants and young children worldwide. It has been estimated that about 39% of childhood diarrhoea hospitalizations are caused by the virus and nearly half a million children die from the infections each year especially in developing countries on the Asian subcontinent, Africa, and Latin America where health care facilities are in short supply. Stool specimens collected from the first to fourth days of illness are optimal for detection of the infection. Diagnosis was originally performed using electron microscopy, which is still occasionally used in centers where it is available. Routine diagnosis is however, now performed by antigen detection on feces using commercially available, simple, rapid immunochromatographic dipstick style kits which have superseded the earlier latex agglutination and enzyme immunoassay methods whose usefulness in diagnosis is still relevant. Reverse transcription polymerase chain reaction of feces is also available in some reference and research centers, and is particularly useful for identification of outbreaks due to serogroups other than group A. Similarly viral culture and serology are also available but do not play much roles in diagnosis of acute disease. Rotavirus infection is indeed a public health problem and early, rapid, accurate and reliable diagnostic techniques are essential for effective patient management and infection control.

Keywords: Laboratory diagnosis, ELISA, Polyacrylamide gel electrophoresis, Polymerase chain reaction, Electron microscopy, Culture methods.

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1. INTRODUCTION

Acute gastroenteritis due to enteric pathogens including bacteria, virus and parasite is responsible for most childhood morbidity and mortality in developing countries. Recently studies have, however, established that enteric viruses are more important as the major etiology of acute diarrhea than enteric bacteria. Of these viruses, rotavirus and norovirus have been recognized as the most common etiological agents of pediatric acute gastroenteritis [1]. Each year rotavirus causes an estimated 111 million episodes of diarrhea requiring only home care, 2 million hospitalizations and 400,000 deaths in children under 5 years especially in developing countries on the Asian subcontinent, Africa, and Latin America which accounts for 82% of the total disease burden [2]. Young children aged 4-24 months, particularly those in group daycare settings, low birth weight, male gender, poor food hygiene, playing with toys, prematurity and bottle-feeding have been associated with increased risk of admission to hospitals as a result of the disease [3]. Nigeria has recently been ranked third among the 10 countries with the greatest number of rotavirus disease-associated deaths per year with an estimated 33,000 deaths in children <5 years old [4].

Rotavirus (RV) is a non-enveloped virus with icosahedral symmetry belonging to the family *Reoviridae*. The virion consists of three layers of protein with the viral genome consisting of 11 segments of double-stranded RNA (ds RNA), which encode six structural proteins, namely, VP1-4, VP6 and VP7 and six non-structural proteins, NSP1-6 [5, 6]. The outer capsid is composed of two independent neutralization antigens namely VP4 which determines P-genotype and VP7 which is denoted as G-serotype. At least 16 G-serotypes and no less than 27 P-genotypes have been reported and 42 P/G genotype combinations that can infect humans have been identified [7]. Four combinations (P[8]G1, P[4]G2, P[8]G3,and P[8]G4) account for nearly 90% of strains worldwide, with P[8]G1 accounting for 60 to 80% of strains in most years [8].

The virus is classified into seven serogroups, A to G, based on the VP6 protein which contains the antigenic determinants and is located on the viral inner capsid. Group A which is endemic worldwide is the most common agent of childhood diarrhea, accounting for more than 90% of rotavirus gastroenteritis in humans [9]. Group B, also called adult diarrhea rotavirus (or ADRV), has caused major epidemics of severe diarrhea affecting thousands of people of all ages in China [10], while group C has been associated with rare and sporadic cases of diarrhea in children in many countries, with outbreaks reported in Japan and England [11].

In addition, electrophoresis of the rotavirus RNA genome allows detection and further classification of the virus into two majors groups, the Long (L) and the Short (S) electrophoretypes based on the migration patterns of gene segments 10 and 11[12]. RV infection can range from mild diarrhea, with limited duration, to a severe case, with fever, vomiting and dehydration [13]. Rotavirus is transmitted from one person to another through the fecal-oral route [14, 15] with boys reported to be twice susceptible and likely to be admitted to hospitals

than girls [16]. Adult infections have also been reported in military populations, hospital workers, travellers and most commonly in parents of infected infants [17].

Access to rapid and accurate diagnostic services for the detection of the virus at a pediatric hospital is important not only for diagnosis of viral gastroenteritis, but also to prevent nosocomial spread of the disease [18]. Since the discovery of rotaviruses by Electron Microscopy (EM), a number of rotavirus detection methods have been developed. Due to the difficulty associated with the use of EM however, other viral and antigen detection methods based primarily on antigen-antibody reactions have been developed. Stool specimens collected from the first to fourth days of illness are optimal for rotavirus detection but virus may be shed for up to 3 weeks depending on the severity of illness. Viral shedding usually coincides with the duration of diarrhea but stooling can continue for an additional few days [19]. The specimens that could be used in diagnosis include stool, serum, cerebrospinal fluid, and throat swab [20].

In surveillance data, basic information is provided on which type of diagnostic test is used in each reported laboratory-confirmed rotavirus infection. Before 1990 most laboratories did not report the method they used for rotavirus detection. However between 1990 and 1997 it was revealed that electron microscopy (EM) was the most frequent diagnostic test used. In 1998, there was a dramatic shift to enzyme-linked immunosorbent assay (ELISA) and rapid immunochromatographic tests (ICTs), which subsequently predominated [21]. Diagnosis is currently based on the identification of the virus in feces or suspension of rectal swab collected early in the illness through direct microscopy, molecular techniques, rapid serological tests, and use of tissue culture technique [18]. Routine diagnosis is however, now performed by antigen detection on feces using commercially available, simple, rapid immunochromatographic dipstick style kits [22]. Rotavirus detection is greatest when diarrhea, vomiting and fever occur together and lowest when each symptom occurred alone [20].

Rates of rotavirus illness in children in developed and developing countries are similar, indicating that good hygiene and clean water supplies are unlikely to have a significant impact on disease prevention [21]. Thus safe and effective vaccination has been suggested as the primary public health strategy to prevent rotavirus infection and reduce the burden of disease [22].

The specific methods used in rotavirus diagnosis include: cultivation technique, electron Microscopy (EM), Enzyme Linked Immunosorbent Assay (ELISA), Latex Agglutination techniques, Immunochromatographic Tests (ICTs), Immunoperoxidase Assay (IPA), Reverse transcriptase polymerase chain reaction (RT-PCR), Polyacrilamide Gel Electrophoresis (PAGE) technique, and serological tests

2. EVALUATION OF ROTAVIRUS DIAGNOSTIC TECHNIQUES

In a recent study, Momenzadeh, et al. [23] compared ELISA, ICT, and RNA-PAGE methods for detection of rotavirus infection in 200 fecal samples from hospitalized children with acute gastroenteritis and reported that rotavirus was detected in the stool specimens by ELISA

(28.5%), ICT (26%), and RNA-PAGE (26%). Furthermore, comparing these methods with age variables yielded P=0.72, P=0.87, and P=0.75 respectively.

Based on their result, the researchers concluded that the sensitivity and specificity rates and positive and negative predictive values of RNA-PAGE are more than ICT, and that of ELISA was more than both, and recommended that the techniques may be suitable for diagnosis of other enteric viral infections.

In a study on pediatric rotaviral diarrhea to compared PAGE and ELISA, Venkatesh, et al. [24] reported that an excellent correlation of ELISA and PAGE results was found in 194 of 200 (97%) specimens with a total of 51 (25.5%) of them found to be positive for rotavirus by either methods and the proportion of ELISA +ve PAGE –ve samples 1/200 lower than the proportion of ELISA-ve PAGE +ve samples (5/200). The report concluded that the modified PAGE technique for the detection of viral RNA was rapid, simple, reliable and far less expensive technique.

In a similar study, three enzyme-linked immunosorbent assays (ELISAs), including Pathfinder, Rotaclone and synthetic oligonucleotide DNA probe (SNAP) were compared with silver-stained polyacrylamide gel electrophoresis (PAGE) of viral RNA for the detection of rotavirus in fecal specimens. Of 286 specimens analyzed by PAGE, SNAP, rotavirus ELISA, Pathfinder, and Rotaclone, 88 were positive by PAGE as well as by the other four assays. Nine specimens that were positive by one or more of the assays were also positive by blocking ELISAs but negative by PAGE. If these nine specimens were considered to be true positives, the final sensitivities and specificities were as follows: PAGE, 91 and 100%; SNAP, 94 and 97%; rotavirus ELISA, 96 and 97%; Pathfinder, 100 and 94%; and Rotaclone, 96 and 97%, respectively [25].

There are several reports comparing Rotazyme and Enzygnost with EM and IEM. Yolken and Leister [26] evaluated Rotazyme I, Enzygnost, and indirect ELISA and compared them with EM. They found the sensitivity of indirect ELISA, Rotazyme I, and Enzygnost to be 100, 93, and 88% and the specificity to be 95, 95, and 89%, respectively. Similarly, a study was conducted to evaluate Rotazyme I and EM. The expected sensitivity was 88.7%, and the negative results had a 91.95% agreement. The tests were read visually, and specimens with high Rotazyme readings correlated 100% with EM [27]. In addition, Rubenstein and Miller [28] compared Rotazyme I with EM and IEM. The levels of sensitivity were 106 particles per ml for simian rotavirus SA11 and 107 particles per ml for human rotavirus. The sensitivity and specificity of Rotazyme I compared with those of IEM were 98 and 92%, respectively. Rotazyme-positive specimens included those specimens that were EM negative but ELISA positive that could be blocked in a blocking assay.

In their study, Keswick, et al. [29] also compared Rotazyme I and EM and found ELISA to be more sensitive than EM and that the Rotazyme test detected SA11 with a titer of 2 x 103 plaque-forming units (PFU)/ml, which was a level of sensitivity greater than that reported by Rubenstein and Miller [28]. They also carried out blocking assays on EM-negative and ELISA-

positive specimens and found the ELISA-positive specimens to be true positives. In a similar study, Chernesky, et al. [30] evaluated Rotazyme II, a version of Rotazyme I with a shorter incubation time and reported that it was 99.4% sensitive and 97.3% specific with an overall agreement of 98.7% when compared with EM on 229 samples from patients aged 6 months to 6 years.

The performance of seven commercially manufactured rotavirus assays was evaluated with 144 pediatric stool specimens and the results were compared with EM findings. In the study four ELISA including Rotazyme II, Pathfinder, IDL rotavirus immunoassay, and Enzygnost (Behring) rotavirus assay and three LA tests including Meritec rotavirus detection test, Virogen Rotatest, and Bartels rotavirus latex test were used. The results of the findings were compared with EM on the basis of sensitivity, specificity, and positive-negative predictive value. Relative to EM, it was reported that 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 [31].

The tests were also compared with each other on the basis the frequency of positive test results, and the frequency of samples in which a test differed from all other tests. Using these measures, they classified the assays 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). The results revealed that LA tests were faster and required less equipment than ELISA, while the Virogen LA assay showed the best overall performance, and was recommended for rapid and accurate rotavirus diagnosis. However in children who have gastrointestinal symptoms with negative rotavirus test results, EM will be useful until such a time as immunological tests for other enteric viruses are available [31].

Similarly Gerna, et al. [32] used 151 specimens and compared the Pathfinder monoclonal antibody ELISA with SPIEM as a reference test. They found Pathfinder to have a sensitivity of 98.7% and a specificity of 98.5%.

In one study with 44 fecal samples, Raboni, et al. [33] reported that all LA-positive samples were also positive by ELISA, and 2 LA-negative samples were positive by ELISA, and that of specimens indeterminate by LA, 67% were positive by ELISA. However LA was 69% sensitive, 100% specific, and 93% accurate. Finally, the researchers concluded that results of LA assay could be as sensitive and specific as the ELISA, and that it could be applied on a large scale for screening stool specimens in suspected rotavirus diarrhea, recommending however that all indeterminate results could also be confirmed by other methods such as ELISA.

In a similar study, Abood [34] examined the performance of ELISA versus LA test in the diagnosis of acute gastroenteritis by rotavirus in children aged between 1-32 months and reported that the highest sensitivity of (92.5%) was obtained with LA followed by ELISA (84.09%), while the highest specificity of (93.6%) was obtained with ELISA followed by LA

(86.3%). The highest predictive positivity value was also obtained with ELISA (92.5%) followed by LA (84.09%). The report concluded that LA is easy to perform and gave high sensitivity with acceptable specificity, thus could be applied successfully for routine diagnosis and epidemiological studies, while ELISA techniques could allow quantitative estimation of rotavirus antigens.

In one study, 100 specimens was used to evaluate four tests which were compared them with EM. The four tests were (i) the Abbott Rotazyme II, a polyclonal antibody-based ELISA; (ii) the Pathfinder ELISA, a monoclonal antibody-based ELISA; (iii) a polyclonal-based ELISA, using reagents obtained from the National Institutes of Health; and (iv) Rotalex LA. The results revealed that the sensitivity of the monoclonal antibody ELISA (95%) to be superior to those of the polyclonal antibody ELISA (73% for Rotazyme II and 57% for the NIH reagent ELISA) and the LA assay (61%). The specificity of the LA assay (98%) was reported to be slightly better than those of the other systems (88 to 96%). The positive and negative predictive values of the monoclonal antibody ELISA (93 and 96%, respectively) were better than those of Rotazyme II (82 and 80%, respectively), the LA assay (96 and 76%, respectively), and the NIH reagent ELISA (93 and 74%, respectively). The visual readings of the monoclonal antibody ELISA correlated better with the spectrophotometric optical density readings than did the visual readings of the polyclonal antibody ELISA; however, the agreement of both with EM results was poor when 1+ or plus-minus readings were observed. They therefore concluded that monoclonal antibody ELISA is more sensitive and predictive than other rotavirus detection systems and second only to the LA assay in specificity in detecting rotavirus in stool specimens [35].

In a similar study, Doern, et al. [36] compared 176 specimens in Rotazyme I, Rotazyme II, and Rotalex LA with a highly sensitive and specific monoclonal antibody ELISA. They found the sensitivities of the Rotazyme I and II and LA to be 97.4%, 100%, and 81.6% respectively, and the specificities to be 88.8%, 83.9%, and 100% respectively. Thus, the Rotazyme II was reported to be more sensitive but less specific than Rotazyme I or LA. The report continued that overall, Rotazyme I and II were highly sensitive, but both lacked specificity. They also noted a problem with a large number of specimens having equivocal results with Rotazyme I and II.

In another study Rotazyme I ELISA and the Rotalex LA test (Finland) were compared with EM. The sensitivity was revealed to be 96% for both while the specificity to be 89% for the Rotazyme ELISA and 86% for the Rotalex LA test [37]. In a similar study, 204 stool samples were for rotavirus using four techniques including two ELISA, Enzygnost and Rotazyme I; and two LA tests, the Slidex Rota-Kit (Biomerieux) and Rotalex (Medical Technology Corp.). The positive rates were 47, 38, 37, and 34%, respectively. However, 12 specimens positive by the Enzygnost test only and 3 specimens positive by the Rotalex test only could not be confirmed positive by EM [38]. In the same way Slidex Rota-Kit were found to be 82% sensitive and 100% specific [39].

In his evaluation studies, Miotti, et al. [40] compared 122 samples by three commercial tests and their own reference microplate ELISA. The three tests were the Rotazyme I ELISA, the Bio-

EnzaBead ELISA, and the Rotalex LA test. The sensitivity was determined to a great extent by the time after the onset of illness during which the specimens were collected. There was no significant difference in the three tests when they were run on specimens collected early in the patients' illnesses. However, lower degrees of sensitivity were seen with the Rotazyme and Rotalex on specimens obtained later in the patients' illnesses. The lower sensitivity of the Abbott Rotazyme was not statistically significant, although the lowered sensitivity of the Rotalex LA was statistically significant. In addition, the authors found that a 104 50% tissue culture infective dose per 0.1 ml of virus suspension was detected at a 1:300 dilution by Bio-EnzaBead, at a 1:30 dilution by the Abbott Rotazyme, and at a 1:10 dilution by the Rotalex LA. The report concluded that there were no false-positive results with any of the three commercial tests, and this was seen with newborn as well as other specimens.

Similarly, ELISA (Premier Rotaclone) was compared with LA test (Bio Kit) for detection of rotavirus in fecal samples from clinically suspected cases of viral gastroenteritis in children. Out of 40 samples 12(30%) were positive for rotavirus antigen by ELISA kit. While 30 samples (75%) samples were positive for rotavirus by LA. All controls were negative for viral antigen by ELISA and LA. ELISA and LA kits were found to be economically sensitive for screening and rapid diagnosis of rotavirus diarrhea. In conclusion, the researchers revealed that LA was clearly a reliable and rapid method for detection of rotavirus but that ELISA was more sensitive than the LA [41].

In a study to compare Rotalex (Finland) with EM, a sensitivity of 92% and specificity of 98% were recorded. However, 19 of 218 specimens could not be evaluated since 10 of them gave equivocal results and 9 of them caused agglutination of the control latex [42]. In addition, Julkunen, et al. [43], using 570 specimens stored frozen at -20°C, compared Rotalex LA and a noncommercial ELISA used in their laboratory with EM results obtained from these samples prior to freezing. They found that their ELISA was more sensitive than EM (168 versus 145 positive), and that of 570 specimens, 30 were EM negative and ELISA positive. The specimens were positive in a confirmatory ELISA. 16 (2.8%) of the LA-positive specimens were negative by both EM and ELISA, and 15 of 16 were only slightly positive. They concluded that the LA test was good for screening, and that the positive results were definitely reliable.

Similarly a comparison was made between Rotalex (Finland), Rotazyme I and EM using 165 stools from children and neonates. Results showed that Rotalex had a sensitivity of 82% and a specificity of 96% compared with EM, and was slightly more sensitive and specific than Rotazyme I. The results also revealed that the sensitivity with Rotalex was dependent on the time of collection of stool samples relative to onset of symptoms. Sensitivities of Rotalex were 100, 96, 60, and 33% during 1 to 4, 5 to 7, 8 to 10, and 11 to 18 days, respectively, after onset of symptoms, and similar results were observed with Rotazyme I. Of 214 EM-negative specimens from asymptomatic newborns, the false-positive rates were 3.3% (7 of 214) for Rotalex and 4.2% (9 of 214) for Rotazyme I [44].

In the examination of stool specimens from 135 children, 0 to 3 years old, referred for fever, abdominal pain, vomiting and/or acute diarrhea, rotavirus antigens were detected from fecal samples by LA, ELISA and PAGE, with a total positivity of 15%, 55%, 12.59% and 11.85%, respectively. With PAGE test as reference, the sensitivity and specificity of LA and ELISA tests was 93.75%, 94.96% and 100%, 99.16%, respectively, The positivity ratio between 13-24 months group was meaningful with all tests (P = 0.042 for LA; P = 0.05 for ELISA; P = 0.031 for PAGE). ELISA and LA use were found to be as sensitive and specific as PAGE in the diagnosis of rotavirus diarrhea [45].

Similarly 293 stool samples collected in the 2001-2008 period were analyzed using ELISA, LA and PAGE. Results revealed that Rotavirus was detected in 34.8% of samples by LA assay, 28.3% of samples by ELISA assay and in 25.6% of samples by PAGE assay. Considering the PAGE method as gold standard, the sensitivity, specificity and accuracy of ELISA were 94.6%, 94.4% and 94.5%, and to LA were 82.6%, 81.6% and 81.9%, respectively. The study concluded that antigen detection by ELISA is a rapid, sensitive and specific method, and could be used in large-scale applications for screening stool samples suspected of RV infection [46].

A comparison was made between ELISA method and shell vial cell culture method for detection of rotavirus in fecal specimens, as well as correlation between laboratory results and clinical scores of patients with gastroenteritis. Among 219 stool samples tested, 107 (48.9%) were determined to be positive. Two of the specimens under study were positive by shell vial cell culture method while negative by ELISA. According to these results the calculated sensitivity, specificity, positive predictive value, and negative predictive value of ELISA were 98.1%, 100%, 100%, and 98.2%, respectively. The mean severity score for the 107 episodes of rotavirus diarrhea was 11.0 +/- 3.6 compared to 4.5 +/- 1.9 for the 112 episodes of non-rotavirus diarrhea in the same population. The study indicated that ELISA, which is easier to perform, faster and cheaper than cell culture methods may be suitable for routine diagnosis of rotavirus infections [47]. In an examination for the comparison of Apolipoprotein H-coated ELISA plates and quantitative real-time PCR, result revealed that the ApoH-ELISA was suitable for the capture of rotavirus-particles and detection down to 1,000 infectious units ($TCID_{50/ml}$). Subsets of diagnostic samples of different G- and P-types tested positive in the ApoH-ELISA in different dilutions. Compared to the quantitative PCR (qPCR) results, the analysis showed high sensitivity, specificity and low cross-reactivity for the ApoH-ELISA, which was confirmed in receiver operating characteristics (ROC) analysis [48].

In a similar study, RT-PCR was applied for the detection of rotaviruses in gastroenteritis episodes encountered in an efficacy trial of rhesus-human reassortant rotavirus tetravalent (RRV-TV) vaccine, in a total of 2398 infants. During a follow-up, covering two rotavirus epidemic seasons, 256 cases of rotavirus associated gastroenteritis were detected by ELISA; 226 were in the primary efficacy analysis period that included children who had received three doses of vaccine or placebo. Results revealed that with RT-PCR, 84 (33%) more cases of rotavirus

gastroenteritis were diagnosed than with ELISA, 65 of these were in the primary efficacy analysis period. Clinically, cases of rotavirus gastroenteritis diagnosed by RT-PCR were much milder (median severity score 6 on a 20-point scale) than those diagnosed by ELISA (median score 11), $P<0.0001$. RT-PCR revealed proportionally more G2 and G4 rotaviruses than ELISA. G1 rotaviruses detected by RT-PCR were almost equally divided between RRV-TV vaccine and placebo groups, whereas an apparent vaccine protective effect was seen in the distribution of G2 (one in the RRV-TV and eight in the placebo group) and G4 rotaviruses (six in the RRV-TV and 14 in the placebo group). The study concluded that RT-PCR is a useful tool in the diagnosis of rotavirus gastroenteritis, particularly for cases associated with serogroups other than the epidemiologically dominant G-type as well as contributing to the overall appraisal of performance of rotavirus vaccine Xiao-Li, et al. [49].

Similarly, real time RT-PCR assay (rRT-PCR) assay was developed for confirmation of infections of Group A or C rotaviruses simultaneously using a total of 54 stool samples obtained from pediatric patients (< 5 years old). All samples were tested for Group A rotavirus by ELISA and result obtained was compared with rRT-PCR assay to determine the test accuracies of both assays. The study revealed that rates of positive testing for Group A rotavirus by ELISA and the rRT-PCR assay were 22.22% and 18.50%, respectively. Forty-two serology-negative specimens for Group A rotavirus were also PCR negative (100% specificity). Two serology-positive specimens for Group A rotavirus was rRT-PCR negative; therefore, rRT-PCR assay represents a decrease of 3.70% in the number of specimens that are positive for Group A rotavirus while for Group C rotavirus, all tested samples were not rRT-PCR positive. The study concluded that ELISA was better than rRT-PCR in terms of sensitivity and specificity [50]

An integrated cell culture and reverse transcription quantitative PCR (ICC-RT-qPCR) assay have been developed to detect infectious rotaviruses based on detection of viral RNA during replication in cells. Cell culturing step before qPCR allows the infectious rotaviruses to replicate and be detected because they are the only ones that can infect cells and produce RNA. The results showed that as low as 0.2 PFU/ml rotaviruses were detected by ICC-RT-qPCR after 2 days of incubation. With samples, the copy numbers of VP7 gene of rotaviruses linearly correlated (with a coefficient (R2) of 0.9575) with initial virus concentrations ranging from 0.2 to 200 PFU/ml. Comparing the tests, showed that ICC-RT-qPCR exhibited higher sensitivity than both the plaque assay and the RT-qPCR when used in the field. ICC-RT-qPCR detected infectious rotavirus in 42% (10/24) of secondary effluents, while only 21% (5/24) and 12% (3/24) of samples were positive with either the plaque counting or the RT-qPCR method, respectively. Concentrations of rotaviruses in secondary effluent samples were shown to be 1–30 PFU/l. The ICC-RT-qPCR method has the advantage of reduced test duration and improved sensitivity towards infectious rotavirus and therefore can be used for detecting infectious rotaviruses in water environments [51].

Similarly a novel reverse transcriptase PCR (RT-PCR) method for the amplification of rotavirus RNA and a reverse hybridization assay on a strip was developed to detect amplimers, identify the specific G and P genotypes present in human stool specimens and permit specific identification of the rotavirus G1P [8] strain, used in the Rotarix vaccine. In a similar way, novel broad-spectrum PCR primers were also developed for both VP4 and VP7 to allow for the amplification of a wide range of rotavirus genotypes. In addition, for the identification of G and P genotypes, two reverse hybridization strip assays were developed. Both the VP4 and the VP7 strip contain universal probes for the detection of VP4 and VP7 sequences and probes to distinguish between wild-type G1 and G1 vaccine strain sequences, irrespective of the G or P genotype. While The VP4 strip contains type-specific probes for P[4], P[6], P[8], P[9], and P[10], the VP7 strip contains type-specific probes for G1, G2, G3, G4, G5, G6, G8, and G9. Results of the analysis of multiple reference strains revealed that both RT-PCR methods allowed the detection of a broad spectrum of genotypes with RT-PCR for VP7 observed to be more sensitive than RT-PCR for VP4. When a confirmatory test was performed using ELISA, it was discovered that all samples positive for rotavirus antigen by ELISA were also positive for both VP4 and VP7. Using sequence analyses and type-specific PCR, the high specificity of the reverse hybridization method could easily be confirmed. The reverse hybridization method has the advantage of permitting accurate identification of mixed infections with different genotypes as well as specifically identifying vaccine strains of rotavirus. Rotavirus genotypes for which no type-specific probes were present on the strip could adequately be identified using universal detection probes after analyzing the specificity, sensitivity, precision, accuracy, and robustness of the assay. When 149 ELISA-positive stool samples were compared with conventional type-specific RT-PCR methods it was revealed that the novel method was more superior especially in the detection of mixed rotavirus infections. This novel method could definitely permit highly accurate detection and identification of human rotavirus infections in stool samples and could therefore be useful for large-scale epidemiological and clinical trials [52].

A new and rapid, qualitative test for rotavirus (TestPack Rotavirus; Abbott Laboratories, North Chicago, Ill.) with another ELISA (Pathfinder Rotavirus; Kallestad Laboratories, Inc., Austin, Texas) was compared with EM to determine its clinical utility in a population of symptomatic hospitalized children using 100 frozen stool samples for the pilot study. The results after resolution with a blocking reagent showed a sensitivity of only 50% and a specificity of 88% for TestPack Rotavirus. In the subsequent study, TestPack Rotavirus was tested on 100 fresh, unfrozen samples. The results (sensitivity/specificity) were as follows: TestPack Rotavirus, 95/90%; Pathfinder Rotavirus, 84/98%; direct EM, 63/100%. Although it was not as sensitive or specific as IEM, TestPack Rotavirus was more sensitive than direct EM or Kallestad Pathfinder Rotavirus. TestPack Rotavirus therefore represents a rapid, qualitative method for the detection of rotavirus in stools of symptomatic children [53].

In a similar study, Von Bonsdorff, et al. [54] evaluated the performance characteristics of three commercial rapid non-microplate ELISA that utilize specific bound monoclonal and/or polyclonal antibodies to capture and detect rotavirus antigens: TestPack® Rotavirus (Abbott Laboratories, Chicago, IL) is a flow-through membrane ELISA; ImmunoCard STAT® Rotavirus (Meridian Diagnostics, Cincinnati, OH) and RotaStrip® (Coris BioConcept, Wépion, Belgium) are recently developed ICT assays. As reference method the scientists used the monoclonal antibody-based Premier Rotaclone® microwell ELISA (Meridian Diagnostics). Fifty rotavirus antigen positive and 50 rotavirus antigen negative stool specimens were tested with the three rapid immunoassays. All three rapid ICT tests were easy to perform, required no specialized laboratory equipment, and could yield results in less than 15 minutes. The specificities reached 100%, 98%, and 92%, and sensitivities were 100%, 98%, and 100% for the Abbott TestPack®, Meridian Immunocard STAT®, and Coris BioConcept RotaStrip®, respectively. All three rapid qualitative methods could therefore represent useful alternatives for the more laborious microplate ELISA procedures. Cost, speed, sample load, and ease of use are likely to influence the decision of the clinical laboratory to implement a rapid rotavirus antigen assay.

Rapid detection of group A rotavirus was again performed by using ImmunoCardStat! Rotavirus (ICS-RV) (which uses immunogold-based, horizontal-flow membrane technology), two commercial enzyme immunoassays (Premier Rotaclone and TestPack Rotavirus), and electron microscopy. The study involves the collction and analysis of a total of 249 stool specimens collected from children with gastroenteritis between February and April 1997. After resolution of 19 of the 22 discordant results by RT-PCR for group A rotavirus, ICS-RV detected 125 positives while Rotaclone and TestPack detected 127 and 129 positives, respectively. Results revealed that the sensitivity, specificity, positive predictive value and negative predictive value were 94%, 100%, 100%, and 93.4% for ICS-RV; 95.5%, 100%, 100%, and 95.0% for Rotaclone; and 97%, 97%, 97%, and 96% for TestPack. ICS-RV was sensitive and specific and was relatively simple to perform and interpret [55].

Enzyme immunoassays (EIAs) for rotavirus were evaluated as potential reference assays for rotavirus testing, using polyclonal and monoclonal antisera (Test Pack Rotavirus [TPK]; Abbott Laboratories), and monoclonal anti-rotavirus antibodies (Rota clone [RTC]; Cambridge BioScience Corporation), and compared with direct negative-staining electron microscopy (EM). 207 stool samples collected consecutively during the winter of 1989 from children with acute diarrhea admitted to a ward for infants from 0 to 2 years of age were tested by the EIAs and by EM. TPK specimens were read visually while RTC results were read spectrophotometric ally. Specimens with conflicting EIA and EM results were further evaluated by fluorescent focus assay. It was observed that specimens showing both negative and positive by EM results and those positive by fluorescent focus assay were considered positive for rotavirus. Of the 207 stools tested, 35 (17%) were positive for rotavirus by these criteria. EM was however only 80% sensitive while the Specificities were 100% for RTC and EM and 89% for TPK. These observations reveal that

EM is very specific in detecting rotavirus but is not as sensitive when compared with a highly sensitive monoclonal antibody-based EIA. An EIA with high sensitivity and specificity, such as RTC, is a more appropriate reference standard for rotavirus testing [56].

The sensitivity and specificity of five detection test kits for rotavirus including LA, ELISA ICT were evaluated in another study. LA was reported to be rapid and easy to perform and showed the lowest sensitivity among the five test kits. ICT showed a good agreement with ELISA and RT-PCR. ELISA was the best in respect of sensitivity and specificity, but difficulty in interpretations of equivocal results and time-consuming procedures were limitations. However, ICT, was easy to perform at a low cost, and may be an optimal method in place of LA for the detection of rotavirus [57].

In another study efforts were made to compare the sensitivity and specificity of assays used routinely in pathology laboratories for the detection of rotaviruses. LA and ICT results were compared with ELISA as gold standard. 90 stool specimens were screened for rotavirus using the Diarlex LAA (Orion Diagnostica, Finland), IDEIA ELISA kit (DAKO, Denmark) and the Coris RotaStrip ICT (Coris BioConcept, Belgium). Results of the study revealed that out of the 90 specimens tested, 83% (75/90) were rotavirus-positive when using ELISA while Diarlex LA showed a very poor sensitivity of 57% (43/75) and a specificity of 93%, and Coris ICT indicated an improved sensitivity of 88% (66/75) and a specificity of 100% compared with the ELISA [57].

While LA provides a rapid result, requires no specialized equipment and is useful for testing single specimens, the Diarlex LA appeared to be relatively insensitive when compared with the Coris ICT (57% v. 88%) which makes it a convenient, cost-effective assay with an equivalent turnaround time that could be adopted for routine, rapid rotavirus detection since it requires no additional equipment and is simple to perform, with easy-to-read results [58].

In a similar study, three different commercial immunologic tests for rapid detection of group A rotavirus (an ICT method, LA, and ELISA) were used to evaluate 228 fecal specimens obtained from Spanish children with acute gastroenteritis. After resolution of 30 (13.2%) discordant results by RT-PCR for rotavirus, the statistical values of ELISA, LA, and ICT method were respectively 96%, 68%, and 99% for sensitivity; 99%, 99%, and 96% for specificity; 98%, 96%, and 92% for positive predictive value; and 98%, 88%, and 99% for negative predictive value. The ICT technique showed high sensitivity and specificity and was rapid and easy to perform in the routine clinical laboratory [59].

In a study involving 127 infants and young children suffering from acute non-bacterial gastroenteritis, diagnosis of rotavirus infection was done by virus detection and serology. Human rotavirus (HRV) detection was performed by direct EM, conventional IEM and/or SPIEM; rotavirus antigens were detected by indirect double-antibody sandwich (DAS). HRV isolation was performed using both MA-104 and LLC-MK2 cell cultures while the serology was done on paired sera from all the patients using indirect immunoperoxidase antibody (IPA) technique for HRV IgG determination, and indirect ELISA method for purified HRV with Wa strain as a solid phase.

HRV particles were detected by EM and/or IEM in 53 cases (41.7%) and by SPIEM in 5 additional cases; HRV antigens were demonstrated by indirect DAS ELISA in the same 53 cases, whereas 40 cases (31.4%) were positive for HRV isolation in cell cultures. Results showed that 64 patients representing 50.3% seroconverted by IPA and ELISA, including the entire cases [55] positive for rotavirus detection in stools and 6 additional cases. Thus, SPIEM appeared to be the most sensitive technique for detecting a few virus particles in stool specimens, but HRV serology is the most sensitive method for diagnosing HRV infections retrospectively, when paired sera are drawn at an appropriate time. However, EM possess the great advantage of detecting in fecal specimens viral agents other than rotaviruses, such as adenoviruses, enteric coronaviruses, small round viruses, astroviruses and others [60].

Rotalex LA test was evaluated and compared with EM, ELISA, and PAGE of viral RNA on specimens frozen at -70^{O}C. Observation made was that LA was the least complex to perform but lacked sensitivity and specificity. They suggested four modifications to improve the test, including diluting the specimens 1:100 rather than 1:10 and reading at 20 min, not at 2 min [61]. Furthermore, [62] compared Rotalex LA with four other methods: PAGE, EM, SPIEM, and a commercial reverse passive hem agglutination test. The positive rates for the five methods were 61% (LA), 63% (PAGE), 59% (EM), 59% (SPIEM), and 57% (reverse passive hem agglutination). In a similar study, Rotalex (Finland) was evaluated in 90 children with diarrhea and found to be 90% (80 of 89) compliant with the established method(s) of EM alone or in conjunction with the Enzygnost ELISA. 10% (9 of 89) were considered false-negative by Rotalex since they were positive by EM alone or by EM with ELISA that could be blocked [63].

The comparative efficacy of different assays for detection of group A rotavirus in pediatric patients was evaluated with a total of 455 fecal samples which were screened by monoclonal antibody based ELISA. The result revealed that 33 (7.25%) samples were positive for group A rotavirus with percentage positivity ranging from 3.22% to 28%. The same samples were also tested by RNA-PAGE, which revealed classical 11 segments with 4:2:3:2 migration patterns in 14 fecal samples showing 3.08% positivity. Virus isolation was successfully done from 21 (4.61%) samples. However, only 15 (3.3%) samples yielded a specific product of 864 and 1,011 bp for VP4 and VP7 genes, respectively, by RT-PCR. The sensitivity and specificity of ELISA, RNA-PAGE and RT-PCR was 100%, 66. 7% and 71.4% and 97%, 100% and 100%, respectively, considering virus isolation as standard test. The report concluded that ELISA being a simple, fast and sensitive assay can be used as routine laboratory test for the diagnosis of group A rotavirus and field epidemiological studies [64].

Peroxidase-labeled monoclonal antibody against rotavirus group-specific antigen (inner capsid) was used for the detection of rotavirus by immunoperoxidase staining (IPS) in trypsin-free MA104 cells within 18 h post-inoculation with clinical specimens. 121 fecal samples from children with acute gastroenteritis were evaluated by IPS, conventional virus isolation in cell culture and a commercially available group A-antigen ELISA (Rotazyme II, Abbott Laboratories). 58 (47.9%)

stool samples were found positive by IPS. In contrast, rotavirus was isolated from only 4 (3.3%) fecal specimens by conventional cell culture (i.e. demonstration of a cytopathogenic effect) while a total of 93 (76.9%) samples were positive by ELISA [65].

Similarly a technique which includes the use of indirect immunoperoxidase antibody (IPA) has been developed for detecting enteric adenovirus and rotavirus antigens in cell cultures and has been compared with immunofluorescence antibody assay (IFA). The IPA technique was as sensitive as the IFA. The number of positive cells detected by both techniques in tissue cultures was the same; false positive results were not observed [66].

2.1. False Positive ELISA Kit Results

Various problems have however been associated with ELISA as revealed by Yolken and Stopa [67] who initially reported problems with nonspecific reactions in the technique. The nonspecific activity was markedly reduced by pretreatment of the specimens with reducing agents, normal goat serum, and anti-human IgM. The authors concluded that it was likely that the specimens contained an IgM antibody capable of reacting nonspecifically with other components of the assay. Although pretreatment with the mild reducing agent N-acetyl cysteine markedly reduced this nonspecific activity, such treatment did not reduce the specific ELISA activity due to rotavirus. A similar study by Hovi, et al. [68] reported that false-negative results might result from fecal protease activity and suggested that the problem could be alleviated by adding 1 to 5% bovine serum to dilution buffers or by using a synthetic broad-spectrum serine-type protease inhibitor. Furthermore Hogg and Davidson [69] evaluated false-positive results and found that, when they incorporated preimmunization serum-coated wells as control wells in their ELISA, 9.7% of specimens which gave positive results were eliminated as false positives.

Several other investigators have also reported problems with false positive ELISA results on specimens from neonates. These occurred especially in earlier studies with Rotazyme I, in which lower positive cutoff values were used. When the cutoff value was raised however, some of the problems appeared to be eliminated. Furthermore it was reported that 22% (79 of 358) of neonatal stool specimens from both asymptomatic and diarrheal neonatal patients were positive by Rotazyme I, although 61 of 79 positives that were analyzed by confirmatory tests, only 7% (4 of 61) were confirmed as positive. This was compared with 77% that were confirmed Rotazyme-positive specimens from children and adults. Suggested causes of these false-positive Rotazyme tests in neonates included nonspecific binding of rotavirus antibody to bacteria or staphylococcal protein A in neonatal stools [70]. However, Pai and Mayock [71], using Rotazyme I to study specimens from infants under the age of 4 months, found that only 9.8% (21 of 214) that were negative by EM were positive by ELISA and thus presumed to be false positives. Only 4.5% (9 of 202) were positive when visual readings of >1+ was considered positive, as recommended by the manufacturer.

Similar results were seen by Rand, et al. [72], who studied stool specimens from diarrhea-free infants in a neonatal intensive care unit with Rotazyme I. None had rotavirus by EM but by ELISA, only 6.8% (10 of 147) were considered either low-level positive or suspect positive. Excluding the suspect positives, which were negative on repeat testing, the false-positive rate was 4.1% (6 of 147). With five repeatedly positive specimens with sufficient quantity to retest, heating to 56°C for 30 min eliminated binding to the Rotazyme bead; heating had no effect on the Rotazyme-positive control. One highly false positive result was not changed by heat or other treatment. Thus, the investigators concluded that heat treatment of positive samples from neonates could eliminate most of the false-positives, although false-positives may result from more than one cause.

Another case of 15% (8 of 53) false-positive rate on specimens from 5-day-old babies was recorded when Rotazyme I was used which showed that in an initial cutoff value specified in early Rotazyme directions and a revised, higher cutoff value, only 1.9% (1 of 53) of the neonatal samples would have been falsely positive [73. 74, 75]. In addition, weakly positive and borderline Rotazyme reactions correlated poorly with direct EM findings[74, 76]. Conversely, Rudd and Carrington [77], in screening babies in a neonatal intensive care unit, found that 2.9% (5 of 170) of babies had specimens' positive by the Rotazyme I test. Two of these babies had necrotizing enterocolitis, one had bloody diarrhea, and two were asymptomatic. Thus, a high false positive rate did not occur in the study.

In a prospective study of 500 fecal specimens from neonates in an obstetrics ward, supernatant fluids of specimens, after 3,000-rpm centrifugation was used in the Enzygnost ELISA, with 5% (25 of 500) positivity. Of these, 52% (13 of 25) were confirmed positive by a blocking ELISA. All (100%) positive specimens from babies with diarrhea were also positive in the confirmatory blocking test, whereas only 33% (6 of 18) from asymptomatic patients were positive in blocking tests. Enzygnost test is therefore suitable only in neonates with symptoms but not asymptomatic neonates. Suggestions have however been made that the Enzygnost and Rotazyme test kits should include a confirmatory reaction in their test kits [78] .

In one study, Herrmann, et al. [79] compared both Rotazyme and their own ELISA, which used a monoclonal detector antibody, with EM. They evaluated specimens from three types of patients: neonates, children, and adults. The sensitivity of their monoclonal ELISA was 100% for all patients, while that of Rotazyme was 100, 86.2, and 50.0% in the three types of patients, respectively. The specificity of the monoclonal ELISA was 100, 95.6, and 96.5%, while that of the Rotazyme was 37, 95.6, and 89.7% respectively. Thus, they concluded that, especially for specimens from children and adults, greater sensitivity and specificity could be achieved with monoclonal antibody as detector antibody as compared with Rotazyme. Also, use of the monoclonal antibodies eliminated the false-positive reactions seen in specimens from neonates when Rotazyme was used. The researchers thought that, by using monoclonal antibodies as detector antibodies, the test sensitivity may be decreased, since these antibodies react with only

one epitope of a given antigen. This problem could be diminished by using monoclonal antibody as a capture antibody and a polyclonal serum as the detector antibody, as these investigators had reported for a rotavirus RIA. The advantage of using the monoclonal antibody for capture is that it may pick up a significant amount of an antigen with several different epitopes. These investigators considered that the polyclonal sera may contain a number of cross-reacting components that may have caused the false-positives in neonates, since false-positives occur in neonatal specimens in other rotavirus ELISAs besides Rotazyme.

The disadvantage of using polyclonal antibodies as a detector is that they may react nonspecifically with antigen adsorbed to the solid phase. This may occur even if inhibitors such as serum, serum fractions, or gelatin are included in the diluents to block nonspecific reactions. For example, they found that in stools containing high titers of rotavirus there was sufficient reactivity to give a positive test with microtiter plates coated with preimmune sera, even though there was two to three times greater reactivity with plates coated with immune sera [79].

In another study, Rotbart, et al. [80] obtained rectal swabs from symptomatic and asymptomatic babies in a neonatal intensive care unit in which an outbreak of necrotizing enterocolitis and hemorrhagic gastroenteritis occurred. A total of 4.0% (19 of 475) of specimens were positive by Rotazyme I, 2.1% (10 of 475) from symptomatic babies and 1.9% (9 of 475) from asymptomatic babies. Confirmatory tests were positive in 80% (8 of 10) of the specimens from symptomatic babies, while confirmatory tests were positive in only 33% (3 of 9) of the specimens from asymptomatic babies. Differences in Rotbart's results and those of Krause, et al. [70] may have been due to Rotbart's use of swabs, which may have contained less inhibitory substances, or inhibitory substances may have been less stable on swabs than in the stool samples used by Krause, et al. [70]. Since all of the studies were carried out at different locales and times, year-to-year and lot-to-lot variations in key reagents might also have accounted for some differences. It was however recommended after several analyses that the current Rotazyme I test should not be used for screening asymptomatic infants while advocating the inclusion of some type of confirmatory testing in the Rotazyme kit, e.g., reaction with nonimmune serum or use of monoclonal antibodies [80].

No study has however found false-negative Rotazyme results in neonates [79].

3. CONCLUSION

Rotavirus detection is greatest when diarrhea, vomiting and fever occur together and lowest when each symptom occurred alone. Diagnosis of the infection is based on the identification of the virus in feces or suspension of rectal swab collected early in the illness through direct microscopy, molecular techniques, rapid serological tests, and use of tissue culture technique. The role of culture is however diminishing as new immunologic and molecular tests are developed that provide more rapid results and are able to detect a larger number of viruses. Routine diagnosis is now performed by antigen detection on feces using commercially available, simple, rapid

immunochromatographic dipstick style kits. Rapid and simple diagnostic techniques indeed influence clinicians in the implementation of effective management and control measures to pediatric rotavirus diarrhea disease.

4. SIGNIFICANCE OF STUDY

This study is one of very few which have investigated and compared results of the sensitivity and specificity of techniques available for the diagnosis of Rotavirus gastroenteritis in pediatric patients.

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