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COMPARISON OF POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE), IMMUNO-ELECTRON MICROSCOPY (IEM) AND ENZYME IMMUNOASSAY (EIA) FOR THE RAPID DIAGNOSIS OF ROTAVIRUS INFECTION IN CHILDREN

H.G. PEREIRA

R.S. AZEREDO
J.P.G. LEITE
O.M. BARTH
F. SUTMOLLER
V. DE FARIAS
M.N.P. VIDAL

Detection of rotavirus RNA by polyacrylamide gel electrophoresis (PAGE) proved to be a highly sensitive and rapid diagnostic test. A comparison of this assay with immuno-electron microscopy (IEM) and enzyme immunoassay (EIA) in 245 faeces from children with gastroenteritis revealed complete agreement between the three assays in 238 (97.14%) samples. Among 75 samples positive in at least one of the three assays, negative results were observed in 5 (6.48%) by PAGE, in 6 (6.76%) by EIA and in none by IEM. Silver staining greatly increased the sensitivity of the PAGE assay.

We conclude that although IEM remains the most sensitive and rapid rotavirus diagnostic assay, the PAGE technique has many advantages in its favour, including the non-requirement of expensive equipment, the use of only chemically defined reagents and the capacity to distinguish virus subgroup and variants and to detect non-crossreactive rotaviruses which are missed in serological assays.

Following the original reports of the detection of human rotaviruses by electron microscopy (Bishop et al., 1973; Flewett, Bryden & Davies, 1973), a number of serological assays such as complement fixation, counter immuno electrophoresis, immunofluorescence, reverse passive haemaglutination, immune adherence haemaglutination, radio and enzyme-immunoassays (see review by Kapikian et al., 1979) have been developed for the rapid diagnosis of infections caused by these viruses. Several comparative studies (e.g. Sarkinen, Tuokko & Halonen, 1980; Brandt et al., 1981; Obert et al., 1981; Yolken & Leister, 1981; Rubenstein & Miller, 1982; Hammond et al., 1982) have demonstrated similar degrees of sensitivities for immuno-electron microscopy, radio and enzyme-immunoassays, the latter, for practical reasons, having become the technique mos commonly used in routine diagnosis. Another technique based not on serology but on electrophoretic analysis of the viral double stranded RNA genome, was first proposed as a

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Instituto Oswaldo Cruz, Caixa Postal 926, 20000 Rio de Janeiro, RJ, Brazil.

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diagnostic test by Espejo, Calderon & Gonzales (1977) and since used by several workers in routine diagnosis (Lourenço et al., 1981; Herring et al., 1982; Avendaño et al., 1982). This technique has also been used to differentiate human rotaviruses into two subgroups (Kalica et al., 1981; Thouless, Beard & Flewett, 1982) and to study the geographical and temporal distributions of strains with differing genome profiles (Espejo et al., 1977, 1978, 1979, 1980a, b; Kalica et al., 1978; Rodger et al., 1981; Lourenço et al., 1981; Mitsusawa et al., 1982; Pereira et al., 1983a).

In the present paper we compare the relative sensitivities of RNA polyacrylamide gel electrophoresis (PAGE), enzyme-immunoassay (EIA) and immuno-electron microscopy (IEM) in the rapid diagnosis of human rotavirus infections.

MATERIAL AND METHODS

Faeces from children with acute gastroenteritis were received from hospitals, outpatients clinics, nurseries and schools in the state of Rio de Janeiro. They were processed as described by Pereira et al. (1983a) and tested immediately or after varying periods of storage at -20^{o}C.

For electrophoretic analysis, 10 to 20% faecal suspensions in 10mM Tris/HCl pH 7.4 buffer containing 15mM Ca Cl_2 (Tris/Ca) were clarified by centrifugation at 1000g after homogenization with Freon 113 and either deproteinized (0.5ml volumes) by extraction with phenol-chloroform and ethanol precipitation as described by Pereira et al. (1983a) or centrifuged (4ml volumes) over a 1ml cushion of 45% (w/v) sucrose in Tris/Ca buffer for 1 hour at 100000g followed by dissociation of the pellet in 15μl of 5M urea, 3% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue. The former treatment was generally used for samples with previous positive results in other assays and the latter for those not previously tested, or in which RNA profiles were absent or indistinct by the phenol-chloroform extraction method.

Electrophoresis was carried out by Laemmli's (1970) technique with slight modifications described by Pereira et al. (1983a). Gels were stained either by ethidium bromide (0.5μg/ml in distilled water) for 30 minutes followed by washing for 1 hour in distilled water and photography in transamitted ultraviolet light, or by silver impregnation as described by Boulikas & Hancock (1981). The greater sensitivity of the latter led to its exclusive use in the later stages of this study. Samples giving negative results or indistinct RNA profiles in gels stained with ethidium bromide were retested by the silver impregnation technique whenever sufficient material was available. Any pattern in which at least the four slower-moving bands were clearly visible were considered positive.

Enzyme-immunoassays were performed by double antibody sandwich techniques (Voller, Bartlett & Bidwell, 1978). The first 88 samples were tested by the technique described by Pereira et al. (1983a) (EIA/IOC) and the remaining 157 by the rotavirus enzyme-immunoassay kit supplied by W.H.O. (EIA/WHO). During a transitional period, 187 samples (not all included in the present study) tested by both techniques gave concordant results in all but 6 (97.86% agreement). Confirmatory tests by IEM, PAGE and EIA/WHO blocking assays revealed that the EIA/IOC test gave 1 false-positive and 5 false-negative results.

Immuno-electron microscopy was performed by the technique described by Almeida et al. (1979). Volumes of 0.5ml of clarified 10 to 20% faecal suspension in Tris/Ca buffer containing guineapig hyperimmune serum against simian rotavirus SA11 at a final dilution of 1/400 were incubated for 30 minutes at 37^{o}C and centrifuged at 12000g for 1 hour. The pellets were resuspended in 0.1ml of Tris/Ca buffer, added to formvar-coated grids, stained with 2% phosphotungstic acid at pH 7.2 for 20 seconds and examined in a Zeiss IOB electron microscope.

RESULTS

Two hundred and forty five samples tested by PAGE, IEM and EIA gave results shown in Table I. The three assays were in complete agreement in 238 (97.14%) samples. Among the 74 samples positive in at least one assay, negative results were observed in 5 (6.48%) by PAGE, in 6 (6.76%) by EIA and in none by IEM. Because the method used for EIA and PAGE varied in sensitivity in successive stages of this study, discrepant results are shown in more detail in Table II. Only one of the 5 PAGE-negative and 2 of the 6 EIA-negative samples were available in sufficient quantities to be tested by the more sensitive silver staining PAGE and WHO EIA assays respectively. It is possible that had the more sensitive assays been used throughout, the number of discrepancies would have been lower.

TABLE I

Combination of results obtained by PAGE, IEM and EIA in human faeces

Assay Results			*Nº of Samples*
PAGE	IEM	EIA	
+	+	+	67
–	+	+	1
+	+	–	2
–	+	–	4
–	–	–	171

+ Positive result
– Negative result

TABLE II

Samples giving discrepant results in three assays

Strain	*PAGE*		*EIA*		*IME*
	EB[1]	*$AgNO_3$*[2]	*IOC*[3]	*WHO*[4]	
CA6	–	NT	+	NT	+
17179	–	NT	–	NT	+
17184	–	NT	–	NT	+
19433	–	NT	–	NT	+
21515	–	–	–	–	+
17266	+	+	–	–	+
19349	+	NT	–	NT	+

[1] Gels stained by ethidium bromide
[2] Gels stained by silver nitrate
[3] EIA performed as described by Pereira et al. (1983)
[4] EIA performed with WHO kit

– Negative result
+ Positive result
NT Not tested

Of the two samples positive by PAGE and IME and negative by EIA, one (strain 19349), previously described by Pereira et al. (1983b) gave an electrophoretic genome profile, which is clearly distinct from those of human rotaviruses of subgroups 1 and 2 and resembles those of a human (Rodger et al., 1982) and a porcine (Saif et al., 1980; Theil et al., 1981) strains which lack the cross-reactive antigen common to most rotaviruses. This may explain the negative EIA result obtained with our strain 19349, which although only tested by the slightly less sensitive EIA/IOC assay, was also negative in this test after 20x concentration by high speed centrifugation. The other EIA-negative but IEM and PAGE-positive specimen may represent another non-crossreactive rotavirus but its genome profile could not be fully characterized due to the small volume and low virus content of the available sample.

Our results are in agreement with those of Boulikas and Hancock (1981), Berry & Samuel (1982) and Herring et al. (1982) demonstrating the greater sensitivity of silver over ethidium bromide for staining RNA in polyacrilamide gels. Figure 1 shows a gel in which high speed pellets (see material and methods) of 10 faecal suspensions were electrophoresed alongside a standard preparation of simian rotaviruses SA11, and stained successively by ethidium bromide and silver nitrate. The former barely reveals RNA bands in only one sample (channel 6) apart from the SA11 standard, whereas two additional positive samples (channels 2 and 3) are detected after silver staining. Furthermore, smearing by impurities, although visible in both staining procedures, is less obscuring after silver staining.

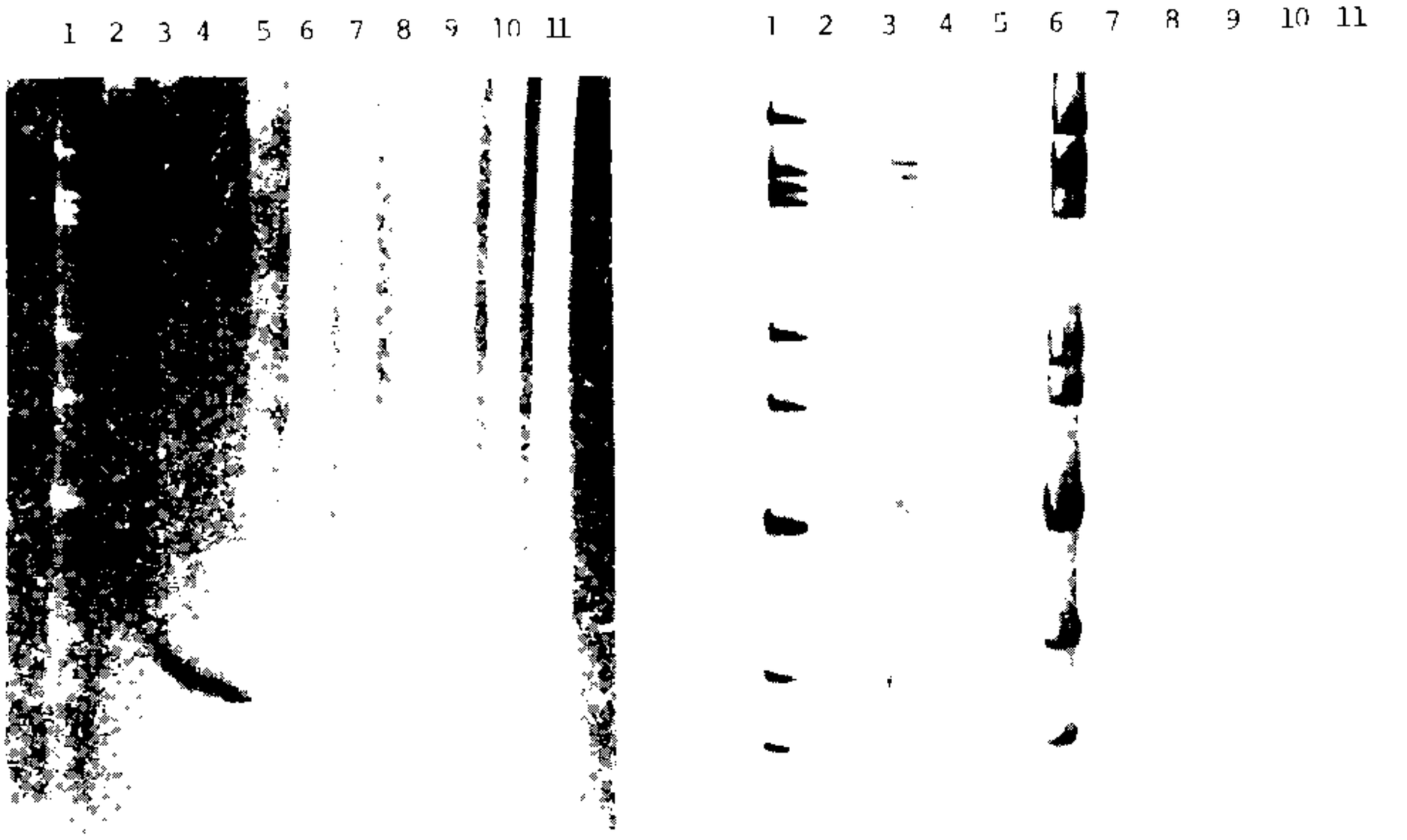

Fig. 1 – Comparison of ethidium bromide and silver staining techniques.

High speed concentrates (see Material and Methods) of simian rotavirus SA11 (channel 1) and of faecal suspensions (channels 2 to 10) stained successively by ethidium bromide (pannel A) and silver nitrate (pannel B).

Classification of PAGE-positive samples into subgroups according to the migration rates of RNA bands 10 and 11 (Kalica et al., 1981; Thouless, Beard & Flewett, 1982) was established in 67 (90.5%) of the 74 PAGE-positive samples. One strain (19349) differed in electrophoretic pattern from both subgroups 1 and 2 as mentioned above and the remaining 6 could not be subgrouped due to absence of visible bands 10 and 11.

Only 2 of the latter were tested by the more sensitive silver staining technique. Of the 67 subgrouped strains, 58 belonged to subgroup 2 and 9 to subgroup 1. All of the latter originated from a single common source school outbreak described by Sutmoller et al. (1982) and showed identical electrophoretic profiles. Subgroup 2 strains showed considerable electrophoretic heterogeneity as previously described (Pereira et al., 1983a).

DISCUSSION

Polyacrylamide gel electrophoresis proved in this as in previous studies (Espejo et al., 1977; Lourenço et al., 1981; Herring et al., 1982; Avendaño et al., 1982) to be a practical and highly sensitive technique for the rapid diagnosis of rotavirus infections and for the distinction of strains with different genome profiles. It has several advantages over other diagnostic techniques. All the elements of the assay, with the obvious exception of the stools under test, are chemically defined and readily available at reasonable cost. The equipment required is generally available in most clinical laboratories. It does not require disposable items which may be expensive or difficult to obtain in developing countries. The use of silver instead of ethidium bromide staining techniques obviates the need of photography in ultraviolet light and greatly increases the sensitivy of the PAGE assays (Boulikas & Hancock, 1981; Berry & Samuel, 1982). Although even higher sensitivity can be achieved by in vitro end-ebeleing of viral RNA (Clarke & McCrae, 1981), this advantage is partially cancelled by limitation in the availability of the necessary radioactive and other reagents.

The time requiered for results to be obtained by PAGE can be as short as 6 hours from receiving a sample. This can be achieved by several technical short-cuts. The phenol extraction procedure can be shortened by eliminating the ethanol precipitation step (Herring et al., 1982) or by precipitating at $-70^{o}C$ for 2 hours instead of at $-20^{o}C$ overnight. Alternatively phenol extraction can be replaced by high speed centrifugation (see material and methods) for 1 hour followed by direct dissociation of the resulting pellet in electrophoresis sample buffer. Electrophoresis can be run either for 2 1/2 hours at 40mA or at 8mA overnight depending on the time of arrival of samples. Silver staining by the method of Boulikas & Hancock (1981) takes about 2 hours.

The sensitivity of PAGE followed by silver staining is brought to levels comparable to or very slightly lower than those achieved by EIA and IEM. PAGE, gives unanbiguous results except for gross errors such as channel overspills or mixing of samples. It has the additional advantage over current serological assays of detecting non-crossreactive rotaviruses such as those described by Bridger (1980), McNulty et al. (1981), Bridger, Clarke & McCrae (1982), Saif et al. (1980), Theil et al. (1981), Rodger, Bishop & Holmes (1982) and Pereira et al. (1983b). Another advantage is that besides detecting rotaviruses it differentiates them into subgroups and electrophoretic variants. Although it is accepted that electrophoretic variation is not necessarily related to charges in antigenic behaviour (Beard, 1982) or to RNA homology (Clarke & McCrae, 1982), it may provide information of epidemiological importance. Finally, the PAGE assay can detect the occurrence of mixed infections (Lourenço et al., 1981) which other methods would not be capable of revealing.

We conclude that although IEM is still the most sensitive and rapid method for the detection of rotaviruses, the PAGE assay has many advantages to reccommended its use.

SUMÁRIO

A evidenciação da presença de ácido ribonucleico (ARN) viral por eletroforese em gel de poliacrilamida (EGPA) foi comprovada como um método altamente sensível e rápido para o diagnóstico de infecções por rotavirus. Uma comparação desta prova com a imunomicroscopia eletrônica (IEM) e com o ensaio imunoenzimático (EIE) no exame

de 245 fezes de crianças com gastroenterite revelou completa concordância entre os três ensaios em 238 (97.14%) amostras. Entre 75 amostras positivas pelo menos em um dos três ensaios, resultados negativos foram observados em 5 (6.48%) por EGPA, em 6 (6.76%) por EIE e em nenhum por IEM. Coloração pela prata aumentou consideravelmente a sensibilidade do ensaio por EGPA.

Concluímos que embora a IEM ainda seja a prova mais sensível e rápida para o diagnóstico de infecções por rotavirus, o ensaio por EGPA tem muitas vantagens em seu favor, sendo as principais as de não necessitar equipamentos caros, de empregar exclusivamente reagentes quimicamente definidos, de identificar grupos e variantes virais e de detectar amostras que não possuindo antígenos comuns à maioria dos rotavirus, não são reveláveis por ensaios sorológicos.

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