Comparative evaluation of the WHO and DAKOPATTS enzyme-linked immunoassay kits for rotavirus detection*

T.H. Flewett,[1] C.F. Arias,[2] L.F. Avendano,[3] A. Ghafoor,[4] M.M. Mathan,[5] L. Mendis,[6] K. Moe,[7] & R.F. Bishop[8]

Faeces obtained from 1163 children (including 66 newborn babies) were analysed in parallel for the presence of rotavirus particles using two enzyme-linked immunosorbent assay kits. The kits had been formulated by the WHO Collaborating Centre for Reference and Research on Rotavirus (WHO-ELISA kit) and by DAKOPATTS (DAKO-ELISA kit) to be suitable for use in laboratories in developing countries. The kits were evaluated in laboratories in Burma, Chile, India, Mexico, Pakistan, Sri Lanka and the United Kingdom. Comparison of the results obtained with the two kits indicated that the DAKO-ELISA had an overall sensitivity of 97% and a specificity of 97% relative to the WHO-ELISA. In individual laboratories the DAKO-ELISA (K349) kit had a sensitivity in the range 90–100%, and a specificity of 85–100%. The kit showed a sensitivity of 100% and a specificity of 98% in assays on faeces obtained from newborn babies. We conclude that the DAKO-ELISA is as sensitive and specific as the WHO-ELISA for the detection of rotavirus antigen in faeces.

Introduction

The enzyme-linked immunosorbent assay (ELISA) is well established as a sensitive and specific technique for the detection of rotavirus antigen in stools. The technique was adopted by WHO as the standard diagnostic technique for use in epidemiological studies in developing countries. Under the auspices of the WHO Diarrhoeal Diseases Control Programme (CDD), the WHO Collaborating Centre for Reference and Research on Rotaviruses at East Birmingham Hospital, England, developed an ELISA kit for distribution to laboratories undertaking studies funded by WHO (*1*). The assay proved to be highly sensitive, specific, and simple to perform. The kit incorporated lyophilized ingredients to ensure the stability of the reagents during the delays and high ambient temperatures that can be encountered during transport to laboratories in developing countries.

Numerous commercially-developed kits based on ELISA are now available for purchase and are widely used in the diagnosis of rotavirus infection in children in developed countries. The DAKOPATTS Rotavirus ELISA kit was originally formulated for use in developed countries and shown to be sensitive and specific and to yield visual readings that identified approximately 98% of positive specimens (*2*, *3*).

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[1] WHO Collaborating Centre on Human Rotaviruses, East Birmingham Hospital, Birmingham, England.

[2] Centre for Investigations in Genetic Engineering and Biotechnology, Universidad Nacional Autónoma de Mexico, Mexico DF, Mexico.

[3] Department of Microbiology and Parasitology, University of Chile, Santiago, Chile.

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[4] WHO Collaborating Centre for Research and Training in Viral Diagnostics, Islamabad, Pakistan.

[5] Wellcome Research Unit and the Department of Gastroenterology, Christian Medical College Hospital, Vellore, Tamil Nadu, India.

[6] Department of Microbiology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka.

[7] Department of Medical Research, Ministry of Health, Rangoon, Burma.

[8] Department of Gastroenterology, Royal Children's Hospital, Melbourne, Australia.

This kit has been modified, at the request of WHO, so that it is suitable for distribution to laboratories in developing countries. Six diagnostic laboratories conducting epidemiological studies of rotavirus infection in young children in Burma, Chile, India, Mexico, Pakistan, and Sri Lanka were chosen for a comparative evaluation of the results obtained using the WHO and the modified DAKOPATTS (K349) Elisa kits. In addition the two kits were evaluated in the WHO Collaborating Centre in Birmingham, England.

Materials and methods

Specimens

Samples of stool were collected from young children presenting at hospital or clinic within 72 hours of onset of acute diarrhoea. The numbers of specimens obtained by each laboratory are listed in Table 1; laboratories in developing countries are identified by a letter code (A to F) that does not correspond to the alphabetical sequence used in this text. Samples collected by laboratory F included 66 stools obtained from newborn babies with and without diarrhoea. Specimens were stored at 4 °C, or −20 °C or −70 °C prior to testing. Specimens collected in each laboratory were assayed in duplicate over a few days by both WHO-ELISA and DAKO-ELISA following the instructions provided with the respective kits.

ELISA assays

The WHO-ELISA is an indirect double-sandwich ELISA, conducted in two stages (*1*). First, a screening test is performed in duplicate on wells coated with post-immune rabbit serum. Specimens giving a positive result in the screening test are further tested by a confirmatory ELISA blocking test using four wells coated with post-immune rabbit serum. The specimen is tested after mixing with rotavirus antibody-negative pre-immune serum, or with hyperimmune rotavirus serum. The WHO-ELISA test is performed by coating polystyrene microtitration plates with hyperimmune antirotavirus (rabbit) antisera. The wells are reacted with 10% (v/v) clarified faecal extracts in phosphate-buffered saline (pH 7.4) containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) and 0.01 mol/l EDTA disodium salt. Rotavirus antigens are detected using hyperimmune guinea-pig anti-rotavirus serum, followed by affinity purified goat anti-guinea-pig IgG conjugated with alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD, USA) reacted with *p*-nitrophenyl phosphate disodium. Optical density of colour reactions is read by spectrophotometer set at 405 nm. No samples are reported as positive until tested in duplicate in the confirmatory blocking tests. The blocking test is considered positive if the mean optical density (OD) reading in the two wells containing post-immune serum is >50% of that in the wells containing pre-immune goat serum. Positive and negative control antigens are included with the kit. The test takes 2 days to perform for screening and an additional 2 days for confirmation.

The DAKO-ELISA is performed by coating polystyrene microtitration plates with duplicate rows of immunoglobulin fraction of rabbit hyperimmune antirotavirus antisera and negative control rabbit serum, followed by the addition of faecal extracts. These were initially prepared as 10% (v/v) suspension in 0.15 mol/l sodium chloride, clarified by low speed centrifugation. Faecal extracts used in the test comprised 1:10 dilution of the supernatant in dilution buffer of PBS (pH 7.4) with added 0.1% Tween 20 and 1% bovine serum albumin. After adsorption and washing, non-specific reactions are blocked by adding normal rabbit immunoglobulin, followed by hyperimmune rabbit antirotavirus antiserum conju-

Table 1: **Comparison of results of rotavirus diagnosis using enzyme-linked immunoassay kits provided by WHO and DAKOPATTS (DAKO)**

Laboratory	No. tested	WHO+ DAKO+	WHO− DAKO−	WHO+ DAKO−	WHO− DAKO+
WHO Collaborating Centre in United Kingdom	133	20	112	0	1
A	92	33	58	1[a]	0
B	159	64	93	1	1
C	219	70	122	6	21
D	238	19	215	2[b]	2
E	210	67	142	0	1[c]
F	112 (66)[d]	34 (24)	77 (41)	0 (0)	1 (1)

[a] DAKO positive when retested.
[b] Positive by electron microscopy.
[c] Confirmed positive by blocking.
[d] Figures in brackets refer to specimens obtained from newborn babies.

gated to peroxidase. Rotavirus antigen is detected by reaction with *o*-phenylene diamine in citric acid and phosphate buffer in the presence of hydrogen peroxide. A positive control antigen is included in the kit. The test can be performed in less than 5 hours. Results can be read visually by comparing the colour of the test well with the colour of the corresponding negative control well. The absorbance of the liquid in the wells can be measured at 492 nm using a spectrophotometer. The criteria for a positive test is that the absorbance of the test well minus the absorbance of the negative control well is greater than 0.1, and the absorbance of the test well divided by the absorbance of the negative control well is greater than 6. If the difference between the absorbance value of the duplicate test wells exceeds 20 per cent, then the test should be repeated.

The WHO-ELISA kit provides all the materials required, including buffers. Pre-immune and hyperimmune rabbit anti-rotavirus serum, guinea-pig anti-rotavirus serum, alkaline phosphatase conjugate, positive and negative rotavirus control antigens are provided freeze-dried. The DAKO-ELISA kit modified for use in developing countries includes lyophilized peroxidase conjugate together with diluent for its reconstitution. Lyophilized positive control antigen is provided sealed under vacuum, and phenylene diamine is provided in tablet form in a vial containing desiccant.

The WHO-ELISA and DAKOPATTS-ELISA kits were sent to each laboratory by air freight with no special provisions for rapid transport, in order to simulate the usual conditions of supply materials to these laboratories.

Confirmation of ELISA results

Electron microscopy of negatively stained faecal extracts[a] was used to confirm the positive and negative ELISA results by laboratories C and D. Gel-electrophoreses of rotavirus genome RNA (*4*) was used to confirm positive and negative ELISA results by laboratories A, B and D. Laboratory E developed a blocking test using DAKO reagents and pre-immune and post-immune human serum in order to evaluate weakly positive results obtained with the DAKOPATTS assay. Mixtures of the weakly positive specimen with non-immune or hyperimmune human serum were added to four wells coated with DAKO rabbit hyperimmune antirotavirus antisera. The blocking test was analagous to that used in the WHO assay.

[a] *Manual for laboratory investigations of acute enteric infections.* Unpublished WHO document, CDD/83.3, Rev. 1, 1987, pp. 74–76.

Results

Comparison of the results obtained with the two kits is listed in Table 1. All results from the total of 1163 specimens were initially read by eye. In addition, results from 953 specimens were read by optical density (OD) measurements using a spectrophotometer.

Disparate results for the same specimen using the WHO and ELISA kits occurred in all laboratories. The number of disparate results ranged from 1 (0.5%) (laboratory E) to 21 (10%) (laboratory C).

In order to estimate the sensitivity and specificity of the results obtained with the DAKOPATTS kit, the results obtained with the WHO kit are regarded as the standard for reference. Table 1 shows that false-negative results were obtained with the DAKOPATTS kit (WHO+ and DAKO−) for 10 of the 307 WHO positive specimens, indicating an overall sensitivity for the DAKOPATTS kit of 96.7%. One specimen from laboratory A proved positive when the DAKOPATTS test was repeated. Two specimens from laboratory D were shown to contain rotavirus particles when examined by electron microscopy. The presence of rotavirus particles in the remaining 11 specimens that were WHO+ and DAKO− could not be confirmed using electron microscopy. Most of these specimens gave weakly positive results, producing little colour when read by eye. In individual laboratories, the sensitivities of the DAKOPATTS kit were calculated as 100% (A, E, F), 98.5% (B), 92% (C), and 90.4% (D). Table 1 shows that the sensitivity of the DAKOPATTS kit recorded by the WHO Collaborating Centre in England was 100%.

Twenty-six (3%) of the total 845 WHO negative specimens gave WHO− and DAKO+ results (Table 1). The presence of rotavirus particles could not be confirmed in any of the apparently false-positive specimens from laboratories A, B, D and E using electron microscopy or RNA electrophoresis. One specimen examined by laboratory B may in fact have contained rotavirus particles since the patient had shed rotaviruses three days previously. The greatest disparity in results obtained between the two kits was observed in laboratory C, and cannot be definitely explained. However at the time, this laboratory was relatively inexperienced in performance of ELISA and discrepant results were probably due to false-positive reactions that occurred with both kits. The overall specificity of the DAKOPATTS kit, taking the WHO result as the reference standard was 96.9%, with individual specificities recorded as 100% (A), 99.5% (E) 99.1% (D), 98.9% (B), 98.7% (F), and 85% (C).

Most of the false positive and negative results

with the DAKOPATTS kit involved specimens giving weak reactions. Table 2 lists the optical density readings for 20 rotavirus positive specimens examined in parallel by the WHO and DAKOPATTS kits in the WHO Collaborating Centre in England. Results are classified as strong, moderate or weak positive readings. The low OD readings observed with some specimens using the WHO kit after the screening test emphasize the need to proceed to a confirmatory blocking test using this kit. With the DAKOPATTS kit there is a clear differentiation between reaction with the positive serum and with the negative serum even when OD readings are both low. Difficulty in distinguishing this difference by eye may explain the false-positive results obtained in some laboratories.

Table 2: **Comparison of optical density readings for 20 rotavirus-positive specimens using enzyme-linked immunoassay kits provided by WHO and DAKOPATTS**

Degree of positive	Optical density[a]		
		DAKOPATTS kit with:	
	WHO kit (screening test)	Positive serum	Negative serum
Strong	1.295	2.000	0.078
	1.308	1.766	0.028
	1.117	2.000	0.001
	1.018	2.000	0.139
	1.012	1.766	0.001
Moderate	0.973	1.508	0
	0.927	2.000	0
	0.907	0.687	0.023
	0.822	0.481	0.001
	0.806	0.875	0.009
	0.681	0.958	0.003
	0.640	1.358	0.001
	0.691	2.000	0.004
	0.556	1.481	0
Weak	0.512	0.293	0.008
	0.283	0.181	0
	0.215	0.563	0.002
	0.224	0.396	0.002
	0.173	0.867	0.012
	0.097	0.185	0.002

[a] All optical density results are the mean of duplicate readings.

Discussion

The ELISA assay adopted by WHO as the standard technique for diagnosis of rotavirus infection has been widely used in developing country laboratories. The format of the kit was carefully adapted to problems likely to be encountered during its use in these countries. The kit incorporated lyophilized ingredients to avoid their deterioration during transport, and included suitable diluents to avoid contamination of sensitive chemicals with impurities often present in water in developing countries. The performance of this kit was extensively monitored against electron microscopy and its sensitivity and specificity were established (*1*).

This comparison of results obtained when faecal specimens were assayed in parallel using the WHO and DAKOPATTS kits in seven different laboratories (Burma, Chile, India, Mexico, Pakistan, Sri Lanka and the United Kingdom) shows good agreement between both kits. The DAKOPATTS kit had an overall sensitivity of 96.7% and overall specificity of 96.9%, using the WHO kit results as the reference standard. This good correlation between the two kits was obtained both in the laboratory of origin of the WHO kit (United Kingdom) and after transport to the distant laboratories. The DAKOPATTS kit showed highly specific reactions when faecal specimens from newborn babies were examined. This is in contrast to experience with other kits where false-positive reactions are more common with assays of faeces obtained in the neonatal period (*5*).

Overall, there was very good agreement between results obtained with the two tests in six of the seven laboratories. Discrepancies in results were observed in one laboratory that had initial difficulty with both tests due to inexperience. This underlines the fact that expertise is an advantage in the performance and reading of ELISA assays, and that laboratory staff need preliminary tuition when commencing these assays.

The DAKO-ELISA was simple to perform, and the results were more rapidly obtained than with the WHO-ELISA. Visual reading was satisfactory in most cases, but weak positive reactions (equivalent to 0.50 OD) needed to be repeated. Occasional duplicate wells showed differences. The manufacturer recommends that the test be repeated if duplicate wells differ by more than 20%. In general, the reading by eye in both assays corresponded with the OD values on the spectrophotometer. The latter was of value in distinguishing positive and negative values when reactions were initially "weakly positive".

Modifications incorporated in the DAKOPATTS kit (K349) apparently overcame problems sometimes encountered with kits used in developing country laboratories. The modified kit contained lyophilized peroxidase conjugate, and positive and negative control antigens. Stability of the enzyme-substrate was ensured by providing tablets of 1,2-phenylene diamine, packaged with desiccant, and shown to be stable for at least 3 years at 4–8 °C (manufacturer's information). The kit also provided a vial of distilled water containing 15 mmol/l sodium azide in order to permit the peroxidase conjugate to

be reconstituted without the risk of trace impurities present in local water supplies interfering adversely with its activity.

The results of this comparative study indicate that the modified DAKOPATTS kit (K349) has high sensitivity and specificity relative to the WHO-ELISA kit. The format makes it particularly suitable for rotavirus diagnosis in etiological studies conducted by laboratories in developing countries.

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Résumé

Evaluation comparative des nécessaires d'épreuves immuno-enzymatiques OMS et DAKOPATTS pour la détection des rotavirus

Le titrage avec immuno-adsorbant lié à une enzyme (ELISA) est une technique sensible et spécifique de détection des antigènes de rotavirus dans les selles. L'OMS l'a adopté en 1980 et en a fait la technique diagnostique classique à utiliser dans les pays en développement pour les études épidémiologiques. Le Centre collaborateur OMS de Référence et de Recherche sur les Rotavirus a mis au point au Royaume-Uni un nécessaire ELISA (OMS-ELISA) conçu pour être acheminé sans danger vers les pays en développement. A l'époque, on trouvait peu de nécessaires de diagnostic dans le commerce. Depuis, la Firme DAKOPATTS a modifié son nécessaire DAKO-ELISA pour le rendre utilisable dans les laboratoires des pays en développement.

L'OMS-ELISA est un ELISA indirect double sandwich effectué en deux temps et sur quatre jours pour le dépistage, suivi d'une épreuve de confirmation par blocage sur les échantillons positifs. Ce nécessaire comprend des ingrédients lyophilisés pour éviter qu'ils ne se détériorent au cours du transport, et les diluants appropriés pour éviter toute contamination des produits chimiques sensibles par les impuretés souvent présentes dans l'eau des pays en développement. Les résultats obtenus avec ce nécessaire ont été largement comparés à ceux obtenus par la microscopie électronique et sa sensibilité et sa spécificité sont reconnues. Les résultats sont lus au moyen d'un spectrophotomètre.

Le DAKO-ELISA s'est montré extrêmement sensible et spécifique dans les pays industrialisés et a été modifié pour pouvoir être utilisé dans les pays en développement: on y a incorporé un conjugué lyophilisé et le diluant nécessaire à sa reconstitution. Le DAKO-ELISA s'effectue en cinq heures, en comparant les résultats obtenus en présence de sérum de lapin avant immunisation (ne contenant aucun anticorps antirotavirus) avec ceux ontenus en présence d'immunsérum antirotavirus hyperimmun. Les résultats se lisent à l'œil nu.

Ces deux nécessaires d'épreuve ont été évalués en parallèle sur un total de 1163 échantillons de selles recueillis dans des laboratoires situés en Birmanie, au Chili, en Inde, au Mexique, au Pakistan, à Sri Lanka et au Royaume-Uni. Ces échantillons provenaient de jeunes enfants admis à l'hôpital ou au dispensaire dans les 72 heures ayant suivi l'apparition d'une diarrhée aiguë. Les titrages ont été effectués en double pendant plusieurs jours, au moyen de l'OMS-ELISA et du DAKO-ELISA, en suivant les instructions accompagnant ces nécessaires.

La comparaison des résultats obtenus avec ces deux nécessaires a montré que le DAKO-ELISA a une sensibilité globale de 97% et une spécificité de 97% par rapport à l'OMS-ELISA. Dans les divers laboratoires, le DAKO-ELISA (K349) a montré une sensibilité allant de 90 à 100% et une spécificité allant de 85 à 100%. Il a montré une sensibilité de 100% et une spécificité de 98% dans les titrages effectués sur les selles de nouveau-nés. On en a conclu qu'en ce qui concerne la sensibilité et la spécificité ces deux nécessaires d'épreuve étaient à peu près équivalents. La possibilité d'obtenir des résultats exacts lisibles à l'œil nu fait du DAKO-ELISA une technique qui convient particulièrement bien aux laboratoires des pays en développement.

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