

Detection of false positives by incorporation of a confirmatory blocking test into a commercial enzyme-linked immunosorbent assay specific for adenovirus antigen

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Abstract

A blocking test was incorporated into the commercial IDEIA™ Adenovirus test (DAKO Diagnostics Ltd., Cambridgeshire, UK) to detect false positive results when faecal specimens were tested for adenovirus antigen. Immune rabbit serum raised against pooled adenovirus particles from human faecal specimens, together with the pre-immune serum, was used. Assessment of positive showed that false positives were produced under two different conditions: when results were based on visual determination instead of a cut-off value determined from photometric reading, and when absorbance values were not immediately read at the end of the test. Under the optimum condition for reading and assessment of test results (immediate reading and photometric determination), 11% of 65 adenovirus-positive samples were checked by the blocking ELISA as false positives. The rest of the specimens showed blocking of positive absorbance values by 70 to 98%. ELISA was found to be more sensitive than immune electron microscopy on samples with lower antigen concentration.

Key words: Confirmation test, ELISA, adenovirus detection.

INTRODUCTION

Adenoviruses is a cause of acute gastroenteritis in humans.¹ Its presence in clinical specimens can be detected by a range of techniques including enzyme-linked immunoassay (ELISA).^{3,4,5} ELISA has several advantages: simple to perform, short assay time for present generation of tests, amenable to automation, objective assessment of results based on photometric determination, high sensitivity. Although high sensitivity is of paramount importance in a detection test, enhancing sensitivity will also increase the probability of false positives caused by non-specific factors in the absence of the specific antigen.⁶ Therefore, there is the need for a confirmation test to differentiate true from false positives. Currently, a commercial ELISA, IDEIA™ Adenovirus test (DAKO Diagnostics Ltd., Cambridgeshire, UK), is available for detecting adenovirus antigens in faecal and cell culture specimens. However, in common with almost all commercial ELISA there is no confirmation test. The objective of this study was to evaluate the performance of the IDEIA™ Adenovirus test by incorporating a confirmatory ELISA blocking test to check all positive-reacting specimens.

MATERIALS AND METHODS

Faecal samples

Faecal samples were from diarrhoeic children admitted to the Kuala Lumpur Pediatrics Hospital. The samples were stored at –20 to –70°C until ready for testing.

Production of rabbit anti-adenovirus hyperimmune serum

Adenovirus-positive faecal samples were pooled in tris buffer (0.1M, pH 8.7). The suspension was clarified by centrifugation at 10 000g for 10 minutes and the clarified supernatant centrifuged at 50 000g for 2 hours at 10°C. The pellets were resuspended in tris buffer and presence of adenovirus particles confirmed by electron microscopy. The virus suspension was purified by cesium chloride gradient centrifugation as previously reported.⁷ The collected fractions were checked for adenovirus particles by electron microscopy and then were pooled. A New Zealand White rabbit previously bled for preimmunized serum was injected intramuscularly and subcutaneously with 1.0 ml of the virus suspension mixed with an equal volume of Freund's complete adjuvant in the

first injection and Freund's incomplete adjuvant in the second injection a month later. The animal was bled 2 weeks after the second immunization and the serum separated and stored at -20°C .

IDEIA™ Adenovirus test procedure

The IDEIA™ Adenovirus test (Dako Diagnostics Ltd., Cambridgeshire, UK) which is a qualitative ELISA for the detection of adenovirus in human faeces or infected cell culture was used. The assay was done according to the manufacturer's procedure except faecal suspensions were clarified by centrifugation at 8,000g for 2 minutes instead of settling on the bench for 10 minutes prior to testing.

Confirmatory ELISA blocking test procedure

Adenovirus-positive samples were suspended in tris buffer and clarified by centrifugation at 8,000 g for 2 minutes. The supernatants then were centrifuged at 50,000 g for 2 hours. Each pellet then was resuspended in 2 drops of tris buffer each and stored at -20°C until ready for testing.

Two 100 μl volumes of test sample were each mixed with an equal volume of either pre-immune or post-immune serum diluted 1/32 in the commercial sample buffer. The mixtures were incubated in a 37°C waterbath for 60 minutes. Each then was tested in duplicate following the IDEIA™ Adenovirus test procedure. A sample was considered positive for adenovirus antigen if the absorbance in wells incubated with pre-immune serum was greater than the calculated cut-off value while the absorbance in wells incubated with post-immune serum was either less than the cut-off value or if greater was less than 50% of the absorbance reading in the pre-immune well. Conversely, a sample was considered negative for adenovirus if absorbance in respective wells incubated with pre- and post-immune sera were less than the cut-off value.

Samples were diluted 1:9 in the commercial sample diluent before testing and negative samples were retested at 1:3 and 1:1 dilutions. On the other hand, samples in wells incubated with pre- and post-immune sera with absorbance greater than the cutoff value but with the post-immune wells having more than 50% of absorbance reading than in pre-immune wells were further diluted and the blocking test repeated. Positive and negative samples were always included in the performance of the blocking test.

Electron microscopy and immune electron microscopy

In electron microscopy, a formvar-coated grid was inverted over a drop of sample on a grease paper for 1 min before excess fluid was removed by touching the edge of the grid with a piece of filter paper. In immune electron microscopy, 25 μl of 1:19 diluted rabbit anti-adenovirus serum (previously determined to clump adenovirus particles without causing their distortion) was dropped on a piece of grease paper placed in a Petri dish lined with a wet piece of filter paper. A formvar-coated grid was inverted over it and the closed dish incubated at 37°C . After 1 h excess fluid on the grid was removed. The antibody-coated grid then was inverted over a 15 μl drop of sample and incubated at 4°C for 1 h before excess fluid was removed. The grid was inverted over a drop of phosphate-buffered saline for 30s and excess fluid removed. Staining in both electron microscopy and immune electron microscopy was accomplished by inverting the grid over a drop of phosphotungstic acid (3% solution, pH 6.5) for 1 min. Excess fluid was removed and the grid viewed at 60,000 magnification. Five squares of each grid were examined: 1 from the center and 1 each from the 4 different sectors.

RESULTS

Assessment of anti-adenovirus antibody preparation used in the blocking test

Table 1 shows that blocking of the adenovirus-positive sample by the rabbit anti-adenovirus serum was detected beginning at 1/8 dilution. Blocking reached the 90% level at 1/32 dilution and remained so until the 1/512 dilution before declining with subsequent dilutions. The anti-adenovirus antiserum did not block the ELISA specific for rotavirus.

Assessment of adenovirus-positive samples using the ELISA blocking test

A total of 69 faecal samples were detected as adenovirus-positive by the commercial ELISA: 3 were positive based on visual observation of a colour change but negative by photometric determination (4.3% of total positive cases); 1 developed a positive photometric reading 20 minutes after the test ended (1.4% of total positive cases); 65 were positive based on photometric reading taken immediately after the test ended.

All 4 samples from the first 2 adenovirus-positive categories gave negative results

TABLE 1: Reaction of pre-immune and anti-adenovirus sera on adenovirus- and rotavirus-specific ELISA

Serum dilution (reciprocal)	Adenovirus-specific ELISA			Rotavirus-specific ELISA		
	serum: pre-immune	anti-adenovirus	% change ^a	pre-immune	anti-adenovirus	% change
4	0.155 ^b	0.158	+2	ND	ND	
8	0.201	0.058	-70	ND	ND	
16	0.266	0.059	-78	ND	ND	
32	0.259	0.015	-94	0.613	0.658	+7
64	0.316	0.027	-91	0.635	0.660	+4
128	0.323	0.013	-96	0.632	0.597	-5
256	0.322	0.026	-92	0.622	0.616	-1
512	0.317	0.023	-93	0.616	0.584	-5
1024	0.274	0.058	-79	0.576	0.606	+5
2048	0.253	0.072	-71	ND	ND	
4096	0.221	0.105	-52	ND	ND	

ND = not done

The adenovirus and rotavirus antigen preparations used were the positive controls from the respective kits

^aA '-' sign before a figure denotes the % decrease in absorbance value of the sample reacted with post-immune serum when compared to the same sample reacted with pre-immune serum. A '+' sign denotes an increase.

^bAbsorbance value

(absorbance values were below calculated cut-off values) with the pre-immune and post-immune sera. Of the other 65 samples from the third category of positives, 7 with absorbance value of between 0.169 to ≥ 2.9 (2 samples, 1.3- to 1.4-fold > cut-off value; 5, 3.8- to -8.9-fold; 1, ≥ 21 -fold) were also retested negative. The remaining 58 gave positive readings with the preimmunized serum. All were blocked by the anti-adenovirus immune serum; 84% of the samples have their absorbance values reduced by 90 to 98%; 9%, 80-89% and 8%, 70-79%.

Comparison of the sensitivity of immune electron microscopy (IEM) with the IDEIA™ Adenovirus test incorporating a blocking test

The results in Table 2 show that all confirmed adenovirus-positive samples with high absorbance values from the IDEIA™ Adenovirus test were detected by IEM. However, the proportion that was detected by IEM declined with decreasing absorbance values of the samples.

All the 7 samples that were positive for adenovirus when first tested by ELISA but negative on retesting with the ELISA blocking test were also negative for adenovirus by IEM. However, 1 sample had virus particles of around

70 nm diameter with surface morphology suggestive of rotavirus.

DISCUSSION

A blocking test was readily incorporated into the commercial IDEIA™ Adenovirus test by preincubation of the samples with pre- and post-immune sera. The only perturbation of the normal procedure was the presence of diluted preimmune and post-immune rabbit sera during the incubation period.

Although the manufacturer states that test results may be assessed visually, this is best avoided as samples with definite but slight colour (in the presence of colourless negative controls) may in fact be negative. Assessment of test results by photometric determination based on a calculated cut-off value overcomes false positives of this nature. The statement that the coloured product of the test is stable for up to 30 minutes after stopping the enzyme reaction may result in no urgency in reading results immediately after a test. However, the observation that a colourless, hence negative, sample developed a colour after standing for 20 minutes suggests results are best read immediately at the end of the test.

Although the manufacturer reported a

TABLE 2: Comparison of immune electron microscopy with IDEIA™ Adenovirus incorporating a blocking test for the detection of adenovirus

Samples	Proportion (%) detected by IEM
adenovirus-positive by ELISA blocking test	
absorbance values: 22.361	10/10 (100)
absorbance values: 0.464 – 2.093 (average, 1.217)	7/10 (70)
absorbance values: 0.149 – 0.268 (average, 0.212)	5/9 (56)
adenovirus-negative by ELISA blocking test	0/7 ^a

^aVirus particles with morphology suggestive of rotavirus seen in 1 sample. The sample was confirmed to be rotavirus-positive by the presence of the typical RNA migration pattern of group A rotavirus.

specificity of 99% when compared with EM,⁸ the results of the ELISA blocking test revealed 11% of positive samples tested under the best condition (photometric determination on tests read immediately) were false positives. Thus the use of the test on its own will result in overreporting of positive cases. This underscores the importance of a **confirmation** test in ELISA.

It is interesting to note that all the 7 false-positives were detected in the ELISA blocking test because they produced absorbance values less than the calculated cut-off values with pre- and post-immune sera (and hence were considered negatives) and not because they had absorbance values assessed as positive that were not blocked. It is unlikely that the negative results obtained on checking were because the samples were low positives and the process of partial purification caused the loss of antigen to a level where they could not be detected. Most of the samples had high concentrations of antigen as reflected in absorbance values 2 to 9 times above cut-off values. It is more likely that the use of cleaner samples helped eliminate these false positives. The presence of rheumatoid factor-like substances in faecal samples reported to produce false positive colour change in ELISA⁶ would have been eliminated in these partially purified samples. Nevertheless, the procedure of ultracentrifugation of samples (previously clarified to remove large particles) to separate virus particles from soluble faecal components is not practical on a routine basis. The best alternative is the incorporation of a confirmatory ELISA blocking test. In this situation, the unmodified IDEIA™ Adenovirus test is considered as the screening test and the blocking test is the confirmation test for samples positive on screening. However, an important factor that needs to be considered is cost as each sample will require 5 antibody-coated wells for

the 2 levels of testing.

Detection of adenovirus by electron microscopy (EM) is definitive as the virus is readily recognizable by its distinctive shape and size. When the sensitivity of IEM was compared to ELISA incorporating a blocking test, it was clear the ELISA test was more sensitive when less virus particles were present. A reason for this could be the ability of ELISA to also detect soluble adenovirus antigens from disintegrated particles.

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