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RS VIRUS DIAGNOSIS: COMPARISON OF ISOLATION, IMMUNOFLUORESCENCE AND ENZYME IMMUNOASSAY

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Two techniques for rapid diagnosis, immunofluorescence (IFAT) and enzyme immunoassay (EIA), have been compared with virus isolation in tissue culture for the detection of respiratory syncytial virus (RSV) in specimens of nasopharyngeal secretions. The specimens were obtained from children under five years of age suffering from acute respiratory illness, during a period of six months from January to June 1982.

Of 471 specimens examined 54 (11.5%) were positive by virus isolation and 180 (38.2%) were positive by immunofluorescence. The bacterial contamination of inoculated tissue cultures unfortunately prevented the isolation of virus from many samples.

Specimens from 216 children were tested to compare enzyme immunoassay and immunofluorescence. Of these 60 (27%) were positive by EIA and 121 (56%) were positive by IFAT. Our results suggest that the EIA technique although highly specific is rather insensitive. This may be because by the time these tests were done the original nasopharyngeal secretions were considerably diluted and contained more mucus fragments than the cell suspension used for IFAT.

Of the three techniques, IFAT gives the best results although EIA may be useful where IFAT is not possible.

Key words: respiratory syncytial virus – rapid diagnosis – immunofluorescence – enzyme immunoassay

Although virus isolation is considered the most sensitive method for the demonstration of infection with respiratory syncytial virus (RSV) this technique involves a delay of several days between inoculation of tissue cultures and evidence of virus replication.

The detection and identification of viral antigens directly in clinical specimens by the fluorescent antibody technique has been found to be a rapid and accurate diagnostic procedure (Gardner & McQuillin, 1980) which has been used successfuly on nasopharyngeal secretions (NPS) to confirm infection with influenza, parainfluenza as well as with respiratory syncytial virus (WHO, 1981).

More recently the use of enzyme immunoassays has been explored for the rapid diagnosis of RSV infections (Chao et al., 1979; Hornsleth et al., 1981; Sarkkinen et al., 1981; McIntosh et al., 1982).

The investigations reported here were designed to examine how well these various tests behaved under day-to-day conditions in a diagnostic virus laboratory in a developing country like Brazil.

MATERIAL AND METHODS

Specimens examined – We examined 471 nasopharyngeal secretions (NPS) collected from children under five years of age suffering an acute respiratory illness, during the period from January to July of 1982 when RSV activity is usually high in Rio de Janeiro (Sutmoller & Nascimento, 1983). Samples were obtained in the first seven days of illness by suction through a nasal catheter according to Gardner & McQuillin (1980). NPS were sent immediatly at 4°C to the laboratory and processed as soon as possible for isolation in tissue culture (TCI) and rapid diagnosis by immunofluorescence (IFAT).

We had sufficient amount of material from 216 NPS to store part of the whole specimen or cell and mucus fraction at -70°C for use in enzyme immunoassays (EIA).

Isolation in tissue culture – NPS were cultured for the presence of RSV in fresh monolayers of Hep-2 cells. Primary Rhesus monkey kidney cells, human embryonic lung and kidney

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cells were used when available. The inoculated monolayers were incubated at 33°C and observed for cytophatic effects (CPE) over a period of 21 days. RSV was identified by immunofluorescence on the cell culture. Where evidence of RSV infection was obtained by IFAT directly from NPS, the tissue cultures inoculated with these samples, although not demonstrating CPE or contaminated, were similary examined by immunofluorescence.

Detection of RSV antigens on NPS by IFAT – Fresh, nonfrozen NPS specimens were examined by IFAT according to the method described by Gardner & McQuillin (1980). A bovine anti-RSV hyperimmune serum and a fluorescein-conjugated rabbit anti-bovine serum (Wellcome) were used. Slides were examined by two investigators using a Zeiss epifluorescence microscope with an halogen lamp as light source and a 40X dry objective.

Detection of RSV antigens on NPS by EIA – EIA were performed by double sandwich technique (Voller, Bartlett & Bidwell, 1978; Yolken et al., 1978) based on the experience of several groups working in antigen detection on NPS by EIA (Chao et al., 1979; Hornsleth et al., 1981; Sarkkinen et al., 1981; McIntosh et al., 1982).

Capture serum – Horse anti-RSV neutralizing serum (Flow Laboratories lot ≠ H83008) was kindly supplied by Dr. K. McIntosh and used at a dilution of 1/10.000.

Detector serum – Rabbit anti-RSV neutralizing serum (Central Public Health Laboratory – lot 1/69) was kindly supplied by Dr. M.S. Pereira and used at a dilution of 1/500.

Swine antibody against rabbit IgG conjugated with horseradish peroxidase (Dako Immunoglobulines A/S lot 120) was used at a dilution of 1/2000. Substrate was ortho-phenilenediamine (Sigma).

Treatment of the samples – RSV infected Hep-2 cells, non-infected Hep-2 cells, RSV positive and negative NPS were treated by 20% (w/v) N-acetyl-cysteine using N-acetyl-cysteine from Sigma. Samples and controls were treated by sonication using a sonicator Branson (Brasonic 12) as an alternative treatment. Final dilutions of NPS were 1:5 for N-acetyl-cysteine treatment and 1:2 for sonication.

Polystyrene flat-bottomed microtiter plates (Nunc, Denmarke) were used as the solid phase. In alternate rows of the microplates, carbonate/bicarbonate buffer 0.25 M ph 9.6 containing the standard dilution of the capture serum or not were added in 100 µl volume. After incubation at 4°C overnight, plates were washed three times, with PBS containing 0.05% (v/v) Tween 20 (PBS/T) and drained.

Previously treated NPS diluted 1/4 and 1/12 in PBS/T were added in 100 µl volumes to two pairs of wells: one pair coated with capture serum and the other uncoated. After overnight incubation at 4°C the plates were washed three times with PBS/T and to each well was added 100 µl of the detector serum diluted in PBS/T with 2% (w/v) bovine serum albumin (PBS/T/BSA). After incubation for two hours at 37°C, the plates were washed three times with PBS/T and 100 µl of conjugate diluted in PBS/T/BSA was added per well. The plates were again incubated for two hours at 37°C, washed three times with PBS/T and to each well was added 200 µl of a freshly prepared solution containing 400 µg/ml ortho-phenylenediamine, 1.5 µl/ml hidrogen peroxide (30%) in 0.1 M citrate-phosphate buffer pH 5.6. The plates were left at room temperature in the dark until the appearance of colour in the wells containing the antigen controls. The reaction was stopped by adding one drop (about 25 µl) of 2M H_2SO_4 per well. The test was read either visually or by measurement of optical density (O.D.) at 492nm in a Titertek Multiskan reader.

RESULTS

Using both IFAT and TCI, RSV was detected in 39.3% (185) of the 471 NPS studied in this paper. Bacterial contamination of specimens (43.9%) unfortunately prevented the virus isolation from many IFAT positive samples (Table I) and this contamination rate was twice as high in the specimens collected from inpatients as in those from outpatients. The typical RSV CPE was noted in 54 NPS (11.4%) although RSV antigens could be detected by immunofluorescence in 25 cell cultures in which CPE was absent or was bacterially contaminated. Despite the TCI results, RSV antigens were detected in 180 specimens (38.2%) by IFAT. 44% of the positive secretions by IFAT collected from out-patients yielded virus by isolation but among the NPS collected from inpatients the yield fell to 24.6% largely because of the bacterial contamination.

Analysing the results of inoculated cell cultures that were not contaminated we found only 49% positive of those found positive by other test. However using immunofluorescence on cell cultures where CPE was absent a further 11 were found positive bringing the total positive

TABLE I

RSV diagnosis by TCI and IFAT on NPS collected between January to July 1982 from children living in Rio de Janeiro

Class of Patients	Number of Specimens	TCI prevented by bacterial contamination	Number of Positive		TCI/IFAT %
			TCI	IFAT	
a) Outpatients	171	47 (27,4)	22 (12,8)	50 (29,2)	44
b) Inpatients	300	160 (53,3)	32 (10,6)	130 (43,3)	24,6
Total	471	207 (43,9)	54 (11,4)	180 (38,2)	30

a) most of them with upper respiratory tract infection, probably not using antibiotics.
b) most of them with lower respiratory tract infection and treated with antibiotics.

TABLE II

Comparison of TCI and IFAT in 264 specimens not contamined in tissue culture

IFAT	TCI		TCI + Immunofluorescence on negative cell cultures		Total
	Positive	Negative	Positive	Negative	
Positive	51	53	62	42	104
Negative	3	157	5	155	160
Total	54	210	67	197	264

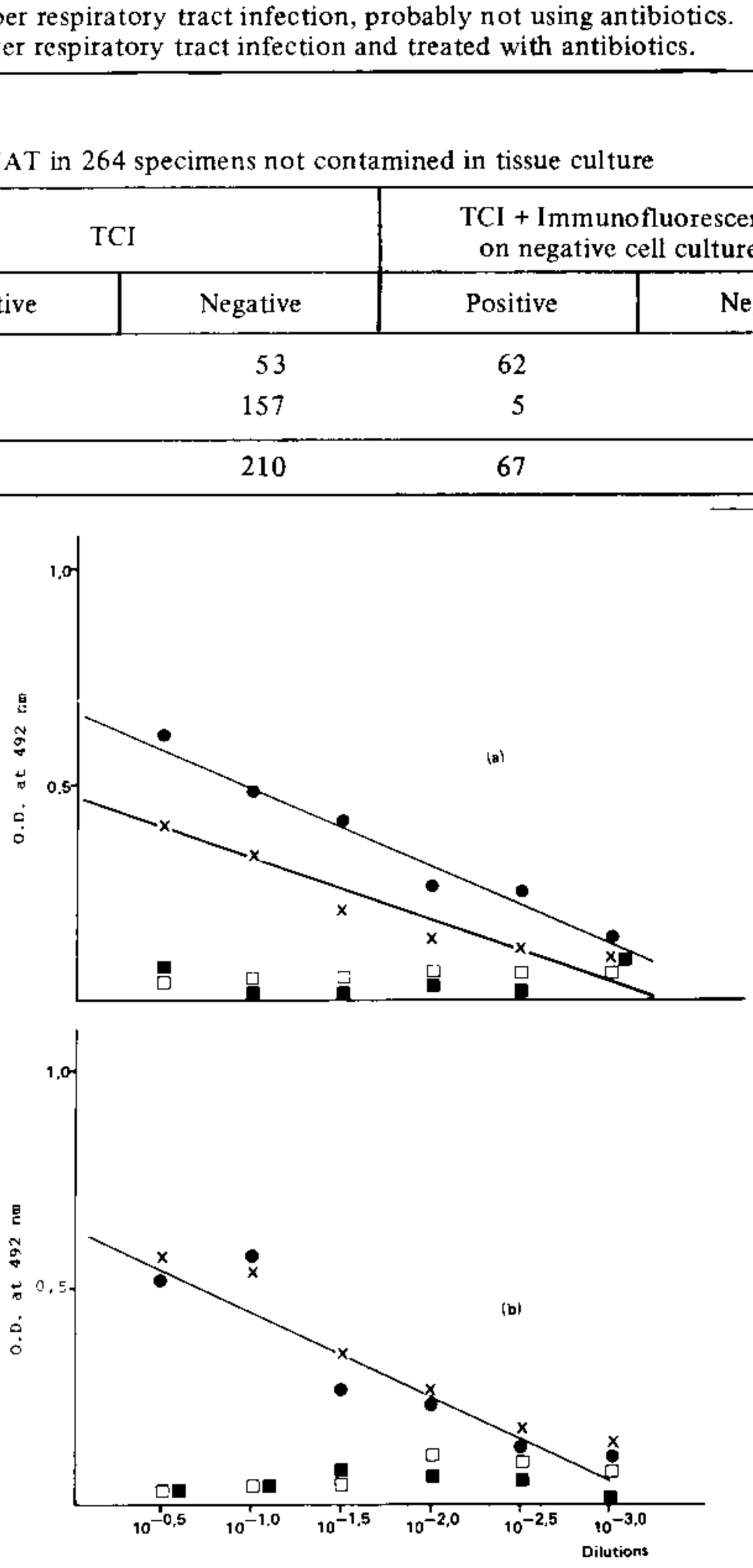


Fig. 1: OD values obtained when dilutions of one NPS-RSV positive (a) and RSV infected Hep-2 cell cultures (b) were tested after two different treatments: sonication (●) and N-acetyl-cysteine (□), and compared with no treatment (X) and negative control [NPS-RSV negative and Hep-2 cell culture (■)].

to 60% (Table II). From one specimen positive for RSV by IFAT only an enterovirus (Echovirus type 19) was isolated. This may have prevented the isolation of RSV. Only five isolates were obtained from specimens that were negative by IFAT.

Fig. 1 shows different treatments applied to NPS or RSV infected Hep-2 cells for EIA tests. 20% N-acetyl-cysteine decreased the absorbance values of the specimens as compared with the untreated controls. Sonication of the specimens was found better for solubilizing the secretions but no difference was found when RSV infected Hep-2 cells were sonicated. We found that N-acetyl-cysteine decreased the absorbance of the RSV infected Hep-2 cells.

Twenty samples collected from children showing ARI during the period when RSV activity is low in Rio de Janeiro (springtime and summer) were used to calculate the cut off value between positive and negative specimens. These specimens were negative for RSV by TCI and by IFAT. Fig. 2 shows the difference in OD values between the EIA tests with and without capture serum of each one of these RSV negative samples, with a mean value ($\bar{x}$) of 0.12 and a standard deviation (S) of 0.06.

When performing the EIA on the 216 suspected samples, these were considered positive when the OD value obtained by the difference between wells with and without capture serum was more than 0.24 [($\bar{x}$ + 2S)] (Fig. 3).

All of 13 NPS negative for RSV but positive for other viruses by IFAT and TCI, were also RSV negative on EIA. Each of 92 NPS representing IFAT negative specimens was negative in EIA except four which had an OD value above the cut off line. Of the 124 RSV positive samples by IFAT and/or TCI 45.5% were also positive on EIA, leaving 54.5% as false negatives. Table III shows a comparison of IFAT and EIA for detection of RSV antigens in NPS. The EIA test presented 53.7% false negatives. Four RSV negative secretions by IFAT and TCI were considered positive by EIA.

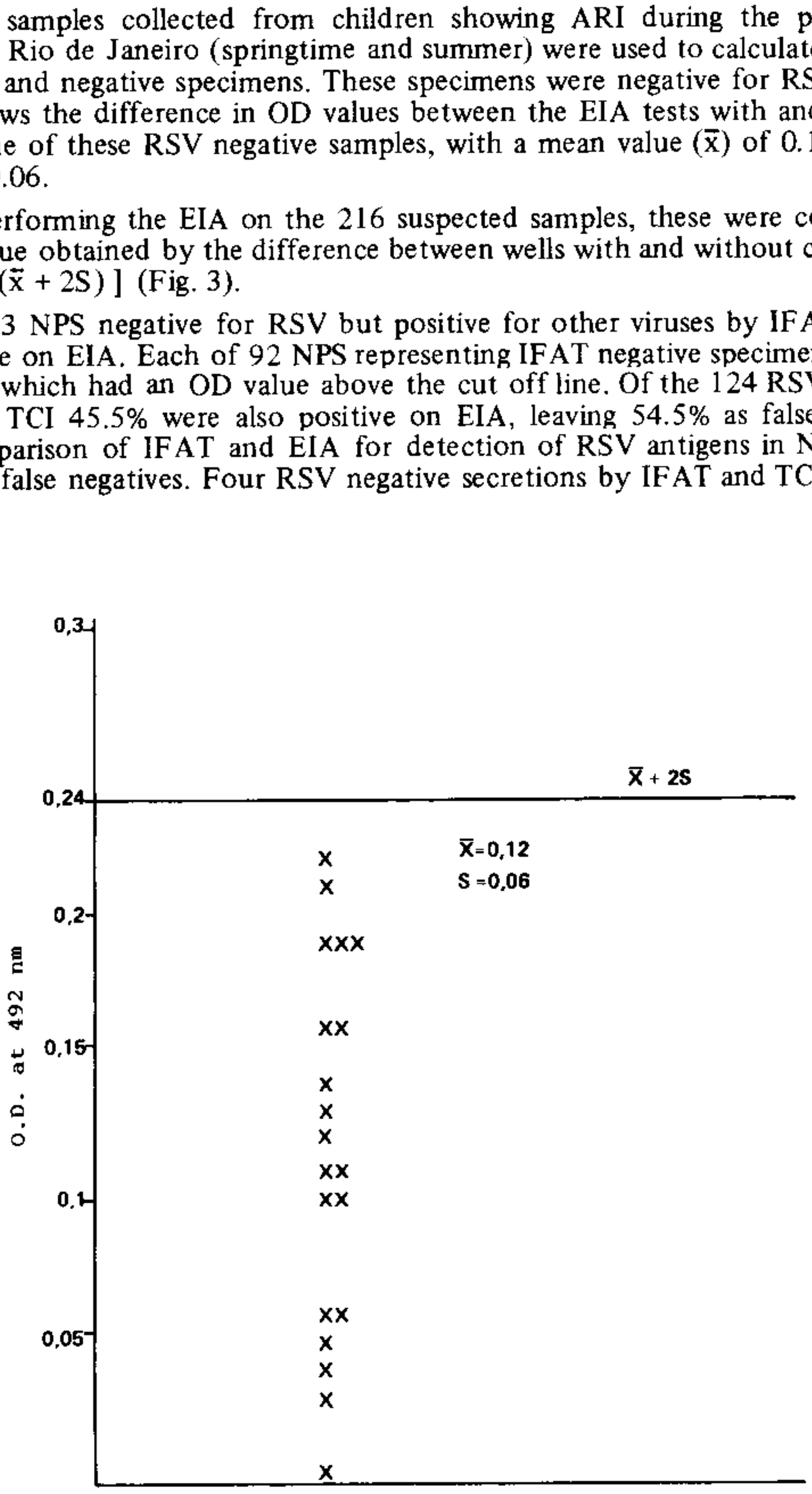
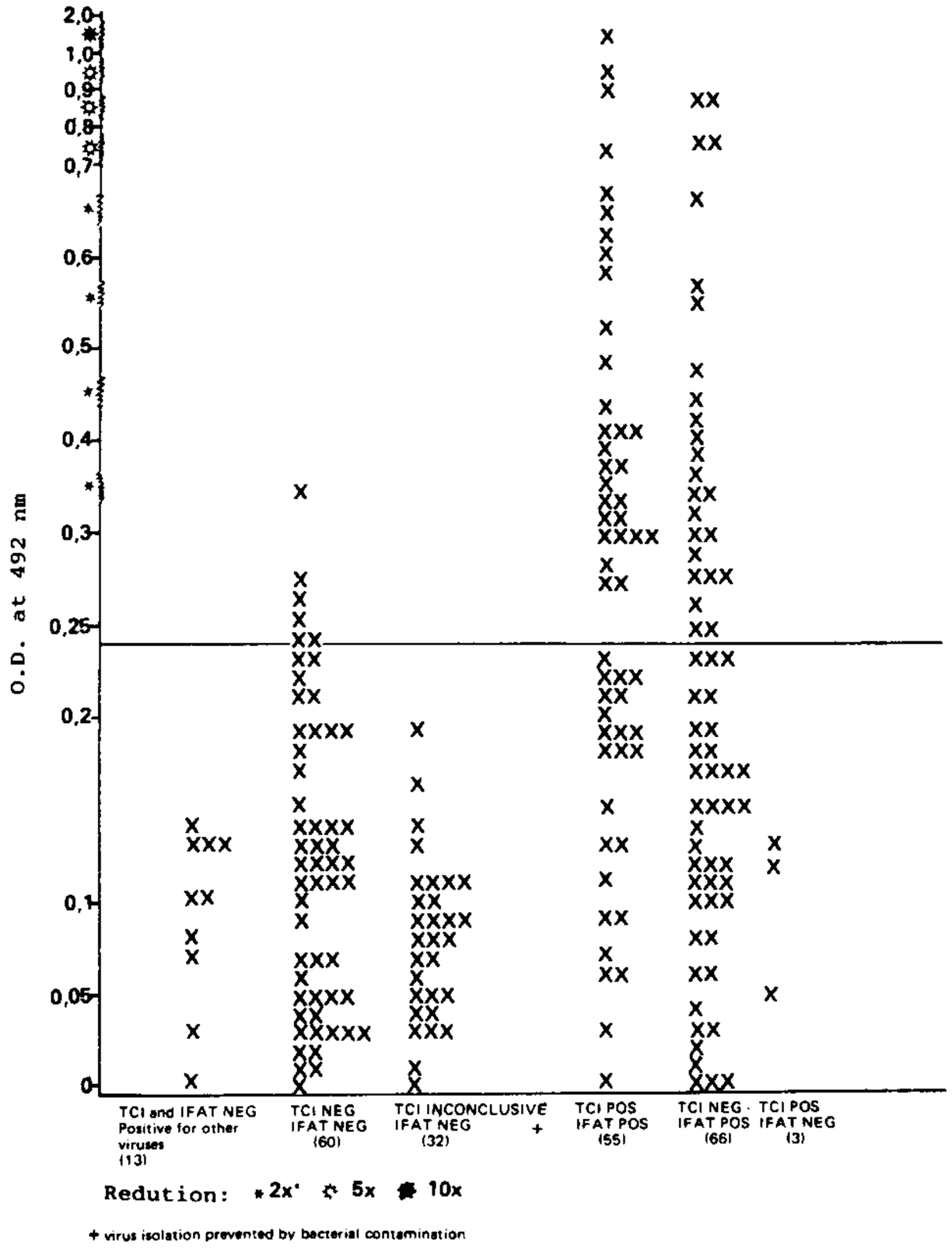


Fig. 2: The cut off value obtained with twenty samples collected from children showing ARI during the period when RSV activity is low in Rio de Janeiro. The difference in OD values between the EIA tests with and without capture serum of each one of these RSV negative samples give a mean value ($\bar{x}$) of 0,12 and a standard deviation (s) of 0,06.



+ virus isolation prevented by bacterial contamination

Fig. 3: EIA results expressed as OD values of NPS obtained from children with acute respiratory diseases. The cut off value between positive and negative samples is indicated. The samples found positive and/or negative by IFAT and/or TCI techniques are also shown. The number of children in the groups is given in parentheses.

TABLE III

Sensitivity comparison of IFAT and EIA for detection of RSV antigens in NPS

IFAT	EIA		Total
	Positive	Negative	
Positive	56	65	121
Negative	4	91	95
Total	60	156	216

DISCUSSION

Techniques for the rapid diagnosis of viral infections have created much interest, including the attention of WHO (1981).

This study has compared two rapid diagnostic methods with standard isolation for the detection of RS virus in nasopharyngeal aspirates from children with acute respiratory diseases.

In many laboratories, virus recovery in tissue culture has been the method of choice for RSV diagnosis. However, tissue culture techniques are time consuming, requiring three to ten days for the isolation of virus and additional time for specific identification of the virus. We observed the typical syncytial formation in Hep-2 cultures in 70% of the samples after the sixth day of inoculation.

Knowing the habit our population has of self medication and the excess of antibiotics used, even under medical orientation, the bacteriological contamination was not unexpected. The high incidence of tissue culture contamination, preventing virus isolation biases our results in favour of direct methods for diagnosis.

Of our 471 unfrozen specimens 180 (38.2%) were positive by IFAT and not all by cell culture, but the fluorescent staining pattern was characteristic. The highly specific immunofluorescent technique has proved to be a test more sensitive than virus isolation in our laboratory. However, many works have shown that the agreement between virus isolation and IFAT is more than 90% for RSV (Kaul et al., 1978; Gardner, 1978). It is likely that the explanation for our discrepant results may be due to neutralization of the virus by a patient's developing antibody, by antigen deterioration (Gardner, McQuillin & McGuckin, 1970) or by differences in the sensitivity of Hep-2 cells employed. However the major cause is probably the bacterial contamination that prevented the isolation of virus from many of the samples.

Actually in the present study IFAT was often applied for the identification of RS virus antigen in tissue culture cells inoculated with clinical specimens where contamination or an uncharacteristic CPE prevented a positive result. Using this technique 25/113 cell cultures were considered positive by isolation.

The EIA test has previously been shown to be a sensitive, accurate and simple assay for the detection of different viruses in body fluids (Yolken, 1982).

The use of nasopharyngeal secretions as specimens for EIA requires the disruption of the mucus present in the secretion. It is necessary to solubilize the mucus and to homogenize the specimens. Two treatments were tried: sonication and N-acetyl-cysteine. Chao et al. (1979) and McIntosh et al. (1982) obtained good results with N-acetyl-cysteine. However Sarkkinen et al. (1981) disagreed with this and found like Hornsleth et al. (1981) and Anderson et al. (1983) that sonication gave the best results.

The specificity of the test was shown by the fact that 13 samples found positive for other respiratory viruses showed no cross reactivities with RSV EIA. We found a large number of "false negatives" with this test. Chao et al. (1979) found only 79% of specimens positive by TCI were positive by EIA. Hornsleth et al. (1981) detected RS virus by inhibition of EIA in 61% of 41 specimens found positive by TCI and in 65% by IFAT. McIntosh et al. (1982) found the sensitivity and specificity in relation to culture to be 82% and 95% respectively for EIA and to be 86% and 96% respectively for IFAT. It is however not possible to compare such results in any meaningful way since there is no uniformity in either the tests or the selection of cases.

The ability of our EIA to detect RS virus antigen in clinical respiratory specimens was not as good as previous reports. The difference between our results and the better results from other groups may reflect differences in the number, type, quality and possibly preparation of clinical specimens. Some of our samples were probably too diluted. The dilution of the specimen, the mucus fractions and the cells present in the original NPS may influence the outcome of EIA. Other workers may have had only small amounts of free antigen present because of the age of the patient or the timing of the sample. Yet, differences in estimation of OD values obtained, might explain the lower sensitivity of our test as compared with others.

In our hands the EIA test was only moderately sensitive. At present we feel the EIA test, as we have used it is not sufficiently sensitive nor rapid (we used an overnight incubation) for routine diagnosis.

EIA has great potential but further studies are needed (PAHO, 1983). Experiments to improve both the sensitivity and the specificity of RSV EIA should examine improved solid-phase supports and more sensitive enzyme substrate systems. The use of monoclonal or affinity-purified antisera should be a further means of improving sensitivity and specificity.

The use of NPS in addition to cough swabs for the isolation of viruses, and RS virus in particular, and the use of IFAT, resulted in a considerable increase in our knowledge of the viral causes in acute respiratory diseases (Sutmoller & Nascimento, 1983) as compared with our previous experience (Sutmoller et al., 1983), and the experience of others authors in Brazil in the past (Candeias & Himelfarb, 1966; Candeias, 1967; Takimoto et al., 1982; Goes, Machado & Couceiro, 1983).

The EIA gives a good specificity, but the clinical sensitivity reported here. 53.7% is lower than we would have wished, but still enough to be used as a rapid test where IFAT is not available. We must consider that for these 216 specimens, EIA show better overall results than TCI.

Another advantage of EIA is that viral antigens may be detected at a central laboratory using specimens collected in localities remote from a virus laboratory, a real advantage in large countries like Brazil which have very few adequately equipped laboratories.

The rapid and accurate laboratory diagnosis of infectious diseases is essential both for the immediate care of the patient and for the introduction of necessary public health control measures.

RESUMO

Duas técnicas para diagnóstico rápido, imunofluorescência (IF) e ensaio imunoenzimático (EIE), foram comparadas com isolamento viral em cultura de tecidos para a detecção do vírus respiratório sincicial (VRS) em secreções de nasofaringe. Os espécimens foram obtidos de crianças abaixo de cinco anos de idade, com doença respiratória aguda, durante um período de seis meses, de janeiro a junho de 1982.

Dos 471 espécimens examinados, 54 (11,5%) foram positivos por isolamento viral e 180 (38,2%) foram positivos por imunofluorescência. A contaminação bacteriana das culturas de tecidos inoculadas prejudicou o isolamento viral em muitas amostras.

Espécimens de 216 crianças foram testados para comparar EIE e IF. Destes, 60 (27,0%) foram positivos pelo ensaio imunoenzimático e 121 (56,0%) foram positivos por imunofluorescência. Nossos resultados sugerem que o ensaio imunoenzimático embora altamente específico é pouco sensível. Isto pode ter ocorrido pois quando estes testes foram feitos as secreções de nasofaringe originais estavam consideravelmente diluídas e continham mais fragmentos de muco do que as suspensões celulares usadas para imunofluorescência.

Das três técnicas a imunofluorescência forneceu os melhores resultados, embora o ensaio imunoenzimático possa ser útil onde a imunofluorescência não for realizável.

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