Comparison of immunofluorescence and immunoperoxidase methods for viral diagnosis at a distance: a WHO collaborative study

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Nasopharyngeal secretions collected in Newcastle were examined in both Newcastle and Stockholm for the presence of influenza virus type A and respiratory syncytial (RS) virus by immunofluorescence and immunoperoxidase techniques. A total of 139 specimens were examined in this way and the agreement between the two centres for immunofluorescence was 94% for influenza virus A, 95% for respiratory syncytial virus, and 95% for negative specimens. This technique can therefore be used for examining specimens taken at great distances from the collecting laboratory. The results of the immunoperoxidase technique were unsatisfactory mainly because of the presence of endogenous peroxidase. Measures taken to remove this also destroyed RS virus antigen. It is premature to introduce the immunoperoxidase technique for rapid virus diagnosis, but in due course, when the problems outlined in this article are solved, it could become a useful technique.

Most developed countries have encouraged the development of diagnostic virological services, and many virologists in these countries now appreciate the need for a rapid service of this kind. Unfortunately, the situation in developing countries, because of the many pressing medical priorities, is far less satisfactory. However, the methods used for rapid virological diagnosis lend themselves to examination of specimens at a distance from the central laboratory (*1*). If reliable methods could be introduced, centres in developing countries could not only examine specimens from their own area, but could also collaborate with more distant centres.

Theoretically, at present, there are two potential methods, immunofluorescence (*2*) and immunoperoxidase (*3*) techniques, and both have advantages and disadvantages. The former has been tried and proven but its main drawback is the need for a fluorescence microscope, which might be a limiting factor in some developing countries. The latter has the advantage that it needs only a light microscope and that, once stained, the preparations are permanent; it is, however, relatively untried on clinical material and the interfering effect of endogenous peroxidase is largely unknown. Both methods are entirely dependent on highly purified reagents.

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With this background, the World Health Organization invited the Department of Virology, Newcastle-upon-Tyne, England, and the Department of Virology, National Bacteriological Laboratory, Stockholm, to evaluate these two methods by interchange of specimens, examining them in parallel under code, and finally coordinating the results.

Among important questions to answer were:

(a) Would it be possible to examine specimens at centres far apart and derive comparable results?

(b) Could the antisera in use and proved satisfactory with the immunofluorescence technique be employed for the immunoperoxidase method?

(c) Do other reagents have any nonspecific activity?

(d) Are the results of the examination of clinical material by the two methods comparable?

(e) Is endogenous peroxidase present in clinical material and, if so, can it be removed without damaging the viral antigen?

MATERIALS AND METHODS

Secretions were taken from all children admitted to Tyneside hospitals, and a series of slide preparations were made and fixed in acetone as described fully elsewhere (2). A minimum of 6 two-square slide preparations were needed for each patient, and in many cases three-square slide preparations were also made. Two fixed slide preparations from each patient were dispatched to Stockholm and 2 were examined in parallel in Newcastle. At least 2 slides were left in reserve for checking any discrepancies that might occur between the two centres. A total of 139 specimens were dispatched and examined in this way.

The fluorescent antibody technique used was that previously described (2), except that for convenience one centre used a 100-W mercury lamp instead of a 200-W lamp; one centre also used a different counterstain.

Material was examined only for respiratory syncytial (RS) virus and influenza virus A, both by the immunofluorescence and the immunoperoxidase methods. Because of many years' experience with immunofluorescence (5), and its proven reliability, RS virus was not routinely cultured when immunofluorescence proved to be unequivocally positive. However, all specimens were cultured for influenza virus A.

Model systems were set up in both Stockholm and Newcastle to test for specificity of the immuno-

peroxidase reagents. In innumerable tests, all reagents were found to be excellent for detecting influenza virus A and RS virus in tissue cultures fixed in acetone. Preliminary experiments with the same reagents on fixed preparations of nasopharyngeal secretions revealed a considerable but variable amount of endogenous peroxidase activity. This seemed to be situated in the inflammatory cells of secretions that appear during the course of respiratory infections.

Methods tried for the removal of endogenous peroxidase activity included treatment with methanol, hydrochloric acid, hydrogen peroxide, acetic acid directly dried on to slides, and acetic acid washed off after various periods of time and at various concentrations, as well as the use of orthotolidine as a counterstain. The technique described below was the result of all these preliminary experiments.

Preparations of tissue culture cells and nasopharyngeal secretions were made and fixed in acetone at +4°C for 5 min and 10 min, respectively. Then 1 drop of 5% acetic acid made up in distilled water was placed on the 2 cell smears in squares on slides for 5 min at room temperature and not allowed to dry. The slides were then rinsed with phosphate-buffered saline, pH 7.9, for 5 min.

Next, a freshly prepared orthotolidine staining mixture was applied. This was prepared using the following reagents: 0.8 ml of 0.03% orthotolidine; 0.08 ml of saturated boric acid solution; 0.02 ml of 20% sodium deoxycholate; and 0.04 ml of 0.3% hydrogen peroxide. The reagents were prepared as follows:

(a) 0.03% orthotolidine: dissolve 0.5 g of orthotolidine in 50 ml of acetone, add 3 ml of this to 100 ml of deionized water, and keep in a dark bottle at 4°C.

(b) Saturated boric acid: dissolve 5.5 g of boric acid in 100 ml of deionized water. Keep at room temperature (22°C).

(c) 20% sodium deoxycholate: dissolve 1 g of sodium deoxycholate in 5 ml of deionized water. Keep at room temperature (22°C).

(d) 0.3% hydrogen peroxide: dilute 0.1 ml of 30% H_2O_2 in 10 ml of deionized water. Prepare freshly each day.

After 15 min at room temperature, the staining mixture was rinsed off with phosphate-buffered saline, pH 7.2, for 5 min. The slides were then dried, and unlabelled specific virus antiserum, at the appropriate dilution determined by prior titration, was applied for 30 min at 37°C. The preparations were then washed 3 times with phosphate-buffered saline,

10 min for each washing. The area of the slide around the preparation was dried but the preparation itself was not allowed to dry completely. Peroxidase-labelled conjugate, appropriately diluted as determined by previous titrations, was applied for 30 min at 37°C, then rinsed 3 times with phosphate-buffered saline for 10 min each.

The peroxidase stain was prepared as follows: 0.1 ml of 1% H_2O_2 and 5 mg of 3'3'-diaminobenzidine tetrahydrochloride was added to 10 ml of 0.5 mol/litre Tris buffer, pH 7.6. It should be almost colourless immediately after making up and should be used at once before it becomes darker. The stain was applied for 10 min at room temperature, then rinsed off in phosphate-buffered saline and finally for 1 min in distilled water before drying.

Three variations of the technique were tried:

(1) Two-square slides were stained after treatment of both with acid; this was followed by the application of RS virus and influenza virus A antisera.

(2) Two-square slides were used without acid treatment. Only a small trial was attempted on 10 specimens at one centre with this method.

(3) Using three-square slides, two squares were treated with acid and RS virus and influenza virus A antisera eventually applied. The third square was not treated with acid but was in due course treated with RS virus antiserum.

Staining procedures were exactly the same in all these experiments.

Method (2) was abandoned because, although RS virus was recognized, non-specific appearances were present in all preparations and great difficulty was experienced in reading the slides.

The trial started with the use of a two-square slide preparation treated with acetic acid, of which one square was examined for RS virus and the other for influenza A. Even with the technique devised, some destruction of RS virus antigen occurred, but influenza virus A antigen was more resistant to this treatment. After approximately half the slides had been examined and analysed, it was decided to try the technique whenever possible using a slide that had 3 preparations on it. These preparations were treated in the following way:

(a) prior acid treatment for examination for RS virus;

(b) prior acid treatment for examination for influenza virus A;

(c) no prior acid treatment for examination for RS virus.

Acid-treated RS virus could then be compared with non-treated RS virus to gauge the destruction of antigen, while the influenza virus A preparation could be compared with the 2 RS virus spots to estimate peroxidase activity.

The results produced by the two centres for the examination of material by immunofluorescence and immunoperoxidase were analysed and found to be remarkably similar.

Immunofluorescence

Both centres examined 139 specimens by the immunofluorescence method for RS virus and influenza virus A. The results are given below.

| | No, of specimens positive | | No. |
	RS virus	Influenza A	negative
Centre X	62	18	59 [a]
Centre Y	60	17	62 [a]

[a] One negative subsequently proved to be positive by culture.

Centre X detected RS virus in 3 more specimens than centre Y and influenza virus A in 1 more specimen; there were very few positive cells in these 3 specimens. Owing to local circumstances, centre Y used a different counterstain and a 100-W lamp. When the 3 specimens were re-examined by centre Y with a new counterstain and a 200-W lamp, the positive specimens previously missed were detected.

Centre Y also obtained one RS virus positive specimen not found by centre X; when this was re-examined, as described above, it was clearly a false positive due to bacteria that were not clearly defined by the 100-W lamp.

All the influenza A immunofluorescence results were confirmed by virus isolation. All virus isolations coincided with the detection of influenza virus A by immunofluorescence.

Agreement between centres X and Y before the investigation of the discrepant results was as follows: influenza virus A, 94%; RS virus, 95%; negative, 95%.

Immunoperoxidase

There were 4 observers (A, B, C, and D) at the two centres.

The results by the immunofluorescence method were used as a standard for comparing the immunoperoxidase results. Table 1 illustrates the results obtained when the 4 observers read immunoperoxidase-stained preparations independently. This first experiment was based on the two-square method.

Table 1. Results obtained by four observers reading immunoperoxidase-stained preparations on two-square slides treated with acid [a]

	Observers							
	A		B		C		D	
RS virus positive	14	(64 %)	17	(63 %)	22	(81 %)	12	(41 %)
Known negative	11	(85 %)	23	(72 %)	26	(81 %)	22	(71 %)
Influenza virus A positive	3	(38 %)	7	(54 %)	11	(85 %)	6	(46 %)
False RS virus	1	(2 %)	7	(9 %)	4	(5 %)	7	(10 %)
False negative	14	(33 %)	16	(20 %)	7	(9 %)	20	(28 %)
False influenza virus A	0		2	(3 %)	1	(1 %)	2	(3 %)
Overall correct results	28	(65 %)	47	(57 %)	59	(77 %)	40	(56 %)

[a] The figures in parentheses show the percentage of the total examined in each category, except for false results when the figures represent the percentage of the total examined. The numbers of preparations read by individual readers varied.

Table 2. Results obtained by three observers reading 10 immunoperoxidase-stained preparations, without acid treatment

	Observers					
	B		C		D	
RS virus positive	3	(100 %)	3	(100 %)	3	(100 %)
Known negative	1	(33 %)	2	(66 %)	2	(66 %)
Influenza virus A positive	0		1	(25 %)	2	(50 %)
False RS virus	1	(10 %)	2	(20 %)	0	
False negative	3	(30 %)	1	(10 %)	2	(20 %)
False influenza virus A	2	(20 %)	1	(10 %)	0	
Overall correct results	4	(40 %)	6	(60 %)	7	(70 %)

Table 3. Results obtained by four observers reading immunoperoxidase-stained preparations on three-square slides

	Observers							
	A		B		C		D	
RS virus positive	18	(66 %)	25	(70 %)	25	(70 %)	30	(81 %)
Known negative	25	(93 %)	20	(71 %)	25	(89 %)	26	(93 %)
Influenza virus A positive	5	(71 %)	6	(86 %)	6	(86 %)	5	(71 %)
False RS virus	2	(3 %)	7	(10 %)	2	(3 %)	1	(1 %)
False negative	16	(24 %)	13	(18 %)	12	(17 %)	9	(13 %)
False influenza virus A	0		3	(4 %)	3	(4 %)	1	(1 %)
Overall correct results	48	(73 %)	51	(71 %)	56	(78 %)	61	(85 %)

Because of the overall poor correlation with immunofluorescence, a second experiment was performed on 10 specimens not treated with acid and read by 3 observers. These results are given in Table 2. Although RS virus could be detected, the preparations were difficult to read and there were many false results.

Table 3 illustrates the final experiment using the three-square system. Overall, the three-square slide method produced better results, particularly for some readers. The diagnosis of influenza virus A tended to improve, but the results for RS virus remained approximately the same as with the two-square method. There was a significant increase in the number of true negative results with the three-square method. The results confirm too that influenza A antigen was resistant to acid treatment, whereas RS virus antigen showed deterioration. However, the main criticism of the immunoperoxidase technique was that the sensitivity did not approach that of the fluorescent antibody technique.

Failure to detect positive cells tended to occur in those preparations in which positive cells, as shown by immunofluorescence, were only present in small numbers.

The results were then compared of 3 readers using the two-square method (influenza A square and RS virus square treated with acid) and the three-square method as previously described (Tables 2 & 4). The 3 readers agreed more frequently with the three-square than with the two-square method. This was of significance for RS virus and in overall agreement for all specimens. But the agreement between the 3 readers never exceeded 58% with the three-square and 39% with the two-square method.

Further comparison of the immunofluorescence and immunoperoxidase techniques

In many preparations dead cells are present and these tend to stain in both immunoperoxidase and immunofluorescence methods. In the latter, there is uniform staining that is completely different from the specific staining. It is more difficult to differentiate such cells in immunoperoxidase-stained preparations.

With immunoperoxidase staining it is also harder to detect the characteristic distribution of antigen. There is a tendency for heavier and more uniform staining, and the positively stained cells do not stand out as sharply as those stained by immunofluorescence. Consequently, each preparation requires much longer study.

Preparations with scanty cells, as detected by immunofluorescence, are rarely detected by immunoperoxidase, and the method appears to have, at this stage, a much lower threshold.

There is, however, evidence that the antisera used for the immunofluorescence method can be used, and are adequate, for the immunoperoxidase method and that the background activity of the conjugate does not affect the results.

DISCUSSION

Our results and those of others (6) show that the immunoperoxidase technique is potentially useful for the detection of virus infection in tissue culture. Attempts have been made to use these methods for the detection of viruses in clinical material, the most impressive results being obtained in the detection of rabies virus in brain material (7); brain material probably contains little endogenous peroxidase activity.

The respiratory secretions of patients with respiratory infections of necessity contain many inflammatory cells that are a good source of endogenous peroxidase. We tested all methods suggested for its removal (8) and found that all removed viral antigen. There was a difference in the rate of destruction of viral antigen by these methods in different viruses; intranuclear influenza virus A appears to be more resistant than the cytoplasmic inclusions of RS virus. It is therefore clear that one of the restricting factors to the introduction of this method is the lack of a technique for the removal of endogenous peroxidase that will not also destroy the virus antigen. Research must be devoted to this problem. With the best method used in this trial, the three-square slide, the sensitivity was only 70–85% of that of the immunofluorescence method. There were also discrepancies between readers, which did not occur with the immunofluorescence method.

There was very good agreement between the two centres in the results with the immunofluorescence method. Minor discrepancies occurred, but these were less than 5% and were corrected when the light sources and counterstains were standardized. The investigation showed that virus-infected material can be transported over great distances and still be suitable for examination by the immunofluorescence method. Despite the problems that may occur with immunofluorescence techniques in developing countries, selected centres could make a start with this method. These countries might act as diagnostic

centres locally, as well as for many outlying areas, and might collaborate with suitable centres in other countries. This, as an immediate step, might be the way to study the epidemiology of virus infections and the causes of everyday virus problems in developing countries.

Although the potential of the immunoperoxidase method is great, it would be disastrous to introduce the technique at this stage for use on clinical material until all the problems described have been solved. It may eventually prove necessary to employ different techniques for different viruses, as has been shown by our experience with influenza A and RS viruses. To introduce prematurely the immunoperoxidase method could only bring disrepute to the method and to virus diagnosis in general, and could retard the progress of virology in developing countries for many years.

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RÉSUMÉ

COMPARAISON DES TECHNIQUES D'IMMUNOFLUORESCENCE ET DE L'IMMUNOPEROXYDASE POUR LE DIAGNOSTIC VIROLOGIQUE A DISTANCE

Des sécrétions rhinopharyngées recueillies à Newcastle ont été examinées à Newcastle et à Stockholm en vue de déceler la présence du virus grippal de type A et du virus syncytial respiratoire (RS) au moyen des techniques d'immunofluorescence et de l'immunoperoxydase. Les deux centres ont appliqué ces deux techniques à un total de 139 spécimens et la concordance des résultats obtenus par immunofluorescence a été de 94% pour le virus grippal A, de 95% pour le virus syncytial respiratoire et de 95% pour les spécimens négatifs. Cette technique peut donc être employée pour examiner des spécimens prélevés à grande distance du laboratoire auquel ils sont transmis. Le fait que la qualité des résultats obtenus par la technique de l'immunoperoxydase ait été moins satisfaisante est dû surtout à la présence de peroxydase endogène, et les mesures prises pour l'éliminer ont aussi détruit l'antigène du virus RS. S'il paraît prématuré d'adopter la technique de l'immunoperoxydase pour le diagnostic virologique rapide, celle-ci pourrait cependant se révéler utile lorsqu'on aura surmonté les problèmes exposés dans le présent article.

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