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Evaluation of a New Enzyme-Linked Immunosorbent Assay (ELISA) in the Diagnosis of Rhinovirus Infection

W. Al-Nakib, C.J. Dearden, and D.A.J. Tyrrell

MRC Common Cold Unit, Salisbury, England (W.A.-N., C.J.D., D.A.J.T.); and Department of Microbiology, Faculty of Medicine, University of Kuwait, Safat, Kuwait (W.A.-N.)

This study describes the evaluation of a newly developed ELISA for the direct detection of rhinovirus antigens in nasal washings. Of 54 volunteers inoculated with 100 $TCID_{50}$ of human rhinovirus type 2 (HRV-2), 50 (96.6%) and 32 (59%) excreted antigen and virus on at least 1 of 3 days investigated, respectively. Thirty-three (61%) had significant rises in rhinovirus-specific IgA by ELISA. Twelve (22%) developed symptoms of colds. Generally the ELISA detected antigen more frequently in volunteers later in the course of infection and provided evidence of infection in a higher proportion of asymptomatic compared with symptomatic volunteers. On the other hand, virus isolation detected virus more frequently earlier in the course of infection and in a higher proportion of symptomatic compared with asymptomatic volunteers. We conclude that rhinovirus antigen detection by ELISA is a simple, rapid, sensitive, and practical test to diagnose a rhinovirus infection and potentially a viable alternative to virus isolation.

KEY WORDS: rapid virus diagnosis, picornaviruses, common cold

INTRODUCTION

Rhinoviruses are responsible for one third to one half of all acute respiratory viral infections [Couch, 1984]. In the majority of individuals, the infection is subclinical or results in only mild upper respiratory tract symptoms characterised by rhinorrhoea, nasal obstruction, some element of pharyngitis, and cough. Fever or systemic reactions are infrequent [Al-Nakib and Tyrrell, 1988]. A number of reports suggest that rhinoviruses also cause lower respiratory tract infections [recently reviewed by Al-Nakib and Tyrrell, 1988]. Indeed, recent community studies in Tecumseh, Michigan, in the United States, confirmed these early reports and showed that rhinoviruses frequently cause lower respiratory tract infections not only among infants and children but also among adults [Monto et al., 1987]. Furthermore, the median duration of illness among those aged over 40 years was as long as 3 weeks [Monto et al., 1987]. Another recent study suggests a significant association between rhinovirus infection and pulmonary disease in hospitalized infants and children [Krilov et al., 1986]. These studies, together with those of Gregg [1983] in which rhinoviruses were implicated in the exacerbation of episodes of bronchitis and asthma in children, confirm that rhinoviruses are capable of causing illnesses that are far more serious than is generally realised and therefore may require specific recognition and treatment.

Recently, there has been significant progress in the prophylaxis and treatment of acute respiratory virus infection, including those caused by rhinoviruses. For example, influenza A viruses can now be treated effectively with amantadine or rimantadine, while influenza B virus and respiratory syncytial virus (RSV) are sensitive to ribavirin [Bektimirov et al., 1985; Knight et al., 1986]. We recently reported a trial of a new synthetic antiviral, R61837, or 3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl] pyridazine, in which we found this new drug to be effective in preventing illness when given before virus challenge in volunteers [Al-Nakib et al., 1987]. It is envisaged that further progress in the field will lead to molecules that will also be effective in treating colds caused by rhinoviruses particularly in patients with lower respiratory tract complications. However, for the antiviral chemotherapy to be effective it would be important to identify rapidly the causative virus in order that the appropriate treatment be prescribed especially since these antivirals are highly specific in their action. Both influenza viruses and RSV can now be rapidly identified in nasopharyngeal aspi-

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Address reprint requests to Professor Widad Al-Nakib, Department of Microbiology, Faculty of Medicine, Kuwait University, P.O. Box 24923 Safat, Kuwait 13110.

rates by either immunofluorescence (IF) or ELISA [Orstavik et al., 1984; Grandien et al., 1985]. Rhinoviruses have "traditionally" been diagnosed either by virus isolation in a sensitive cell culture or by showing significant antibody rises by neutralisation tests or both [Hamparian, 1979]. However, these procedures are laborious, require considerable expertise, and take some 2–3 weeks before a diagnosis is reached. We have recently developed a new ELISA system capable of detecting viral antigens directly in nasal secretions [Dearden and Al-Nakib, 1987]. In this study we report on the diagnostic potential of this new method when compared with that of virus isolation.

MATERIALS AND METHODS

Clinical Specimens

Serial nasal wash specimens and paired sera were collected from 54 volunteers who participated in therapeutic trials involving zinc gluconate lozenges, at the MRC Common Cold Unit, Salisbury [Al-Nakib et al., 1987]. In these trials the volunteers were challenged with 100 $TCID_{50}$ of human rhinovirus type 2 (HRV-2). Details of these trials, methods of sample collection, clinical assessment, virus isolation, and procedures for measurement of antibody rises by neutralisation assays have been described [Al-Nakib et al., 1987]. In this study we investigated three consecutive nasal wash specimens from each volunteer (which usually corresponded to the first 3 days of symptoms of cold or days 3 to 5 after virus inoculation) for the presence of virus by isolation in Ohio Hela cells and for antigen by ELISA. A volunteer was considered infected if at least one of the three nasal wash samples was positive for the presence of virus or antigen and/or showed significant antibody rises in paired sera by ELISA (see below). Nasal washes from 40 volunteers who had been infected with other respiratory viruses such as influenza virus A (10 volunteers) or B (10 volunteers) or coronavirus (10 volunteers) or given only saline instead of virus (10 volunteers) were used as controls to check the specificity of the rhinovirus antigen assay. These were treated in exactly the same way as those obtained from rhinovirus-infected volunteers.

Direct Detection of Rhinovirus Antigen in Nasal Washings by ELISA

The procedure for detecting rhinovirus antigens in nasal washings has recently been described [Dearden and Al-Nakib, 1987]. Briefly, wells of an ELISA plate were coated with 100 μl of either a rabbit antirhinovirus type 2 (HRV-2) immune (postimmunization) or normal (preimmunization) serum. After an overnight incubation at 4°C, plates were washed three times with PBS–Tween-20, and 100 μl of a nasal washing or control antigen (uninfected tissue culture fluid) was added directly to each of a set of duplicate ELISA plate wells that had been precoated with either the normal or the immune rabbit anti-HRV-2 serum. After a further overnight incubation period at 4°C, plates were washed with PBS–Tween-20 and 100 μl of streptavidin wash (Amersham), diluted 1:200 with PBS, was added for 10 minutes at room temperature to all wells to block any nonspecific binding to endogenous biotin in the specimens. After washing with PBS–Tween-20, 100 μl of biotinylated anti-HRV-2 serum diluted to its optimal working dilution with the ELISA diluent (PBS–Tween-20 containing 0.1% v/v BSA and 5% v/v control antigen) was added to all the wells of the plates. After incubation at 37°C for 2 hours, plates were washed as before with PBS–Tween-20 and 100 μl of streptavidin–B-galactosidase preformed complex (Amersham) diluted 1:200 with PBS–Tween-20 containing 0.1% v/v BSA was added to all the wells of the plate and incubated again at 37°C for 2 hours. Plates were then washed five times with PBS–Tween and 100 μl of substrate (Ortho nitrophenyl–B-D-galactosidase [ONPG]) was added to all test wells plus a row of wells, not used in the test, as blank.

Plates were read at 410 nm when the color intensity of the standard positive controls reached its maximum. Test results were calculated by subtracting the mean optical density of a specimen tested in the duplicate wells coated with normal rabbit (preimmunization) serum from the mean optical density of the same specimen tested in duplicate wells coated with immune rabbit (postimmunization) serum. A nasal washing was considered positive when the net optical density of the test sample was >1.5 times that of the "negative" control wells plus one standard deviation. The negative controls contained uninfected tissue culture fluid instead of a clinical sample, and these were tested in exactly the same way as the test sample. As previously reported, this calculation was found to be the most appropriate for controlling the considerable variation in background optical densities obtained with different nasal wash specimens. The cut-off value was arrived at after we tested a series of well-characterised rhinovirus-positive and -negative nasal wash samples [Dearden and Al-Nakib, 1987].

Measurement of Rises in Rhinovirus-Specific IgA in the Serum

Details of the ELISA used in measuring rhinovirus-specific IgA antibody and the criteria used to identify significant specific IgA antibody rises have recently been described [Barclay et al., 1988]. We chose rises in specific serum IgA rather than in IgG to indicate a recent infection since these gave better correlation with neutralising antibody rises [Barclay et al., 1988]. Indeed, in this study the ELISA detected rises in four additional volunteers in whom the neutralization test failed to detect a rise even though these volunteers excreted virus.

Statistical Analysis

The association between variables were tested by means of the chi-square (χ^2) test or Fisher's exact test.

TABLE I. Rhinovirus Antigen Detection by ELISA in Relation to Other Tests

Test	No. of volunteers[a] showing a positive test (%)	
	On any 1 of 3 days	On any 2 of 3 days
ELISA-Ag	50 (92.6)	30 (55.6)
Virus isolation	32 (59)	26 (48)
Antibody rises	33 (61)	—

[a]A total of 54 volunteers were inoculated with HRV-2.

RESULTS

Specificity of the ELISA for Rhinovirus Antigen Detection

A nasal wash from each of 40 volunteers who were either infected with influenza A (10 volunteers) or influenza B (10 volunteers) virus or coronavirus (10 volunteers) or who were given saline instead of virus (10 volunteers) and that was positive for the respective virus by isolation in culture was tested by the ELISA for the presence of rhinovirus antigen. None of these samples gave positive results for rhinovirus by ELISA, while nine rhinovirus type 2-positive specimens used as standards were consistently positive. These results indicated that the rhinovirus antigen detection by ELISA antigen was highly specific.

Rhinovirus Antigen Detection by ELISA in Relation to Other Tests

Of the 54 volunteers inoculated with HRV-2, 50 (92.6%) were found to have antigen by ELISA and 32 (59%) excreted virus as detected by isolation in their nasal secretion on at least 1 of the 3 days investigated, respectively (Table I). Thirty (55.6%) volunteers excreted antigen and 26 (48%) excreted virus on any 2 of the 3 days investigated. Thirty-three (61%) had significant rises in rhinovirus-specific serum IgA by ELISA (Table I). As shown in Figure 1, 48, 50, and 61% of volunteers excreted antigen on days 1, 2, and 3 of the investigation (corresponding to days 3, 4, and 5 after virus inoculation, respectively) while 50, 54, and 48% excreted virus on days 1, 2, and 3, respectively, suggesting that the ELISA detected antigen more frequently later during the course of infection.

Of the 54 volunteers inoculated with HRV-2, 39 (72%) were considered infected since they either had virus isolated from their nasal secretions on at least 1 day and/or had significant antibody rises. Among this infected group there was a highly significant association between antigen detection by ELISA and virus isolation by culture on the first day of the investigation ($P = 0.0076$). Significant associations were also seen between antigen detection by ELISA on any 2 of 3 days investigated and evidence of infection by other laboratory tests ($P = 0.04$) and with virus isolation on 3 consecutive days ($P = 0.05$).

There were 14 volunteers who were inoculated with HRV-2 and who excreted antigen on at least 1 of the 3 days investigated but who did not excrete virus on any day or showed significant antibody rises. Of these, four excreted antigen for 2 days and one for 3 consecutive days.

Rhinovirus Antigen Detection in Asymptomatic Versus Symptomatic Volunteers

Of 54 volunteers inoculated with HRV-2, 12 (22%) developed symptoms of cold (Table II). Eleven of 12

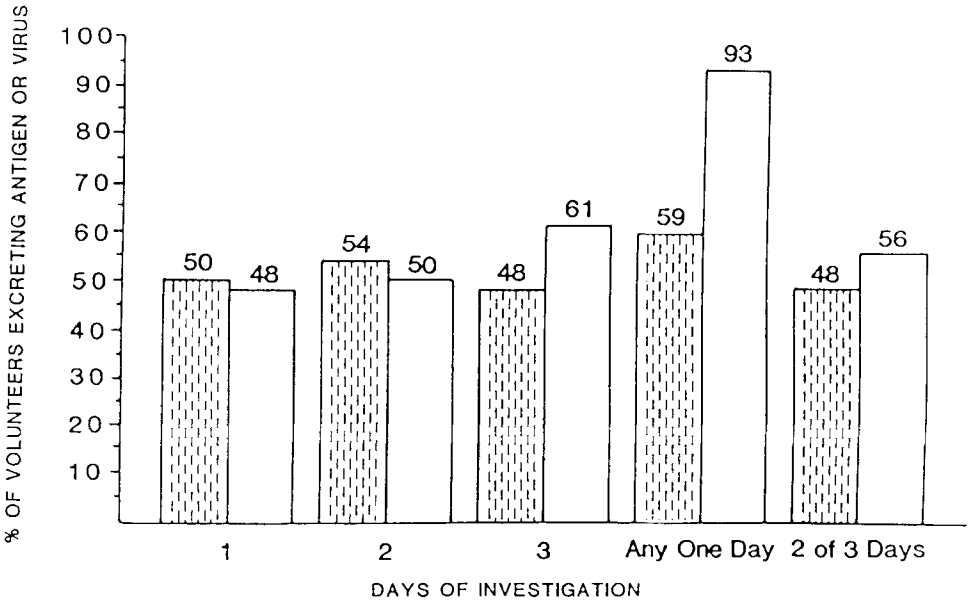

Fig. 1. Percentage of volunteers excreting antigen (open bars) or virus (stippled bars) on different days of the investigation.

TABLE II. Summary of Results in Volunteers With Colds

Category	No. (%) of volunteers
Symptomatic	12/54 (22)
Infected[a]	11/12 (92)
Excreted virus[b]	10/12 (83)
Excreted antigen[b]	10/12 (83)
Excreted antigen and infected[a]	9/12 (75)
Excreted antigen but had no other laboratory evidence of infection	1/12 (8)

[a]Excreted virus on at least any 1 of 3 days and/or had significant antibody rise.
[b]On any 1 of 3 days.

TABLE III. Detection of Rhinovirus Antigen and Virus in Symptomatic and Asymptomatic Volunteers

Test	Volunteers[a]	Percent of volunteers showing a positive test: On any 1 of 3 days	On any 2 of 3 days
ELISA-Ag	Infected	96	71
	Symptomatic	82	45
Virus isolation	Infected	78.5	57
	Symptomatic	90	90

[a]N = 28 infected volunteers; N = 11 symptomatic volunteers.

(92%) had laboratory evidence of infection since they excreted virus on at least 1 of the 3 days investigated and/or had significant rises in rhinovirus-specific IgA. Of these 11 volunteers, 10 (83%) excreted virus and antigen on at least 1 day. Nine (75%) of the symptomatic volunteers who had antigen also had evidence of infection by other tests. One volunteer with symptoms of cold, therefore, had antigen in his nasal secretions although he did not excrete virus on any day nor did he show significant antibody rises (Table II).

Table III shows a trend in that the ELISA seems to detect antigen more frequently in volunteers who were asymptomatic (96% were positive on any 1 day and 71% were positive on any 2 days) than in symptomatic volunteers (82 and 45%, respectively). In contrast, virus isolation in culture seems to detect virus more frequently in symptomatic than in asymptomatic volunteers (90% were positive on any 1 or any 2 days vs. 78.5 and 57%, respectively).

DISCUSSION

The data presented in this study show that the recently developed ELISA for rhinovirus antigen detection is generally more sensitive than virus isolation. Furthermore, it seems to detect antigen more frequently later during the course of infection. This finding is similar to that reported during the course of influenza virus infection. Berg et al. [1980] showed that an enzyme-linked fluorescent immune assay detected influenza antigen in nasal samples from only 6 of 12 (50%) volunteers on the second day after virus administration, whereas virus isolation was positive in 9 of 12 (75%). By 7 or 8 days after inoculation, however, all samples were negative by virus isolation, yet antigen was still detectable in nasal specimens from 6 (50%) and 5 (42%) of 12 volunteers, respectively [Berg et al., 1980]. Using synthetic oligonucleotides as probes, we, too, have recently found that although rhinovirus RNA could be detected as efficiently as virus by culture during the first 2–3 days after virus inoculation, more volunteers were positive for viral RNA by hybridization than for virus by culture on subsequent days [Bruce et al., 1989]. Furthermore, 54 of 57 (95%) volunteers inoculated with HRV-14 were positive on at least 1 day by hybridization, whereas virus was isolated from only 41 (74%) [Bruce et al., 1989]. These results are similar to those obtained by the ELISA in this study and show that both antigen and RNA detection may be more sensitive than virus isolation in establishing evidence of infection in a higher proportion of inoculated volunteers. Indeed, the ELISA seems to detect antigen more often in asymptomatic than in symptomatic volunteers (96% positive vs. 82% on any day). These results suggest that a very low-grade reproduction of virus may have taken place in the nasal epithelium of these individuals although virus was undetectable by isolation.

Rhinovirus antigen was detected by ELISA in the nasal washings of 14 volunteers even though they did not excrete virus or show significant rises in rhinovirus-specific IgA. Four of these volunteers excreted antigen for 2 days and one for 3 consecutive days. Although it is possible that the ELISA may have falsely detected antigen in these individuals, this, we feel, is unlikely since the test was shown to be highly specific for rhinovirus and did not detect antigen falsely in any of the nasal washes from individuals who had been infected with influenza A or B virus or coronavirus and that were positive for the respective virus by culture. Moreover, all 10 nasal washing samples from the 10 individuals given saline instead of virus were also consistently negative for rhinovirus by this ELISA. It is, therefore, conceivable that antigen may have been present especially since these volunteers were in fact inoculated with HRV-2 (and one of them actually developed symptoms of cold without excreting virus or showing serological evidence of infection). The infection, however, may have been limited to little infectious virus being formed or neutralized by low titres of nasal antibody. If so, the antigenic load may not have been sufficient to stimulate a measurable immune response.

We conclude that the ELISA described in this study is potentially a viable alternative to virus isolation. It is sensitive, rapid, does not require considerable expertise, and could be adapted to large-scale epidemiological studies. However, we believe that further research is required to investigate optimal methods for specimen collection and processing, e.g., whether nasopharyngeal aspirates might be a better sample than washings. Moreover, release of adequate antigen in the sample by mechanical or chemical methods prior to testing may improve sensitivity. The antibody element

in the test might be standardized by using monoclonal antibody reagents instead of polyclonal sera. Furthermore, since the present test is type specific, it would be important to identify a monoclonal antibody or a panel of monoclonal antibodies that detect C-type reactivity or other common epitopes found on most of the 100 rhinovirus serotypes.

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