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Diagnosis of Human Coronavirus Infections in Children Using Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay (ELISA) was developed for diagnosing human coronavirus (HCV) infections in children. One hundred and seventy seven nose swabs, throat swabs, and nasopharyngeal aspirates were collected from 30 children suffering from acute respiratory infections. These samples were tested for HCV antigens by ELISA and 28.2% of the samples were shown to be HCV positive. These results indicate that our ELISA should prove useful in the diagnosis of HCV infections in children. Further studies are in progress to extend the ELISA to detect HCVs in experimentally and naturally acquired infections in adults.

Key words: HCV infections, HCV diagnosis, HCV in children, ELISA for HCVs

INTRODUCTION

Human coronaviruses (HCVs) cause mild upper respiratory tract infections or common colds in both adults and children: up to 30% of colds have been associated with HCVs [Larson et al, 1980; McIntosh, 1974; Monto, 1974]. However, most previous studies have used changes in serum antibodies to confirm HCV infections [Bradburne and Tyrrell, 1971; McIntosh, 1974; Monto, 1974]. Few studies have reported the isolation or detection of HCVs in nasal secretions due to the fastidious growth requirements of most HCV isolates in tissue and organ culture [Hamre and Procknow, 1966; Larson et al, 1980; McIntosh et al, 1967; Tyrrell and Bynoe, 1965]. Nevertheless, one study has used immunofluorescence to detect HCV antigens in epithelial cells shed from the nasopharynx of volunteers inoculated with HCVs [McIntosh et al, 1978]. However, no HCVs were detected by this method in similar cells shed in the nasal secretions of children with naturally acquired respiratory infections [McIntosh et al, 1978].

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In this paper, we describe an ELISA to detect HCVs, from both HCV antigenic groups, the HCV 229E and HCV OC43 groups [Mcnaughton, 1981; Macnaughton et al, 1981; McIntosh, 1974; McIntosh et al, 1969], in nose swabs, throat swabs, and nasopharyngeal aspirates taken from children suffering from acute respiratory infections.

MATERIALS AND METHODS

Children Specimens

Thirty children aged 6 months to 6 years with recurrent upper and lower respiratory tract infections were studied between October 1979 and February 1981. Details of these children and their infections have been described elsewhere [Isaacs et al, 1981; Isaacs et al, 1982]. 159 samples, comprising 111 nose swabs, 11 throat swabs, and 55 nasopharyngeal aspirates, were collected from the children during episodes of respiratory tract infections. Nose and throat swabs were taken with sterile wool swabs and broken into viral transport medium containing penicillin, streptomycin, and amphotericin. A neonatal mucous extractor and mucous trap (Chilton Surgical ME/30) was used to take nasopharyngeal aspirates, to which were added 2 ml of viral transport medium. Specimens were transported to the laboratory on solid carbon dioxide and stored at −70°C. The samples used represented over 40% of all samples collected from children with respiratory tract infections, and included samples from which other respiratory viruses had been isolated as well as samples from which no viruses had been isolated. There was no deliberate selection of which specimens to test although specimens from which other respiratory viruses had been isolated were less likely to be used as these specimens had sometimes been used up in other tests. These experiments were approved by the Ethical Committee of Northwick Park Hospital, Harrow, England.

Viruses

Representative members of the two HCV antigenic groups were used. These were HCV 229E, the prototype virus of the HCV 229E group [Hamre and Procknow, 1966], and CV Paris, of possible human origin isolated from the feces of a neonate with necrotizing enterocolitis [Sureau et al, 1980], and a member of the HCV OC43 group.

Growth and Purification of Viruses

HCV 229E was grown in MRC continuous (MRCc) cells as previously described [Macnaughton and Madge, 1978]. CV Paris was grown in HRT 18 cells in RPMI 1640 with 10% fetal calf serum [Hasony and Macnaughton, 1982]. For both viruses, the cells were frozen and thawed once and the resulting suspension was clarified at 2,000 g for 30 min at 4°C. The virus preparations were pelleted at 75,000 g for 1 hr at 4°C and then resuspended in 1 ml aliquots of Dulbecco's phosphate-buffered saline "A" (PBSA). The resuspended virus samples were overlaid on to linear 25 to 55% (w/w) sucrose gradients in PBSA and centrifuged at 90,000 g for 16 hr at 4°C. The virus peaks at 1.18 gm/ml were collected and the concentration of virus particles/ml in the samples determined by electron microscopy particle counts [Macnaughton et al, 1980].

Preparation of Antisera

Immune sera against HCV 229E and CV Paris were prepared in adult New Zealand white rabbits [Kraaijeveld et al, 1980a]. A 0.5-ml amount of purified virus at a concentration of 10^{10} virus particles/ml, as determined by electron microscopy particle counts [Macnaughton et al, 1980], was mixed with an equal volume of Freund's complete adjuvant and injected intracutaneously at up to 20 different places in the shaven back of a rabbit. Two days before immunization animals received 0.5 ml Bordetella pertussis vaccine (The Lister Institute of Preventive Medicine, Elstree, England) intracutaneously as an additional adjuvant. Each animal was bled before and 6 weeks after immunization. The sera were absorbed with an equal volume of newborn calf serum and stored at −20°C.

ELISA

The ELISA method used was based on a method described previously for detecting antibodies to HCVs [Kraaijeveld et al, 1980b]. Flat-bottomed polystyrene microtiter plates (Dynatech) were coated with duplicate 0.2-ml amounts of antigen diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at room temperature. After incubation the plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20 and 0.02% sodium azide (PBST) and shaken dry. Portions of 0.2 ml of sera diluted in PBST were added to the wells and incubated for 4 hr at room temperature. After incubation, the plates were washed four times in PBST and shaken dry. Anti-rabbit immunoglobulin G, directed against heavy and light chains, and labeled with alkaline phosphatase conjugate (Miles Laboratories) at a dilution of 1:800, was added in 0.2-ml quantities and left overnight at room temperature. After four additional washes with PBST, 0.2 ml of phosphatase substrate, consisting of a 0.1% solution of p-nitrophenylphosphate in 10% (wt/vol) diethanolamine buffer (pH 9.8) with 0.02% sodium azide and 0.01% $MgCl_26H_2O$, was added to each well. Absorbance values were read after 30 min at 405 nm in a Flow Titertek Multiscan photometer.

RESULTS

Detection of HCV Antigens by ELISA

Figure 1 shows the ELISA absorbance values obtained for a number of HCV 229E concentrations and PBSA using 1:20 and 1:200 dilutions of a positive HCV 229E rabbit antiserum and negative antiserum from the same rabbit. We have called the ratios of positive to negative HCV 229E antisera absorbance values at the same serum dilutions, ELISA ratios. Table I shows the ELISA ratios calculated from Figure 1. The ELISA ratios for serum dilutions 1:20 and 1:200 against PBSA were 1.8 and 1.3, respectively. In each case the lower antigen concentrations tested produced similar ELISA ratios to those obtained with PBSA.

It might be expected that the ELISA ratios should be approximately 1.0, ie, that there would be similar nonspecific binding of both sera to the ELISA plates. However, there was increased nonspecific binding of the positive sera to the plates relative to the negative sera, presumably reflecting a higher immunoglobulin content in the positive sera.

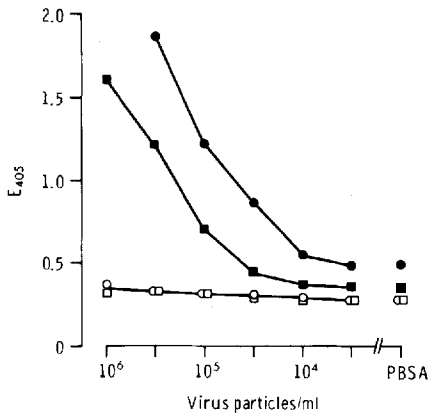

Fig. 1. ELISA absorbance values obtained with HCV 229E against rabbit antiserum to HCV 229E. HCV 229E dilutions were made from an antigen preparation containing 10^{10} particles/ml as determined by electron microscopy particle counts [Macnaughton et al, 1980]. ● and ■, positive HCV 229E antiserum at dilutions of 1:20 and 1:200, respectively; ○ and □, negative HCV 229E antiserum at dilutions of 1:20 and 1:200, respectively. Absorbance values were read at 405 nm after 30 min.

TABLE I. Detection of HCV 229E Using ELISA Ratios

Serum dilution	Antigen concentration in virus particles/ml[a]						
	10^6	5×10^5	10^5	5×10^4	10^4	5×10^3	PBSA
1:20	ND[b]	5.8[c]	3.9	2.7	1.9	1.8	1.8
1.200	5.0	3.8	2.3	1.5	1.3	1.3	1.3

[a]Determined by electron microscopy particle counts [Macnaughton et al, 1980].
[b]ND, not done.
[c]Figures are ELISA ratios (ratios of positive to negative HCV 229E antiserum absorbance values) and are obtained from Figure 1.

We suggest that the lowest HCV 229E concentrations that can reliably be detected are those with ELISA ratios that are at least twice those obtained with PBSA, ie, concentrations with ELISA ratios of 3.6 and 2.6 or more with 1:20 and 1:200 serum dilutions, respectively. Table I shows that 10^5 particles per ml could be detected with 1:20 serum dilutions, while 5×10^5 particles per ml could be detected with 1:200 serum dilutions. Chequerboard titrations showed that 1:20 serum dilutions were generally the most satisfactory in detecting HCV 229E antigens.

Similar studies were done to detect CV Paris by ELISA using positive and negative CV Paris antisera produced in rabbits. Chequerboard titrations showed that 5×10^5 particles/ml could be detected using 1:20 serum dilutions. In this case ELISA ratios of 1.5 were obtained against PBSA and we suggest that samples were positive for CV Paris antigens when they had ELISA ratios of at least twice this value, ie, 3.0 or more.

Detection of HCV Antigens in Children's Nasal Secretions

The criteria for detecting HCV antigens, described above, were used in the analysis of 177 samples taken from 30 children with respiratory infections. The

samples comprised mostly nose swabs but also included throat swabs and nasopharyngeal aspirates. Chequerboard titrations on several of these samples showed that 1:200 dilutions of the nasal samples produced the highest absorbance values with 1:20 dilutions of the rabbit serum samples, and these dilutions were therefore used in the following experiments.

Figure 2 shows that ELISA ratios obtained with 1:200 dilutions of the children's nasal specimens using 1:20 dilutions of antisera to HCV 229E (Fig. 2a) and CV Paris (Fig. 2b). A wide range of ELISA ratios were obtained for both HCV 229E and CV Paris antisera. As described above, ELISA ratios of 3.6 or more with HCV 229E antisera, and 3.0 or more with CV Paris antisera, were considered to contain HCV 229E and CV Paris antigens, respectively. We have shown previously that HCV 229E and CV Paris are representative members of the HCV 229E and HCV OC43 antigenic groups and that all HCVs can be detected by ELISA using one or the other of these antigens [Hasony and Macnaughton, 1982; Kraaijeveld et al, 1980b; Macnaughton, 1981; Macnaughton et al, 1981]. Thus, 46 or 26.0% of the samples were positive for HCV 229E group viruses, and 7 or 4.0% of the samples were positive for HCV OC43 group viruses. Three of the samples were positive for both HCV antigenic groups, so that 50 or 28.2% of the samples were positive for one or other of the HCV antigenic groups.

Table II shows the number of nose and throat swabs, and nasopharyngeal aspirate specimens used in this study, and the percentage detection of HCVs in them. The highest detection rate of HCVs was in the nose swabs: HCVs were detected in 34.2% of the nose swabs and in 18.2% of the throat swabs and nasopharyngeal aspirates.

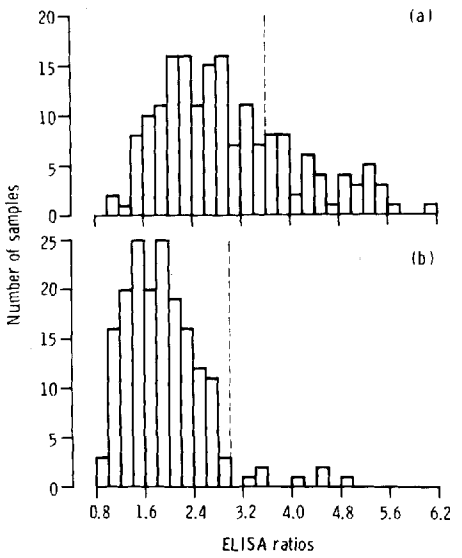

Fig. 2. ELISA ratios obtained with 159 nasal secretion samples using rabbit antisera to a) HCV 229E and b) CV Paris. Samples were used at dilutions of 1:200 and antisera at dilutions of 1:20. Absorbance values were read at 405 nm after 1 hr.

TABLE II. Detection of HCVs in Different Nasal Secretion Specimens Using ELISA

Specimen	Number of samples		Percentage positive for HCVs
	Total	Positive for HCVs	
Nose swab	111	38	34.2
Throat swab	11	2	18.2
Nasopharyngeal aspirate	55	10	18.2

DISCUSSION

This is the first study to report the detection of HCV antigens by ELISA in nose swabs, throat swabs, and nasopharyngeal aspirates taken from children with naturally acquired respiratory infections. In the past, ELISA has been shown to be one of the most sensitive methods for detecting HCV antibody rises in volunteers inoculated with a number of HCV isolates [Kraaijeveld et al, 1980b; Macnaughton et al, 1981]. The ELISA used previously has now been adapted to detect HCVs in clinical specimens: specimens containing at least 10^5 HCV particles per ml can be detected. At present it is difficult to compare the detection of HCV antigens in such specimens by ELISA with other methods, as isolation of HCV isolates in organ and/or tissue cultures is difficult and tedious [Hamre and Procknow, 1966; Larson et al, 1980; McIntosh et al, 1967; Tyrrell and Bynoe, 1965].

The detection of HCVs in over 28% of the samples is higher than that previously observed in children using seroepidemiologic methods [Kaye et al, 1971; McIntosh et al, 1970]: this presumably reflects the high sensitivity of our ELISA method. However, the number of samples containing HCVs is probably an underestimate as the criteria used for deciding that a sample contains HCV antigens are very rigorous and samples containing less than 10^5 HCV particles per ml would not be detected.

Forty six of the samples were positive for HCV 229E group viruses while only seven were positive for HCV OC43 group viruses. This result is not unexpected as the incidence of both HCV 229E and OC43 isolates in the population follows a complex cyclic pattern, and the samples studied were taken over a period of 17 months. Infections with both groups of HCVs occur in 2 to 3 yr cycles [McIntosh, 1974; Monto, 1974].

It is interesting that 34.2% of the nose-swab specimens contained HCV antigens, while HCVs were detected in only 18.2% of throat swabs and nasopharyngeal aspirates. Thus it appears that although HCVs replicate throughout the upper respiratory tract, most HCV replication probably occurs in nasal epithelial cells.

It would have been useful to confirm the identification of HCV antigens in nasal secretions by observing HCV antibody rises in children's sera after HCV infections. However, it has proved difficult to obtain the appropriate preinfection serum samples. This is because HCV infections occur frequently in the population often producing subclinical infections [Macnaughton, 1982] and the children in this study had recurrent upper and lower respiratory tract infections where in some cases HCV antigens appeared to persist in nasal secretions for periods of several months [Macnaughton, unpublished results].

In conclusion, we have developed an ELISA for detecting both HCV 229E and OC43 isolates in nose swabs, throat swabs, and nasopharyngeal aspirates from children with respiratory infections: nasal swabs proved the most useful specimens

for detecting HCVs by ELISA. Further studies are in progress to extend our ELISA to detect HCV antigens in nasal washings and nose swabs from volunteers inoculated with HCVs and from adults with naturally acquired HCV infections.

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