The Tecumseh Study of Respiratory Illness. VI. Frequency of and Relationship between Outbreaks of Coronavirus Infection

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Specimens of blood collected in Tecumseh, Michigan over a four-year period were studied for rise in antibody titer against coronavirus OC43. Peaks of infection were found in the winter and spring of 1966, 1968, and 1969; at other times, infections occurred sporadically. All age groups were involved, especially the very young. Rises in titer by CF and by HAI tests frequently did not occur together in the same individual. Agreement between the two tests was better in 1966 and 1969 than in the other years. A portion of the paired specimens showing rises in CF and/or HAI titer was tested by neutralization. Rises in neutralizing antibody were usually found in pairs collected in 1966 and 1969 but not in those collected in 1967 and 1968. The infecting viruses in 1966 and 1969 thus appeared more closely related to OC43 than did those in 1967 and 1968.

Coronaviruses are significantly involved in respiratory infections of man, especially in those occurring in the colder months of the year [1–4]. However, neither the total number of different coronaviruses nor their relative importance is known. Understanding of the behavior of these viruses has been hampered greatly by the difficulties in propagation encountered in the laboratory. Only one coronavirus, 229E, can be isolated in cell culture, and this is difficult [5]. A major contribution has been the adaptation of viruses originally isolated in organ culture to animals or cell cultures [6, 7]. Several serologic tests have thus been made possible, and infections can now be identified by rise in antibody titer [8, 9].

As part of the study of respiratory infections in Tecumseh, Michigan, specimens of blood were collected on a regular basis from families under surveillance [10]. By testing of specimens collected in early 1967, a large-scale outbreak of infection with coronavirus 229E was detected [11]. All segments of the population were involved, and infection was significantly associated with respi-

ratory illness. Specimens collected over four years have now been tested for infection with coronavirus OC43. The present report describes the occurrence of coronavirus infections during this period as determined by different serologic techniques.

Materials and Methods

Population studied and processing of specimens. Details of the surveillance methods used in Tecumseh have been described [12]. Families for study were selected randomly from those with children whose parents were under 46 years of age. Each family was followed for one year. Specimens were obtained for isolation of virus when a respiratory illness was reported within two days of onset [13]. Samples of blood were collected at time of recruitment, six months later, and at the end of the year of surveillance [10]. Because recruitment of families was staggered, specimens of blood were collected during all months of the year. Blood was drawn by venipuncture from individuals of approximately five years of age and older, and the specimens of serum were stored at −20 C. The specimens were collected from younger children on filter paper disks by finger stick. At least four disks were obtained at one time, and they were dried and stored at 4 C until use.

For CF and HAI tests, two disks were placed in 0.5 ml of veronal-buffered saline, and the blood was eluted for 18 hr at 4 C. Thereafter, the fluid

was completely expressed from the disks and inactivated at 56 C for 30 min. The eluate was centrifuged for 1 hr at 650 g, and the supernatant was considered to represent a 1:4 dilution. The specimens of serum were inactivated at 56 C for 30 min before dilution for CF, HAI, or neutralization tests.

Antigens used. OC43 antigen was prepared from a mouse brain-adapted strain obtained through the courtesy of Mr. H. S. Kaye, Center for Disease Control. The seed was propagated by intracerebral inoculation of suckling mice. Antigen was prepared as a 10% suspension, in phosphate-buffered saline for HAI tests, and in tryptose-phosphate broth for neutralization tests. For CF tests, antigen was prepared as a 20% suspension in veronal-buffered saline. The identity of each pool of virus was confirmed by CF with a homologous and a hyperimmune antiserum to murine hepatitis virus. The seed of 229E virus was originally obtained from Dr. Dorothy Hamre and was passed serially in WI-38 cell culture. Antigen for CF tests was prepared by inoculation of WI-38 bottles or tubes with undiluted virus. After incubation for 34–38 hr at 33 C the cultures were frozen and thawed three times, fluids were centrifuged, and the supernatant was used as antigen [11].

Serologic tests. CF and HAI tests were performed on all specimens of serum collected from members of 269 families. These families were selected randomly from within each of the four years of study so that all periods would be represented. The standard CF test was performed by a microplate technique with sheep erythrocytes, 4 units of antigen, and 1.8 units of complement. The HAI test was performed in microplates by the method of Kaye et al. [8]. Sera were serially diluted in phosphate buffered saline from the initial 1:4 dilution, and 4 units of antigen were added. After incubation for 30 min at 24 C 0.4% chick erythrocytes were added. The test was read after incubation for an additional 1 hr at 24 C. For both CF and HAI, all sera from the same individual were tested simultaneously. A significant rise in titer was considered to have occurred if antibody appeared in a 1:8 dilution when prior specimens did not have demonstrable antibody at a 1:4 dilution. A fourfold rise from a previous antibody titer was also considered significant.

Approximately 40% of serum pairs demonstrating a significant rise in antibody titer by CF and HAI were also tested by neutralization [9]. The

neutralization tests were performed in cultures of BS-C-1 cells. Cells were grown in Eagle's minimal essential medium (MEM) made in Hanks' salts with 10% fetal bovine serum. Before use, the cultures were washed three times with balanced salt solution, and they were maintained on serum-free MEM in Earle's salts. Virus was used at a dose of 100–320 $TCID_{50}/0.1$ ml. After inactivation at 56 C for 30 min the sera were serially diluted. To 0.3 ml of diluted sera, 0.3 ml of the appropriate dilution of virus was added, and the mixture was incubated at 24 C for 1 hr. Thereafter 0.2 ml of each mixture of serum and virus was inoculated into each of two cell-culture tubes. The tubes were placed in a roller drum and incubated at 33 C. After four days, rat erythrocytes were washed three times, and 0.2 ml of a 0.4% solution was added to each cell-culture tube. Tubes were examined microscopically after 45 min at 24 C, and end points were determined based on the presence of virus-specific hemadsorption.

Results

Occurrence of coronavirus infection 1965–1969. All specimens of serum collected from members of 269 families were studied by CF and HAI for rise in titer of antibody to coronavirus OC43. These specimens had been collected from November 1965 to June 1969. In nearly all cases, three specimens were obtained from each individual during each period of surveillance. To determine the temporal occurrence of infection with OC43-related viruses, the three specimens collected from each person were divided into two component pairs, each spanning a six-month period. The time of each pair was identified by the month of collection of the second serum of that pair. Results of the serologic tests are shown in figure 1. Rises in CF and HAI titers frequently were independent of each other. Therefore, each rise was counted without regard to the presence or absence of the other and is indicated as a separate curve in the figure. Bars indicate the percentage of total specimens tested in which the rises in titer by CF and HAI occurred together.

It has been reported that the principal periods of transmission of coronavirus are in the winter and early spring [2, 3]. This pattern could be seen when the CF and HAI curves for OC43 were examined. Frequent infections were detected by CF and HAI in sera collected during the six-
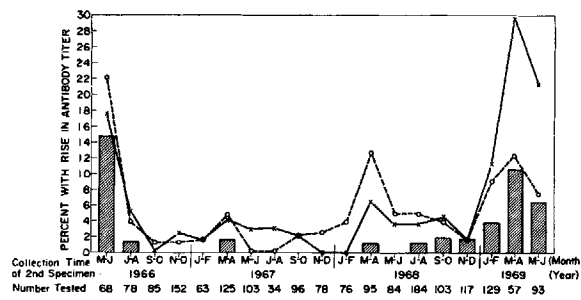
Figure 1. Incidence of coronavirus OC43 infection as determined by CF (O– – – –O) and HAI (X–——X) tests separately, and by both tests combined (▨), from 1966 through 1969.

month period ending May–June, 1966. These were the first specimens available for study, and it is possible that the period of infection began earlier. Thereafter the rates dropped, showed a suggestion of an increase in March–April 1967, the period of the 229E outbreak [11], and then remained low until March–April 1968. This peak of infection was detected by both serologic tests with the rate by CF twice that by HAI. After this point the rates dropped only to peak in 1969, again in March–April. During this outbreak rates of incidence were very high, with considerably more infections detected by HAI than by CF.

During the four years studied, 156 paired specimens showed a rise in titer by CF and/or HAI; of these, only 37 (23.7%) had a rise in titer by both techniques. This divergence in serologic results, which has been noted in previous studies of OC43 virus [4], was not uniformly spread over the full period. As shown by the relative height of the bars in figure 1, agreement between the two serologic tests was an infrequent occurrence except in May–June 1966 and again in 1969. During these periods the percentage of positive paired specimens in which the rises occurred together was 39.2%, but in the rest of the four years it was 9.8%. Even during March–April 1968, when a peak of infections occurred, there was still little correlation between the two tests.

Age-specific occurrence of infection. Results of past investigations have suggested that age may be a factor in producing differences in sensitivity of the CF and HAI tests; CF was found to be most sensitive when the population studied was composed of adults [9], and HAI was most sensitive when the population was made up exclusively of children [4]. The ages of individuals who exhibited the rises in titer were therefore examined,

as one means of exploring the divergence in serologic results. Results are given in table 1. The overall rate of infection for each age group was calculated by determining the total number of individuals who showed a rise in antibody titer by CF and/or HAI. The proportion of these infected individuals who had a rise in CF antibody was then calculated as was the proportion of infected individuals with a rise in HAI antibody. Since the intent was determination of the relative frequency of rise in titer detected by each test, persons with increases in both CF and HAI titer were counted in both categories. As shown in table 1, age did not appear to be a factor in determining the type of response. At all ages below 40 years, rises in titer were more frequently detected by HAI than by CF, but the difference was often not great. Only among individuals 40 years and older was this pattern reversed slightly. The total rates of infection (table 1) fell gradually as age increased, but not as precipitously as with other respiratory pathogens [10, 13]. A high annual rate of infection was observed among all adults studied.

Study of specificity of antibody response. Agreement between the HAI and CF tests was better in the first part of 1966 and in early 1969 than at other times, including the peak of infections in 1968. It seemed possible that agents similar to OC43 might have been responsible for the 1966 and 1969 outbreaks and more distantly related agents for activity at other times. Such a hypothesis could be tested by evaluation of the specificity of observed rises in titer through use of the recently described neutralization test [9].

Approximately 40% of the serum pairs that

Table 1. Significant rises in titer of antibody to coronavirus OC43 during 1966–1969, and proportion of rises detected by CF or HAI tests.

Age group (years)	No. tested (person-years)	No. infected* (%)	% with CF rise†	% with HAI rise†
5–9	158	35 (22.2)	54.2	74.2
10–14	186	38 (20.4)	60.5	68.4
15–19	75	12 (16.0)	58.3	75.0
20–29	152	23 (15.1)	47.8	65.2
30–39	254	34 (13.4)	58.8	61.8
40+	85	14 (16.5)	64.3	50.0
Total	910	156 (17.1)	57.1	66.7

* Number of persons with rise in antibody titer by CF alone, HAI alone, or both.

† Number of rises by indicated test/number of persons infected × 100.

showed rises in antibody by CF and HAI were tested by neutralization. They were selected randomly from within individual respiratory years so that all periods would be represented; the respiratory year has been defined as extending from September to the following August and is identified by the date in which most of the year fell. Results are given in table 2. Period of collection was the most important predictor of presence or absence of rise in titer of neutralizing antibody. Since the 1966 and 1969 periods, on the one hand, and the 1967 and 1968 periods, on the other, resembled each other, they have been combined. In serum pairs in which the second specimen was collected in 1967 and 1968, and in which there was a rise in CF titer alone, no rises in titers of neutralizing antibody were found. In the pairs from this period with a rise in HAI antibody, there was an occasional rise in titer of neutralizing antibody, and among the few pairs showing an increase in titer by both CF and HAI, 28.6% had a rise in titer by neutralization. The situation with the 1966 and 1969 specimens was quite different. The specimens with rises in CF or HAI titer both showed 66.7% agreement with the neutralization test. All specimens with a combined CF and HAI increase also had a rise in titer by neutralization. Thus, the 1966 and 1969 period was distinct from the 1967 and 1968 period in having not only more frequent combined CF and HAI rises in antibody titer, but also because all rises in antibody titer were more likely associated with rises in neutralization titer. These findings confirm that the 1966 and 1969 outbreaks were caused by viruses more closely related to OC43 than the viruses involved in infections during the 1967 and 1968 period.

During 1967, a large-scale outbreak of 229E

virus was detected in Tecumseh [11]. It is possible that some of the rises in titer of antibody to OC43 during 1967 might represent cross-reactions from an actual infection with 229E. Therefore, the 21 sera with HAI or CF rises in titer of OC43 antibody in 1967 were tested by CF against a 229E antigen. Of those tested, only two (9.5%) showed a rise in titer. Since this CF test is sensitive when used with sera collected close in time to infection, it is probable that the agent involved in producing the rises in OC43 titer during 1967 was other than 229E.

Study of the 1969 outbreak. The outbreak detected serologically in late 1968 and the first part of 1969 not only was large scale, but was also probably caused by an agent related to OC43. To define better the occurrence of infection during this period, serologic data from the 106 families whose second and third specimens of blood were collected during the period from August 1968 to June 1969 were examined separately. To complete the study of these families the blood specimens collected on filter paper disks from the younger children were also tested by CF and HAI against OC43 antibody. Persons were identified as infected if they had a significant increase in antibody titer by CF or HAI test, since during the period both were equally likely to agree with the neutralization test. The results, shown in table 3, calculated on the basis of person-years of observation, indicate the great frequency of infection with the agent. Those under five years of age showed the highest rates of infection, and the rates fell slightly as the age of children increased. Among adults, the 30–39-year age group had the highest rates. The very high rate of infection in adults was similar to that observed in the commu-

Table 2. Agreement of neutralization test with rises in titer to coronavirus OC43 by CF and HAI tests.

Type of rise in antibody titer	1967 and 1968		1966 and 1969		Total	
	No.	%	No.	%	No.	%
CF only	0/11*	0	6/9	66.7	6/20	30.0
HAI only	2/11	18.2	10/15	66.7	12/26	46.2
Both CF and HAI	2/7	28.6	12/12	100.0	14/19	73.7
Total	4/29	13.8	28/36	77.8	32/65	49.2

Header note: Respiratory years in which serum was collected

* Number of rises in titer by neutralization test per number in group.

Table 3. Age-specific occurrence of infection with coronavirus OC43 during 1968–1969 as determined by CF and HAI tests.

Age (years)	No. tested (person-years)	No. infected* (%)
0–4	72	21 (29.2)
5–9	84	24 (28.6)
10–14	59	16 (27.1)
15–19	17	4 (23.5)
20–29	71	13 (18.3)
30–39	84	22 (26.2)
40+	27	6 (22.2)
Total	414	106 (25.6)

* Number of persons with rise in antibody titer by CF alone, HAI alone, or both CF and HAI.

nity during the 229E outbreak of 1967 [11]. However, the marked involvement of children under age five was not noted at that time nor has it been appreciated in other studies of coronavirus infections. Relative frequency of CF and HAI response among these small children was like that of the rest of the population during this period, with approximately two and a half times as many rises in titer detected by HAI compared with those detected by CF.

Familial aggregation of infection was frequent, as has been observed with other respiratory viruses [10]. As many as four infections were observed in a single family. During the 229E outbreak in 1967, in spite of the high rate of infection and the fact that infection was significantly associated with illness, no peak in incidence of illness could be identified [11]. Similarly, in the present outbreak no peak of incidence of illness could be identified in February or March 1969, the likely period of peak dissemination of virus.

Discussion

Investigations on the behavior in populations of respiratory pathogens ideally use as methods both isolation of infecting viruses and testing of sera. An isolate can be used as antigen in the serologic procedures and rises in antibody titer related directly to the activity of that virus. Unfortunately, with most of the coronaviruses isolation is difficult [14, 15]. Seroepidemiology is thus the only technique available for study of infections caused by these agents in large numbers of people. The relation between the virus chosen for antigen and the virus actually infecting the population can thus be determined only by inference.

It is known that rises in titer of heterologous antibody do occur [16–18]; however, the exact nature and frequency of these cross-reactions have not been determined. Only limited numbers of natural infections have been studied by both isolation and serology, a situation in which the critical observations on antibody response should be possible; thus basic gaps in essential knowledge exist.

Use of parallel CF and HAI procedures was based on the possibility that heterologous cross-reactions might be encountered; it seemed more likely that there would be agreement between the two tests when the infecting organism was closely related to OC43. Disagreement could be a result of either lack of close relation between the infecting organism and OC43 or the fact that one of the serologic tests was more sensitive than the other. However, in terms of differences in agreement from year to year, the former factor was operative. The viruses in circulation in 1966 and 1969 were clearly more closely related to OC43 than those in circulation in 1968, a difference that would not have been detected had both CF and HAI not been used. Neutralization tests confirmed the difference, and indicated that in the individual case during a period of prevalence of agents related to OC43, even if CF and HAI do not agree, it is likely that an infection with the agent actually did occur. It may also be speculated that some minor differences existed between the 1966 and 1969 viruses, since CF was more frequently positive in the former period and HAI in the latter.

The annual periodic pattern of coronavirus infections in general is clear from the Tecumseh data when the 1967 229E outbreak is included, and is in agreement with the findings of others [2, 3, 8]. The outbreaks take place in the period of late winter through early spring, with sporadic infections at other times of the year. A longer cycle for a specific virus type can also be deduced from the data. The period between the OC43-like outbreaks was three years. Periods of high incidence of 229E infections have also been reported to recur on a two- or three-year cycle [19]. The repetitive activity of specific coronaviruses and the fact that they cause large-scale outbreaks of infection is in marked contrast to the situation with the rhinoviruses. Cycling of these agents occurs but not on any sort of a regular or predictable basis. However, the number of infections caused at any one time is low, and many agents take part in a single peak of illness [20]. The fact that coronaviruses individually seem to cycle more regularly and cause many infections suggests that there are a reasonably small number of these agents in existence. This has positive implications in terms of control.

However, reinfection with coronaviruses is a very frequent event. In the present study, 81.5% of the infections studied by the neutralization tests occurred despite prior neutralizing antibody. Others have also noted that antibody is usually present before a rise in titer and have thus called into question the protective value of this circulating neutralizing antibody [9, 19]. The occurrence of reinfections explains, in part, the age-specific

patterns observed with OC43 infection in Tecumseh. Antibody is acquired early in life, as shown by high incidence of infection in children less than five years of age. Subsequently, reinfection is frequent in older children and in adults. This frequency of infection in adults may explain the relation of coronaviruses to exacerbations of chronic bronchitis [21].

The proportion of the OC43 infections detected serologically that were expressed as clinical illness is difficult to assess. Kaye et al. found that 47.3% of infections detected in children by HAI were accompanied by illness [4]. If such a ratio prevailed in Tecumseh in the 1969 outbreak, it would mean that 12.1% of all individuals experienced a symptomatic infection during the course of a single season. Clearly the coronaviruses make a major contribution to the overall burden of respiratory illnesses. Although illnesses caused appear to be mild, their frequency and possible relation to chronic respiratory disease makes further understanding of their behavior essential.

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