*Epidemiol. Infect.* (1994), **113**, 143–152



# Measurement of IgA responses following Norwalk virus infection and other human caliciviruses using a recombinant Norwalk virus protein EIA

S. P. PARKER AND W. D. CUBITT

*Department of Virology, Institute of Child Health and Hospital for Sick Children, 30, Guilford Street, London WC1 1EH*

(*Accepted 8 February 1994*)

## SUMMARY

An enzyme immunoassay employing recombinant Norwalk virus capsid protein was evaluated for the measurement of IgA responses. Tests on 23 volunteers and patients known to have been infected with Norwalk virus (NV) showed that 19 developed significant IgA responses, 2 had unchanging levels of IgA and 2 failed to respond. There was no evidence of IgA responses to NV following infection with Hawaii or Snow Mountain-like viruses.

Tests on sera from patients involved in outbreaks associated with eating contaminated shellfish suggest that some patients may have been infected with more than one strain of calicivirus. The use of the rNV EIA for measuring IgA and IgG responses in patients involved in a major outbreak of food poisoning affecting hospital staff indicated that the causative agent was probably NV.

## INTRODUCTION

Although Norwalk virus (NV) was first recognized as a cause of extensive outbreaks of diarrhoea and vomiting over 20 years ago [1] diagnosis of infections was confined to a few research laboratories in the USA which had access to antigen and sera obtained from human volunteer studies. The recent success in sequencing the entire NV genome [2, 3] and subsequent expression of self assembling NV capsids in baculovirus [4] has enabled enzyme immunoassays (EIA) to be developed to measure antibody responses to NV [5]. Previous studies with human reagents indicated that anamnestic responses to NV occurred in some individuals infected with other strains of small round structured viruses (SRSV) [6] and caliciviruses (HuCV) [7]. The recombinant NV enzyme immunoassay (rNVEIA) has been shown to be highly sensitive and specific for NV [5] but slight IgG responses to NV were demonstrated in volunteers challenged with Hawaii and Snow Mountain agent when sera were tested at low dilutions [8]. In contrast IgA responses only occurred in the volunteers challenged with NV [8].

The present study describes the evaluation of an rNVEIA to measure IgA responses in volunteers challenged with NV, and its application to the study of community outbreaks of gastroenteritis associated with SRSV and HuCV which have occurred in the United Kingdom.

## MATERIALS AND METHODS

### *Norwalk virus reference sera*

*Volunteers.* Acute and convalescent sera were obtained from 14 adult volunteers who had been challenged with NV.

*Community outbreaks.* Acute and convalescent sera were available from adults infected with NV in an oyster-associated outbreak which occurred in Australia in 1978 [9], an outbreak in a hospital in Manchester in 1979, and two outbreaks which occurred in the USA in 1987 at the White Pine Ranch and in Philadelphia.

*SRSV UK3/BARNET/86/UK.* Acute and convalescent sera from two elderly patients in a geriatric home involved in an outbreak of diarrhoea and vomiting which occurred in 1986 [10].

*SRSV/CAMBRIDGE/92/UK.* Sera from adults involved in an oyster-associated outbreak in which 50/100 participants presented with symptoms. Sequence analysis of the RNA dependent RNA polymerase region of this strain (SRSV-925/CAMBRIDGE/92/UK) showed more homology (88%) with Snow Mountain agent than with NV (60%) [11].

*HUCV UK2/PORTSMOUTH/80/UK.* Acute and convalescent sera from five patients aged 77–92 years infected with morphologically typical caliciviruses during an outbreak in a residential home for the elderly [12].

*HUCV UK4/COLCHESTER/82/UK.* Acute and convalescent sera from adults involved in an oyster-associated outbreak in which 10/17 patients became ill [13].

*HUCV UK4/TOWER HAMLETS/83/UK.* Acute and convalescent sera from adults involved in an oyster-associated epidemic in which $> 500/1700$ people attending a series of receptions became ill [14].

All the sera above had previously been tested for the presence of NV specific IgG using a rNV EIA [8].

*SRSV/LONDON/88/UK.* Paired sera were available from 19 medical and nursing staff who became ill 24–48 h after attending a dinner in January 1988. Examination of stools from affected patients revealed the presence of SRSV (Dr H. Appleton, Central Public Health Laboratory, Colindale, UK, personal communication). Sera had been stored at $-20$ °C because no diagnostic tests were readily available at the time of the outbreak.

### *Recombinant EIA for Norwalk IgG*

Serum samples from the outbreak caused by SRSV/London/88/UK were tested for the presence of specific IgG using a recombinant NV protein as antigen in an EIA as described previously [15]. Sera were tested at a dilution of 1/1000 in antigen coated and uncoated wells of a microtitre plate. NV-specific IgG was detected using horseradish peroxidase conjugated rabbit anti human IgG (Dako Ltd) and TMB substrate. Samples with an OD $\geqslant 0{\cdot}1$ (coated-uncoated well) were considered to be positive.

### *Removal of IgG from serum using protein G*

Sera were initially treated with Protein G Sepharose (Pharmacia LKB) to remove IgG. 100 $\mu$l of a 1/5 dilution of serum in phosphate buffered saline (PBS) was shaken with 200 $\mu$l of a 1/5 dilution of prewashed protein G in a sealed

microcentrifuge tube overnight at room temperature. Each sample was then spun at 13000 rpm in a microfuge for a period of 5 min and the supernatant removed and stored at 4 °C until tested. The final dilution of the sera was approximately 1/15. All samples were run in the rNV IgG EIA to ensure that IgG had been removed by the protein G treatment.

*Recombinant EIA for Norwalk IgA*

Sera treated with protein G were further diluted in diluent buffer (1·0% Marvel skimmed milk in PBS) to give a final dilution of 1/50 of the original serum and 100 $\mu$l aliquots were added to coated and uncoated wells and incubated at 37 °C for 2 h. After plates had been washed three times in PBS/Tween 20, 100 $\mu$l of a 1/2000 dilution of horseradish peroxidase conjugated rabbit anti-human IgA (Dako Ltd) was added to each well and incubated for 2 h at 37 °C. Plates were washed three times as before and once in distilled water before 100 $\mu$l of TMB substrate was added to each well. The reaction was stopped after 10 min by the addition of 50 $\mu$l 2N $H_2SO_4$ and the optical densities read at $A_{450}$ nm. Samples with ODs of $\geqslant$ 0·2 were considered to be positive.

Pre- and post-challenge sera from volunteers were titrated out to an end point to investigate the relationship between OD at 450 nm and concentration of NV IgA.

## RESULTS

*Volunteers*

The results of tests for NV IgA responses in the 14 volunteers challenged with Norwalk virus are shown in Table 1. Eleven of 14 volunteers showed a significant rise in NV IgA. By plotting the OD at 450 nm of each convalescent volunteer sample titrated to end point, a straight line relationship was observed between the log dilution and absorbance over the OD range 0·2–1·4. A prozone effect was observed at a dilution of 1/50 in some samples with OD > 1·4 which titrated out to $\geqslant$ 30000.

Volunteers 1, 7 and 8 who seroconverted following challenge developed significant IgA responses. The others had evidence of IgG in their pre-challenge sera; eight of them showed strong IgA responses. Volunteers 10 and 11 who had high levels of pre-existing IgG (8000, $\geqslant$ 64000) and pre-existing IgA and showed no significant IgA response. Repeated tests on the sera from volunteer 13 showed a significant IgG response but we failed to detect evidence of an IgA response.

*Community outbreaks of Norwalk virus infection*

The IgA responses in 9 individuals involved in 4 outbreaks are shown in Table 2. Eight patients showed significant IgA responses, but patient 7 involved in the White Pine Ranch outbreak developed a significant IgG response but failed to mount an IgA response.

*Outbreaks associated with other strains of SRSV/HuCV*

High levels of IgG (8000–16000) and IgA (OD 0·88–0·96) were detected in paired sera obtained from one patient involved in the Cambridge outbreak. The other patient had unchanging levels of NV IgG (2000–2000) and showed no evidence of an IgA response (OD 0·18–0·15).

Table 1. *IgA responses in pre and post challenge sera from adult volunteers infected with NV*

| Source | Case | Code* | rNV IgG titre | rNV IgA $A_{450}$ nm† | rNV IgA titre‡§ |
|---|---|---|---|---|---|
| Baylor USA | 1 | 505–1 | < 1000 | 0·02 | < 50 |
| | | 505–4 | 64000 | 1·04‡ | INS |
| | 2 | 555–1 | 2000 | 0·06 | < 50 |
| | | 555–4 | 128000 | 1·07‡ | INS |
| Mass. USA | 3 | MC | 1000 | 0·73 | 500 |
| | | | ⩾ 64000 | 1·31‡ | ⩾ 30000 |
| | 4 | BE | 4000 | 0·62 | 1000 |
| | | | ⩾ 64000 | 1·17‡ | 15000 |
| | 5 | DO | 1000 | 0·08 | < 50 |
| | | | ⩾ 64000 | 1·34‡ | ⩾ 30000 |
| | 6 | PE | 2000 | 0·11 | < 50 |
| | | | ⩾ 64000 | 1·51‡ | ⩾ 30000 |
| | 7 | FR | < 1000 | 0·09 | < 50 |
| | | | ⩾ 64000 | 1·39‡ | ⩾ 30000 |
| | 8 | WE | < 1000 | 0·12 | < 50 |
| | | | 32000 | 1·30‡ | 15000 |
| Roch. USA | 9 | 6 | 4000 | 0·08 | < 50 |
| | | | 256000 | 1·27‡ | ⩾ 30000 |
| Atlanta USA | 10 | GVS1731 | 8000 | 0·52 | 500 |
| | | | ⩾ 64000 | 0·61 | 500 |
| | 11 | GVS1973 | ⩾ 64000 | 1·30 | 15000 |
| | | | ⩾ 64000 | 1·23 | 4000 |
| | 12 | VGL4349 | 4000 | 0·68 | 500 |
| | | | ⩾ 64000 | 1·22‡ | 4000 |
| | 13 | VGL4361 | 2000 | 0·08 | < 50 |
| | | | ⩾ 64000 | 0·12 | < 50 |
| | 14 | VGL4367 | 4000 | 0·53 | 250 |
| | | | ⩾ 64000 | 0·97‡ | 2000 |

* Coded sera previously tested in NV assay [5].
† OD at 1/50 serum dilution; ⩾ 0·2, IgA positive.
‡ Significant rise in IgA.
§ Highest dilution with an OD of 0·2.

There was no evidence of an IgA response in sera obtained from patients involved in the Barnet outbreak which was caused by a virus antigenically related to Hawaii agent. However, both patients had evidence of unchanging levels of NV specific IgG (32000, 4000) and IgA (OD 0·76, 0·33) in acute and convalescent phase sera. A similar situation was found in the elderly patients involved in the Portsmouth outbreak where all the patients were shown to have IgA in their acute phase sera but there was no evidence of significant IgG or IgA responses following infection.

In the Colchester outbreak the only symptomatic individual who developed an IgG response to NV (2000–32000) also produced an IgA response (OD 0·05–0·45). The other 3 symptomatic patients and 6 asymptomatic adults who attended the meal had evidence of unchanging levels of IgG and IgA in sera collected immediately after the outbreak and 10–14 days later. The highest level of IgA (OD 0·77–0·78) was detected in the patient with highest titres of NV IgG (64000–

https://doi.org/10.1017/S0950268800051566 Published online by Cambridge University Press

Table 2. *IgA responses in community outbreaks of NV*

| Source | Case | Code | rNV IgG titre | rNVIgA $A_{450}$nm* |
|---|---|---|---|---|
| Melbourne, Australia | 1A | 9177 | 8000 | 0·28 |
| | C | 10577 | 256000 | 0·87† |
| | 2A | 9176 | 2000 | 0·09 |
| | C | 10584 | 128000 | 0·26† |
| Manchester, UK | 3A | 4712 | 4000 | 0·06 |
| | C | 11023 | 64000 | 0·30† |
| | 4A | 10425 | 4000 | 0·29 |
| | C | 11024 | 32000 | 0·90† |
| White Pine, USA | 5A | 3934 | < 1000 | 0·04 |
| | C | | ⩾ 16000 | 0·64† |
| | 6A | 3935 | < 1000 | 0·08 |
| | C | | ⩾ 16000 | 0·66† |
| | 7A | 3998 | 2000 | 0·0 |
| | C | | ⩾ 16000 | 0·05 |
| Philadelphia, USA | 8A | 602 | 1000 | 0·03 |
| | C | | ⩾ 16000 | 0·43† |
| | 9A | 621 | 8000 | 0·28 |
| | C | | ⩾ 16000 | 0·60† |

* OD ⩾ 0·2 IgA positive.
† Significant rise in IgA.

64000). There was one asymptomatic individual who developed a significant IgG response (2000–64000) but had no evidence of NV IgA in his sera (OD 0·06/0·03–0·04/0·06).

The results of tests on paired sera from symptomatic and asymptomatic individuals in the Tower Hamlets outbreak are shown in Table 3. Six of the 8 symptomatic group showed rising levels of IgA between their acute and convalescent phase sera and 1/6 of the asymptomatic group. There was one symptomatic patient (Case 6) who developed a significant IgG response but failed to develop IgA.

The results of tests for IgG and IgA responses in 19 symptomatic individuals involved in the London outbreak are shown in Table 4. Seventeen of 19 individuals had evidence of NV IgG and NV IgA in their initial serum samples collected between 7 and 11 days after the meal. Significant IgG responses to NV occurred in seven patients. Six of these patients also showed marked increases in the levels of IgA between their first and second samples but only a trace of IgA (OD = 0·2) could be detected in the convalescent sample obtained from the other person. No significant increase in NV IgG or IgA levels was found in the other 12 symptomatic individuals but many of them had high levels of both classes of antibody in their sera.

## DISCUSSION

The demonstration of serum IgA responses in adult volunteers challenged with NV was in agreement with the results of Erdman and colleagues [16] who used antigen from volunteers and Treanor and colleagues [8] who used rNV capsid antigen and confirms the validity of our assay. However, in contrast to the results

Table 3. *IgA responses in the HuCV/UK4/TH/83 outbreak*

| Case | Code | rNV IgG titre | rNV IgA $A_{450}$nm* |
|---|---|---|---|
| Symptomatic group | | | |
| 1A | 1206 | < 1000 | 0·13 |
| C | 2459 | 1000 | 0·45† |
| 2A | 1316 | 2000 | 0·10 |
| C | 1914 | 32000 | 0·61† |
| 3A | 1204 | 4000 | 0·31 |
| C | 1908 | 8000 | 0·69† |
| 4A | 1506 | 1000 | 0·03 |
| C | 2198 | 8000 | 0·49† |
| 5A | 1504 | 1000 | 0·23 |
| C | 2197 | 4000 | 0·38† |
| 6A | 1209 | 4000 | 0·31 |
| C | 2018 | 128000 | 0·38 |
| 7A | 1213 | 32000 | 0·34 |
| C | 1915 | 256000 | 0·51† |
| 8A | 1503 | 4000 | 0·00 |
| C | 2196 | 16000 | 0·15 |
| Asymptomatic group | | | |
| 9A | 1499 | 1000 | 0·05 |
| C | 2193 | < 1000 | 0·04 |
| 10A | 1265 | 1000 | 0·05 |
| C | 1927 | < 1000 | 0·02 |
| 11A | 1260 | 8000 | 0·40 |
| C | 1930 | 8000 | 0·38 |
| 12A | 1259 | < 1000 | 0·11 |
| C | 1918 | < 1000 | 0·04 |
| 13A | 1269 | 8000 | 0·28 |
| C | 1925 | 16000 | 0·51† |
| 14A | 1313 | 2000 | 0·30 |
| C | 1913 | 4000 | 0·27 |

* OD $\geqslant$ 0·2 IgA positive.
† Significant rise in IgA.

of Treanor and colleagues [8] we found one volunteer who mounted a significant IgG response but IgA could not be detected in pre-challenge or post-challenge sera. A similar situation was observed in one patient involved in a community outbreak of NV in the USA and one patient in the Tower Hamlets outbreak. This indicates that not all patients infected with NV produce detectable levels of IgA in their sera.

The study of patients infected with Hawaii-like and Snow Mountain-like agents and HuCV strain UK2 demonstrated that there was no anamnestic IgA response when challenged with strains antigenically distinct from NV although the majority had evidence of pre-existing NV specific IgA in their acute phase sera. Similar observations have recently been reported by Treanor and colleagues [8] in volunteers challenged with Snow Mountain or Hawaii agents.

The results of tests on patients involved in outbreaks associated with consumption of shellfish were less clear cut. In the Colchester outbreak which was associated with HuCV(UK4) the one symptomatic patient who developed a significant IgG response also developed an IgA response. However an asymptomatic individual who had a significant IgG response showed no evidence of IgA in

Table 4. *IgG and IgA responses in the SRSV/LONDON/88/UK outbreak*

| Code | Days post challenge | rNV IgG titre | rNV IgA $A_{450}$ nm* |
|---|---|---|---|
| 1A | 7 | 2000 | 0·35 |
| C | 18 | 128000 | 0·94† |
| 6A | 7 | 4000 | 0·14 |
| C | 24 | 64000 | 0·82† |
| 7A | 8 | 1000 | 0·12 |
| C | 32 | 4000 | 0·20 |
| 8A | 8 | 1000 | 0·41 |
| C | 18 | 4000 | 0·91† |
| 10A | 12 | 8000 | 0·14 |
| C | 16 | 32000 | 0·32† |
| 12A | 8 | 8000 | 0·42 |
| C | 18 | 32000 | 0·73† |
| 23A | 8 | 8000 | 0·55 |
| C | 15 | 64000 | 1·12† |
| 3A | 8 | 128000 | 0·71 |
| C | 22 | 64000 | 0·85 |
| 4A | 7 | 4000 | 0·06 |
| C | 18 | 8000 | 0·13 |
| 21A | 8 | 4000 | 0·78 |
| C | 18 | 8000 | 0·90 |
| 5A | 11 | 32000 | 0·91 |
| C | 18 | 32000 | 0·85 |
| 11A | 11 | 64000 | 0·81 |
| C | 24 | 64000 | 0·52 |
| 15A | 7 | 32000 | 0·74 |
| C | 25 | 32000 | 0·53 |
| 17A | 7 | 4000 | 0·26 |
| C | 23 | 8000 | 0·44 |
| 18A | 11 | 16000 | 0·59 |
| C | 23 | 16000 | 0·44 |
| 19A | 10 | 128000 | 0·54 |
| C | 24 | 128000 | 0·47 |
| 22A | 8 | 4000 | 0·18 |
| C | 21 | 4000 | 0·16 |
| 24A | 11 | 32000 | 0·75 |
| C | 22 | 64000 | 0·67 |
| 13A | 7 | < 1000 | 0·03 |
| C | 18 | < 1000 | 0·03 |

* OD $\geqslant$ 0·2 IgA positive.
† Significant rise in IgA.

her sera. In view of the results obtained from the volunteer studies the results are highly suggestive of dual infection with HuCV(UK4) and NV. A similar situation was observed in the Tower Hamlets outbreak in which the majority of symptomatic patients developed responses to both viruses. A point of interest is that two individuals who showed no significant IgG response when tested by RIA and rNV EIA [5] showed a significant IgA response. One of these patients who

https://doi.org/10.1017/S0950268800051566 Published online by Cambridge University Press

displayed symptoms was shown to be excreting HuCV UK4 [14]. Recently Monroe and colleagues [17] have reported a similarly complex pattern of IgA and IgG responses to NV in patients involved in the outbreaks in the USA.

In the London outbreak, 7/19 symptomatic individuals developed significant IgG and IgA responses which enabled the causative agent to be diagnosed as NV. The majority of the other patients had high levels of IgG and IgA in samples collected approximately 1 week after the onset of symptoms and 10 days later. The delay in collecting the initial serum samples makes it impossible to be certain whether the other 12 people developed a response to NV or not. The measurement of IgA responses to NV provides a useful confirmatory test to the rNV IgG EIA in diagnosing NV infections and in differentiating between outbreaks caused by other caliciviruses.

The presence of pre-existing IgG and IgA in many patients makes it essential that paired samples are collected which often proves impossible to achieve. An alternative approach to diagnosis is reverse transcription PCR for detection of antigen in faeces but initial results show there is considerable genomic variation within the human caliciviruses, even within the conserved RNA polymerase region of ORF1 [11, 18].

## ACKNOWLEDGEMENTS

We would like to thank Professor Estes and Dr Jiang for providing us with recombinant Norwalk virus capsid antigen and Drs R. Glass, J. Herrmann, D. Lewis, P. Madore, and R. Schnagl for providing us with sera from volunteers and community outbreaks.

## REFERENCES

1. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Channock RM. Visualization by immune electronmicroscopy of a 27 nm particle associated with acute non bacterial gastroenteritis. J Virol 1972; **10**: 1075–81.
2. Jiang X, Graham DY, Wang K, Estes MK. Norwalk virus genome and characterization. Science 1990; **50**: 1580–3.
3. Jiang X, Wang M, Wang K, Estes MK. Sequence and genomic organization of Norwalk virus. Virol 1993; **195**: 51–61.
4. Jiang X, Wang M, Graham DY, Estes MK. Expression, self assembly and antigenicity of the Norwalk virus capsid protein. J Virol 1992; **66**: 6527–32.
5. Parker SP, Cubitt WD, Jiang X, Estes MK. Efficacy of a recombinant Norwalk virus protein enzyme immunoassay for the diagnosis of infections with Norwalk virus and other human 'candidate' caliciviruses. J Med Virol 1993; **41**: 179–84.
6. Madore HP, Treanor JJ, Baja R, Dolin R. Antigenic relationships among the Norwalk-like viruses by serum antibody rises. J Med Virol 1990; **32**: 96–101.
7. Cubitt WD, Blacklow NR, Hermann JE, Nowak NA, Nakata S, Chiba S. Antigenic relationships between human caliciviruses and Norwalk virus. J Infect Dis 1987; **156**: 806–13.
8. Treanor JJ, Jiang X, Madore HP, Estes MK. Subclass specific antibody responses to recombinant Norwalk virus capsid antigen in adults infected with Norwalk, Snow Mountain, or Hawaii virus. J Clin Microbiol 1993; **31**: 1630–4.
9. Murphy AM, Grohmann GS, Christopher PJ, Lopez WA, Davey GR, Millsom RH. An Australia-wide outbreak of gastroenteritis from oysters caused by Norwalk virus. Med J Aust 1979; **2**: 329–33.
10. Lewis DC. Three serotypes of Norwalk-like virus demonstrated by solid-phase immune electronmicroscopy. J Med Virol 1990; **30**: 77–81.

11. Cubitt WD, Jiang X, Wang J, Estes MK. Sequence similarity of human caliciviruses and small round structured viruses. J Med Virol 1994. In press.
12. Cubitt WD, Pead PJ, Saeed AA. A new serotype of calicivirus associated with an outbreak of gastroenteritis in a residential home for the elderly. J Clin Pathol 1981; **34**: 924–6.
13. Cubitt WD. Human caliciviruses. Characterization and epidemiology. [dissertation]. London: Middlesex Hospital, London University, 1985: 159–63.
14. Gill ON, Cubitt WD, McSwiggan DA, Watney BM, Bartlett CLR. Epidemic of gastroenteritis caused by oysters contaminated with small round structured viruses. B M J 1983; **287**: 1532–4.
15. Parker SP, Cubitt WD, Jiang XJ, Estes MK. Seroprevalence studies using a recombinant Norwalk virus protein enzyme immunoassay. J Med Virol 1994; **42**: 146–50.
16. Erdmann DD, Gary GW, Anderson LJ. Serum immunoglobulin A response to Norwalk virus infection. J Clin Microbiol 1989; **27**: 1417–18.
17. Monroe SS, Stine SE, Jiang X, Estes MK, Glass RI. Detection of antibody to recombinant Norwalk virus antigen in specimens from outbreaks of gastroenteritis. J Clin Microbiol 1993; **31**: 2866–72.
18. Green J, Norcott JP, Lewis D, Arnold C, Brown DWG. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction sequencing. J Clin Microbiol 1993; **31**: 3007–12.