Research article

Gastroenteritis outbreaks associated with Norwalk-like viruses and their investigation by nested RT-PCR

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Abstract

Background: Norwalk-like viruses are the most common cause of gastroenteritis outbreaks and sporadic cases of vomiting and diarrhoea. In healthy individuals infection is often mild and short-lived but in debilitated patients infection can be severe. It is essential that the virus laboratory can offer a sensitive and specific test, delivered in a timely manner.

Methods: We have developed a nested reverse transcriptase PCR based on published primers against the RNA polymerase gene and after comparison with electronmicroscopy used the assay to investigate 31 outbreaks of gastroenteritis. These were in diverse situations including nursing homes, small district hospitals, large general hospitals, a ferry ship, hotels, restaurants and staff canteens.

Results: A positive diagnosis was made in 30/31 outbreaks investigated giving an overall outbreak positive detection rate of 97%. At an individual patient level there was a positive diagnostic rate of 11.5% in a large hospital environment to 100% in smaller outbreak situations. The average patient positive rate was 34%. In addition we investigated 532 control faecal specimens from adults. Of these 530 were negative and 2 were repeatedly positive.

Conclusions: It is essential that insensitive electronmicroscopy is replaced with the more sensitive reverse transcription PCR assays. These tests should be made available "on call" at weekends and public holidays. It is also important that outbreaks of NLV infection are monitored using sensitive RT-PCR assays so that the laboratory information can be used in ascertaining the spread and duration of the outbreak

Background

Norwalk-like viruses (NLV) formerly known as small round structured viruses (SRSV) are the commonest cause of epidemic gastroenteritis outbreaks and also sporadic cases of community acquired vomiting and diarrhoea in adults and children [1–3]. They are highly infectious with transmission being linked to contaminated food, water and environmental contamination the latter often caused by vomiting [4]. In healthy individuals NLV infection is often mild and short-lived but in debilitated

hospitalised patients infection can be severe or occasionally life threatening; reports suggest that the incidence of NLV infection is increasing [5–7]. It is not unusual for NLV outbreaks to occur simultaneously in different hospital and community institutions, leading to a period of sustained demand on the local virus laboratory. A definitive diagnosis is particularly important in the hospital environment where cost implications and the associated disruption of planned procedures can be particularly onerous. Effective management of community and hospital outbreaks of gastroenteritis commonly requires the formation of a multi-professional infection control team and can result in ward and hospital closure [7,8]. An urgent response from the virus laboratory is often pivotal to the investigation of a given outbreak. Therefore it is essential to use a sensitive, specific test with a rapid turn around time. Nested reverse transcriptase polymerase chain reaction (nRT-PCR) is a highly sensitive technique for the detection of NLV [8]. The genome of NLVs contain a positive-sense polyadenylated single-stranded RNA of approximately 7.6 kb with 3 major open reading frames (ORF). Although there is considerable diversity among strains of NLV the RNA dependent RNA polymerase gene of NLV in ORF1 is highly conserved [9–11]. We have developed a nRT-PCR based on published primers against the polymerase gene and after comparison of the assay against electronmicroscopy (EM) have introduced it for the routine investigation of NLV associated gastroenteritis. We report the use of this assay for the management of gastroenteritis outbreaks associated with NLV.

Results

Validation of nRT-PCR

The 14 known NLV positive specimens associated with 4 different outbreaks of NLV infection were detected by nRT-PCR. Eight were first and second round positive and 6 were second round positive. The 4 adult specimens known to be NLV RT-PCR negative (Central Public Health Laboratory, Colindale, and London) and the 4 specimens from children with rotavirus infection were confirmed negative by nRT-PCR.

Virological investigation of non-outbreak specimens

Faecal specimens from 532 adult hospitalised patients who were not involved in outbreak situations were investigated at the same time as specimens from outbreak situations. These were included and used as protocol controls. Of these 530 were negative by nRT-PCR and 2 were repeatedly positive.

Virological investigation of outbreaks

The results for 31 outbreaks of gastroenteritis are presented in Table 12. The first 2 outbreaks were tested by nRT-PCR and EM. Subsequent outbreaks were investigated by nRT-PCR alone. Of the 31 outbreaks investigated for NLV by nRT-PCR 30 were positive with 2 or more patients. One was negative giving an overall positive detection rate for outbreaks of 97%. At an individual specimen level we tested a total of 647 specimens from patients involved in outbreaks and found 221 positive by nRT-PCR (34%). Most of the outbreaks occurred between January and May with a peak in March and April but cases were detected in every month. Outbreaks 10, 17 and 22 occurred in the same large hospital. All other outbreaks were associated with different establishments. Illustrative outline details of some of the outbreaks are given below.

Outbreak 1 was associated with a roll-on-roll-off ferry ship. Vomiting and diarrhoea affected more than 14 crew out of a total of 53 over a period of several days. No passengers were affected. Eight of 10 faecal specimens were positive for NLV by nRT-PCR. All 10 specimens were negative for viruses by EM. An environmental inspection of the ship revealed no concerns about food preparation and handling but identified poor sanitation in the crew facilities and sleeping quarters.

Outbreak 2 was in a country hotel and involved staff and the guests of 5 wedding functions that had taken place over a 6-day period. Vomiting and diarrhoea affected individuals with an onset within 72 hours of their meal. The case attack rate varied from 8.3% to 52% depending on the function attended. In total at least 172 guests and 14 staff were affected giving an overall attack

Table 1: NLV primers and references

Primer		Primer Sequence	Primer	Reference
A*	Outer +	5'-ATA CCA CTA TGA TGC AGA TTA-3'	JV12	[3]
B	Outer-	5'-CGA TTT CAT CAT CAC CAT A-3'	SM31	[18]
C	Inner +	5'-GAA TTC CAT CGC CCA CTG GCT-3'	N1	[17]
D	Inner-	5'-ATC TCA TCA TCA CCA TA-3'	E3	[17]

First round primers A+B yielded a product of 333 bp. Second round nested primers C+D yielded a product of 114 bp.

rate of 33%. We tested 17 specimens from symptomatic staff and guests. Fourteen were positive by nRT-PCR and 2 were positive by EM.

Outbreak 3 involved 23 children and 2 staff of a nursery school. The index case was identified as a child who had been ill at home 24 hours before vomiting in the nursery school sandpit. We received specimens from 12 children with 7 testing positive.

Outbreak 4 involved 23 cases of vomiting and diarrhoea among guests and staff of a city hotel. On investigation a member of staff had vomited in the hotel kitchen during the preparation of the meals. Three catering staff with symptoms were tested by nRT-PCR and they were all positive.

Outbreak 6 which occurred in a restaurant also involved a member of the kitchen staff being taken ill during the preparation of the meals. Seven cases of vomiting and diarrhoea among diners and staff were reported and investigated. All 7 were positive by nRT-PCR for NLV.

Outbreak 7 took place in a large hospital. The outbreak was reported to have affected 70 patients and staff from 4 different ward areas (maternity, medical, elderly and surgical). The hospital was closed to all new admissions. During the first nine days of the outbreak 58 specimens were examined and 14 were positive (from 13 patients). The perception in the hospital was that the outbreak continued for a further 14 days. During this time we tested 58 specimens which were all negative.

Outbreak 10 was spread over 4 wards of a large hospital. There were 16 cases positive out of 58 tested during a 3 week period of March 1999. Although the last positive case in this outbreak was on March 29 and the outbreak was declared over in early April we continued to receive specimens from this hospital for the investigation of gastroenteritis. Over the next 9 months of 1999 we received and tested 171 specimens from patients in this hospital. These additional specimens were all negative.

Outbreak 11 was confined to a medical ward in a district general hospital and involved 21 patients and 9 staff. There symptoms were vomiting and diarrhoea which affected them over the course of an 8 day period. We tested 14 patients with 8 positive and 3 members of staff with one positive. The index case in this outbreak was almost certainly infected in the community before entering hospital. Two of the patients affected, including the index case, reported similar illness among family members.

Outbreak 15 occurred in a nursing home for elderly adults where 28 of the 33 residents and 7 members of staff were affected by vomiting and diarrhoea. We received and tested only 2 specimens from the outbreak and both were positive.

Outbreak 17 spread to 4 wards over a 3 week period during which we tested specimens from 39 patients with 9 positive. The perception in the hospital was that the outbreak continued for a further 4 weeks with diarrhoea being the most frequent symptom. We tested a further 39 specimens in this period and all were negative. A further outbreak occurred in this hospital 2 months later (outbreak 22).

Outbreak 20 developed 2 days after the index case became ill with nausea, vomiting and diarrhoea. In total 13 patients and 3 staff were affected giving a 63% attack rate. We tested 5 specimens and 2 were positive.

Outbreak 27 involved 485 hotel guests and their families over the new year holiday period. The hotel guests were responsible for a large number of secondary cases among family members when they returned home after the holiday. Twelve specimens from hotel guests were submitted for virological investigations and 6 were positive.

Outbreak 28 occurred in one wing of a large hospital. Forty-two specimens were tested over a 6 day period with 12 positives. A further 25 specimens were then tested over the following 9 days and all were negative. The outbreak came to our attention via a telephone call from an infection control doctor on a Friday afternoon. Approximately 24 staff and patients were affected with vomiting and diarrhoea and the index case appeared to be a patient who was admitted 3 days previously and had vomited profusely in the ward. This index case was subsequently shown to be positive. We agreed to test 8 samples on call on Friday evening. This involved specimen extraction and first round RT-PCR on Friday evening with second round nested PCR and gel electrophoresis on Saturday morning. Six of the 8 specimens were positive. On the Saturday morning cases of diarrhoea were reported in the main hospital block and for optimal management of the outbreak it was agreed to test 4 specimens from representative cases within the main hospital block. These were set up as before with the final result available on Sunday. All 4 proved negative and on the basis of these results it was decided not to close any of the wards in the main hospital block.

Outbreak 30 involved 47 identified cases out of approximately 750 people who attended several functions at a regimental reunion which was held over a weekend.

We investigated 11 specimens, 9 of which were positive. The first case occurred within 16 hours of the suspected meal and the last identified case occurred 48 hours later.

Outbreak 31 involved 3 groups of teenagers who were attending a residential leisure centre specialising in sports activities. Out of 94 teenagers present 61 had symptoms of vomiting and diarrhoea giving an attack rate of 65%. Six specimens were investigated virologically for nRT-PCR and 4 were positive. An index case could not be identified but common to all 3 groups was that they were engaged in water sports activities in seawater. The seawater subsequently was shown to be contaminated with raw sewage.

Discussion

In common with others we have used a nested approach for the diagnosis of NLV infection [8,12,13]. Nested PCR adds increased sensitivity and specificity to PCR protocols [14]. The increased sensitivity is thought to be in the order of 10 to 1000 fold [8]. In our laboratory it was also relatively straightforward to introduce the NLV nested protocol into our molecular diagnostic unit where nested PCR is used extensively for the detection of other viruses from routine clinical specimens [15,16]. The main argument against nested PCR protocols is that because of their exquisite sensitivity they are subject to the possibility of carryover or amplicon contamination. Cross contamination is possible with a wide range of laboratory protocols, but we feel that where separate areas are strictly adhered to for the various processes of PCR and attention paid to sterile technique as routinely taught and practised in virology laboratories, the possibility of sample carryover contamination is considerably reduced. This assessment is underlined by the low positivity rate observed in 532 control faecal specimens (2/532) processed, resulting in a specificity rate of at least 99.6%.

During the development of the nRT-PCR we evaluated several different combinations of primer sets specific for the polymerase gene of Norwalk-like caliciviruses [3,17,18]. The combination of primers in our nRT-PCR were selected for their broad range reactivity, specificity and sensitivity when used to detect NLV in specimens from patients involved in outbreak situations. The outer primer pair are broadly reactive against all NLV strains and are a combination of primers which are known to detect about 90% of strains of NLV [3,18]. The inner primer pair is also broadly reactive and detect at least 93% of known NLV strains [17].

In the first 2 outbreaks we investigated, both were tested in parallel using nRT-PCR and EM. We found the molecular approach to yield a greater than 80% positive rate compared to EM which detected 2/17 (11.7%) in one outbreak and none in the other. Thereafter we used the nRT-PCR test alone, applying EM to nRT-PCR negative outbreaks only. This is in contrast to the decision made by other units where EM is regarded as the front line test [1,19]. EM is relatively insensitive and non-specific [20–23]. Even in expert hands it can be difficult to ascertain virus morphology and therefore to distinguish between certain virus groups [23]. With the more sensitive and specific nRT-PCR the diagnostic window is much greater [1]. During the past 3 years we have investigated 31 outbreaks of gastroenteritis which were clinically and epidemiologically consistent with NLV infection. Using nRT-PCR we have achieved a positive diagnosis in 30 of these outbreaks. This is a positive diagnostic rate in outbreak situations of 97% using nRT-PCR alone whereas the best estimated diagnostic rate achieved by EM is less than 50% [1,19]. Before the introduction of nRT-PCR our outbreak diagnostic rate was also less than 50%. The one outbreak in our series that was negative by nRT-PCR (outbreak 18) and was also negative using EM. The investigation of this particular outbreak which was associated with a staff canteen was compromised by specimen quality. The faecal samples received were from individuals who were all more than several days into convalescence in the asymptomatic stage of their illness when virus was possibly no longer present or detectable.

A striking observation from our data is that there was a dramatically higher positivity rate when the assay was applied to clearly defined outbreaks associated with hotels and restaurants, small hospital areas and nursing home units compared to the large hospital outbreaks. It is clear to us that in large hospitals it is difficult to distinguish between actual cases of gastroenteritis associated with a given outbreak and other background causes of diarrhoea. In our experience this is a particular problem in wards containing elderly patients. In the large hospital environment there is a tendency to investigate all cases of diarrhoea for NLV whether they are directly linked to an outbreak or not. The identification of an outbreak tends to increase surveillance for patients with gastrointestinal symptoms. This is a reason for the impression that hospital outbreaks last longer than they actually do. The "long-tail" of cases that are negative for NLV is associated with a change from vomiting and diarrhoea to diarrhoea alone as seen in outbreaks 7 and 10. The absence of vomiting in new cases is probably a good indication that the outbreak has ended. However it must be noted that not all cases of NLV infection are associated with vomiting [1].

The duration of illness associated with NLV infection is generally 1–3 days and the incubation period is 1–2 days. Knowing this it should be possible for infection control teams to declare outbreaks over at 5 days after the onset

Table 2: Outbreaks of NLV investigated from August 1998 to May 2001.

Outbreak	Type of Outbreak	Date	nRT-PCR +ve/nt[1]	nRT-PCR (% +ve[2])	Number of individuals involved
1	Ferry Ship	Aug 1998	8/10	80%	14
2	Country Hotel	Aug 1998	14/17	82%	514
3	Nursery school	Nov 1998	7/12	50%	25
4	City Hotel	Jan 1999	3/3	100%	23
5	Restaurant	Jan 1999	8/32	25%	
6	Restaurant	Mar 1999	7/7	100%	7
7	Large Hospital	Mar 1999	14/116	12.1%	70
8	Psychiatric Hospital	Mar 1999	27/35	77%	
9	Restaurant	Mar 1999	5/5	100%	
10	Large Hospital	Mar 1999	16/58	27%	58
11	Medical Ward	Apr 1999	9/17	53%	30
12	District Hospital	Apr 1999	8/32	25%	
13	Medical Ward	May 1999	3/5	60%	
14	Nursing Home	Jun 1999	2/2	100%	
15	Nursing Home	Nov 1999	2/2	100%	35
16	Care of Elderly Ward	Feb 2000	5/15	33%	
17	Large Hospital	Feb 2000	9/78	11.5%	
18	Staff Function	Mar 2000	0/7	0%	109
19	Care of Elderly Ward	Apr 2000	8/22	36%	
20	Nursing Home	Apr 2000	2/5	40%	16
21	Nursing home	Apr 2000	2/2	100%	
22	Large Hospital	Apr 2000	7/37	19%	
23	District Hospital	May 2000	2/2	100%	
24	Care of Elderly Ward	May 2000	9/12	75%	
25	Nursing Home	Jun 2000	2/5	40%	
26	Hotel	Aug 2000	8/10	80%	
27	Hotel	Dec 2000	6/12	50%	485
28	Large Area Hospital	Jan 2001	12/67	17.9%	
29	Hotel	Mar 2001	3/3	100%	
30	Regimental Reunion	Apr 2001	9/11	82%	750
31	Leisure Centre	May 2001	4/6	66%	94

[1]Number positives/number tested [2]Percentage positive

of the last laboratory diagnosed case. To achieve this it is important that environmental contamination has been eliminated as it is known that NLV can remain infectious for at least 12 days on contaminated surfaces such as floor carpet [24].

The standard advice given to hospital staff and food handlers who have been affected by NLV infection is that they should not return to work until 48 hours after resolution of symptoms. In one case in this study a chef was found to be nRT-PCR positive 12 days after the onset of symptoms. The excretion of virus more than 3 days after onset has been demonstrated previously using sensitive RT-PCR tests [25,26]. Parashar et al demonstrated the presence of NLV in the stool of a food handler 10 days after the resolution of his illness[26]. On at least one occasion an outbreak of gastroenteritis has been traced to a post-symptomatic food handler [27]. While it is not necessary to advise staff to stay away from work until they are clear of the virus they should be alerted to the continuing risk of spreading the virus which they pose in the immediate period following cessation of symptoms and instructed accordingly about the importance of personal hygiene measures.

Since we have introduced this assay into routine use there has been an increase in demand for its availability. This is due in part to the generally recognised increase in the number of NLV outbreaks for which we have no explanation at present [5–7]. However the increased demand on the service is also due in part to the sensitivity and specificity and therefore usefulness of the nRT-PCR assay. Outbreak 28 illustrates the impact that the application of a sensitive assay can have when used in the real

time management of an outbreak. The decision not to close acute wards in a major referral hospital was based on the results of the assay used out of normal working hours confirming the outbreak had not spread. Such a scenario has to occur only very occasionally to make the service cost effective, as hospital outbreaks of NLV have major cost implications [1,28].

There have been many published papers on the sensitivity and specificity of RT-PCR for the detection of NLV [3,17,18,29]. However also in the scientific literature there are often reasons stated justifying its non-introduction into routine practice. We do not intend to review the literature here, but among the stated reasons for non-introduction are poor primer specificity, presence of possible PCR inhibitors, it is not a "catch-all" technique, the necessity of specimen extraction and the requirement to use nested RT-PCR for sensitivity leading to the possibility of carry-over contamination. It has been established in this work and by others that RT-PCR is capable of detecting and diagnosing between 90–97% of outbreaks associated with NLV infection [3,17,18]. This is considerably more sensitive in spite of the above listed potential problems than the alternative of EM [19,30]. The time has now come for a different strategy to be used in the investigation of presumptive outbreaks of NLV. Front-line investigation should involve nRT-PCR. nRT-PCR negative outbreaks should be investigated by EM but they should also be the stimulus for investigating alternative primer combinations. This would be best achieved at a supra-regional level such as public health virology reference laboratories. This would facilitate the quick diagnosis and early implementation of appropriate action by infection control teams while paving the way for a more comprehensive ability to fully investigate these costly outbreaks.

Conclusions

We have shown that protocols for nRT-PCR can be devised and introduced into routine practice for the investigation of viral infections. To date these have been in routine use over a three year period with no evidence of amplicon contamination.

Amplification techniques such as the nRT-PCR described here should be used as front line tests for the investigation of cases of gastroenteritis which are clinically and epidemiologically consistent with NLV infection. These tests should also be made available "on call" at weekends and public holidays.

Nested RT-PCR is capable of detecting and diagnosing at least 97% of outbreaks associated with NLV infection whereas the best that can be expected with EM is 50%. Outbreaks of NLV infection should be monitored using sensitive RT-PCR assays so that the laboratory information can be used by infection control teams in ascertaining the spread and duration of outbreaks. Only RT-PCR negative outbreaks should be investigated by the less sensitive electronmicroscopy technique. Positive findings by this method (EM) should then be used as the stimulus for investigating alternative primer combinations.

In large hospital outbreaks it is difficult to distinguish between actual cases of gastroenteritis associated with a given NLV outbreak and other background causes of diarrhoea. In our experience this is a particular problem in wards containing elderly patients. The identification of an outbreak tends to increase surveillance for patients with gastrointestinal symptoms. This leads to the impression that hospital outbreaks last longer than they actually do.

NLV infections were detected in all age groups and were concentrated in the early months of the year with a peak incidence during the months of March and April. However outbreaks have been detected in all 4 seasons therefore putative NLV infections should be investigated all year round.

Materials and Methods

Specimens and patients

Faecal specimens were received at the Regional Virus Laboratory from patients with gastroenteritis. The specimens were prepared in 10% suspensions in Eagles medium with Earles salts, centrifuged at 4,000g for 10 min and the clear supernatant either stored at -20°C or tested before freezing. For nRT-PCR the specimens were further prepared using the QIAGEN DNA Blood extraction kit. This kit is the standard extraction method for PCR and RT-PCR in this laboratory and it copurifies RNA and DNA. The QIAGEN protocol was adapted to use a vacuum manifold developed in house and RNA was eluted from the spin columns using 50µl of nuclease free water. The extracts were tested by nRT-PCR immediately or stored at -20°C until tested the following day.

Nested multiplex RT-PCR (nRT-PCR)

The nRT-PCR was developed after evaluating various combinations of published primers against EM positive faeces from outbreaks of gastroenteritis associated with NLV infection [3,17,18]. The primers were specific for the polymerase gene of NLV. The primers selected were chosen for their broad range specificity and sensitivity against NLV and are listed in Table 21. The outer primer set yielded a product of 333 bp and the nested inner pair yielded a product of 114 bp.

The nRT-PCR was developed using Access RT-PCR (Promega) for the first round and conventional PCR for the second round in 10 µl volumes. The nRT-PCR was systematically optimised with respect to primer and magnesium ion concentrations in the presence of 0.25 mM cresol red and 15% sucrose. The latter were added to facilitate direct gel loading. The optimum primer and magnesium ion concentrations for first and second round are given in the respective mastermixes below. The reverse transcription step in the first round was also optimised. It was found that 48°C/10 min were sufficient for this step in the protocol. The optimum annealing temperature for both first and second rounds was found to be a broad temperature band within the range 30°C to 54°C. An annealing temperature of 37°C was selected which is similar to that used by others for NLV RT-PCR.

For first round nRT-PCR 2 µl of extracted sample RNA was added to 8µl of master-mix to give a final volume of 10 µl. The first round final 10 µl volume contained Access RT-PCR buffer (Promega), 0.6 µM outer primers A and B, 1.5 mM MgS04, 1 unit AMV, 1 unit Tfl, 0.2 mM NTP, 0.25 mM cresol red and 15% sucrose. The nRT-PCR first round cycling conditions were as follows; 48°C/10 min, 94°C/3 min followed by 35 cycles of 94°C/30 s, 37°C/30 s and 72°C/30 s. This was followed by 72°C 5 min and finally a hold step at 20°C.

For second round PCR 0.2 µl of first round product was added to 9.8µl of second round mastermix to give a final volume of 10 µl. The second round contained Tris buffer, 0.25 units TAQ in storage buffer B (Promega), 0.2 mM NTP, 3.5 mM $MgCl_2$, 0.2 µM nested primers C and D, 0.25 mM cresol red and 15% sucrose. The second round PCR cycling conditions were as follows; 94°C/2 min followed by 25 cycles of 94°C/30 s, 37°C/30 s and 72°C/30 s. This was followed by 72°C 5 min and finally a hold step at 20°C.

Mastermixes for first and second round nRT-PCR were made up in batches, labelled with a unique identifier, aliquoted and stored at -70°C. For use an aliquot of mastermix was thawed and appropriate volumes were dispensed into PCR tubes.

The nRT-PCR tests were routinely run on either GeneAmp 2400 and 9700 thermal cyclers. Both first and second round products were run on 2.5% agarose gels in TAE buffer for 45 mins in a 15 cm gel at 75 volts. The gels were stained with ethidium bromide (1 µg/ml) for 30 min and photographed using a Polaroid land camera.

Validation and application of nRT-PCR

The nRT-PCR was developed and validated using a panel of 14 faecal specimens, which were positive for NLV by EM. They were representative specimens from 4 different outbreaks, which had been collected over a 3-year period. Eight faecal specimens were also tested as negative controls. Four of these were from adults known to be NLV RT-PCR negative (Central Public Health Laboratory, Colindale, and London) and 4 were from children with rotavirus infection. The nRT-PCR was used in parallel with EM on clinical specimens from the first 2 outbreaks of gastroenteritis associated with NLV infection. Thereafter it was used without comparison with EM.

Electron microscopy

Two millilitre volumes of 10% faecal specimens were centrifuged at 40,000 rpm for one hour and the resulting pellet was resuspended in 25 µl distilled water. The suspension was then adsorbed onto a glow discharged formvar coated EM grid for a minimum of 3 hours and stained with 2% methylamine tungstate for 10 s. The grid was viewed in a Phillips CM 10 EM at a magnification of 45,000 for approximately 10 min.

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