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Characterisation of the bovine enteric calici-like virus, Newbury agent 1

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Abstract

The bovine enteric calici-like virus, Newbury agent 1 (NA1) was characterised to determine if it is a member of the *Caliciviridae* and to establish its antigenic relationship to the established bovine enteric calicivirus Newbury agent 2 (NA2). Solid phase immune electron microscopy (SPIEM) allowed quantification of NA1 virions and identification of faecal samples with optimal virus levels. NA1 particles were 36.6 nm in diameter, had an indefinite surface structure resembling that of human small round structured viruses (SRSVs), and a buoyant density of 1.34 g ml^{-1}. A single capsid protein of 49.4 kDa was detected by Western blotting in purified NA1 preparations prepared from post-infection but not pre-infection faecal samples and with post- but not pre-infection sera. NA1 was antigenically unrelated to the bovine enteric calicivirus NA2 by SPIEM. These properties were consistent with classification of NA1 within the *Caliciviridae* but demonstrated heterogeneity in the capsid composition of bovine enteric caliciviruses.

Keywords: Calicivirus; Diarrhoea; Cattle; Newbury agent

1. Introduction

In the last decade, caliciviruses have become recognised as a major cause of non-bacterial gastroenteritis in human adults (reviewed in [1]). Calici-like viruses have been identified in association with calf diarrhoea, first in the UK [2,3] and subsequently in Germany [4–6]. Unlike the human viruses, they have not been well characterised, in part due to the lack of a cell culture system and the apparently low numbers of particles in faeces. Two of the three reported bovine viruses, one from the UK (Newbury agent 2, NA2) [7] and one from Germany (Jena virus) [8], were shown recently to be members of the family *Caliciviridae*. In the present paper, we characterise the second bovine enteric calici-like virus, Newbury agent 1 (NA1), identified in the UK to examine the hypothesis that NA1 is also a member of the family *Caliciviridae* and to explore its relationship to NA2.

2. Materials and methods

2.1. Viruses, antisera and immunoglobulin concentrates

The bovine viruses, Newbury agent 1 (NA1) and Newbury agent 2 (NA2) originated from two herds in the UK with a history of calf diarrhoea and were initially described as Newbury agent SRV-1 and SRV-2 [2,9]. The original faecal samples also contained astroviruses, rotaviruses or coronaviruses which were removed as described previously. Pre- and post-inoculation NA1 antisera were raised in a 50 days old gnotobiotic calf (P143) inoculated orally with 3 ml of a faecal filtrate of NA1 prepared by passage of a 25% faecal emulsion in PBSα through a 0.45 μm membrane filter. The calf developed diarrhoea and recovered. Calici-like virus particles were identified in faecal samples by electron microscopy but no other viruses were seen. Ten weeks later, a similar faecal filtrate was inoculated intravenously and the calf was bled out 5 days later to provide antiserum. The antiserum was free of antibodies to bovine astrovirus, the UK strain of bovine rotavirus, bovine enteric coronavirus, bovine viral diarrhoea virus, feline calicivirus and the Haden strain of bovine parvovirus (J.C. Bridger, personal observations).

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An NA1 immunoglobulin concentrate was kindly prepared from the calf antiserum by Dave Harkins, The Moredun Research Institute, Edinburgh using a protein A Sepharose fast flow column (Pharmacia Biotech). The NA2 immunoglobulin concentrate was described previously [7].

Feline calicivirus (FCV, NADC strain) and hyperimmune rabbit anti-FCV NADC serum with a neutralising titre of 1:40 000 was provided by Dr. John Neill from the National Animal Disease Centre (NADC), IA, USA. It was grown in the crandel reese feline kidney (CRFK) cell line supplied by Ruth Ryvar, Faculty of Veterinary Science, University of Liverpool. Recombinant Lordsdale virus capsid antigen and rabbit antiserum were kindly provided by Dr. Ian Clarke, Molecular Microbiology Group, University Medical School, Southampton General Hospital, Southampton, UK. The concentration was estimated to be 5–10 μg μl^{-1} (Dr. Ian Clarke, personal communication).

2.2. Calf inoculation

Faecal samples were obtained from three gnotobiotic calves (1424, 2042, 2010) inoculated with NA1 [9]. Gnotobiotic calves 1424 (41 days old) and 2042 (45 days old) were given 3 ml of the same faecal emulsion by mouth. Faecal samples from calf 1424 were used to inoculate calf 2010 (49 days old). Faecal samples were collected before inoculation and daily afterwards for 5 days.

2.3. Solid phase immune electron microscopy (SPIEM)

SPIEM was conducted with 25% faecal suspensions or purified virus preparations as described previously using a 1:20 (NA1) or 1:40 dilution (NA2) of the immunoglobulin concentrate [7]. A uniform and well-stained area of the grid was selected at 150× magnification and particles counted at 50 000× instrumental magnification. For each grid square, virus particles were counted in five fields and a total of three or five grid squares were counted for each test sample. A positive control sample was included in each assay to ensure reproducibility.

2.4. Particle size and buoyant density determination

NA1 particles, trapped onto electron microscope grids by SPIEM, were negatively stained with a mixture of equal volumes of 4% PTA pH 6.0 and catalase crystals (Boehringer Mannheim Corp.) which were used as an internal measurement standard. The diameters of 20 particles were measured on negative exposures by calculating the mean value from two measurements taken at right angles to each other. The distance between 10 consecutive lattice spacings (taken as 87.5 Å) of catalase crystals in the same electron microscope field was used as an internal measurement standard.

A clarified freon-extracted day 2 faecal sample from calf 1424 was diluted to 10 ml, centrifuged at 83 000 × *g* for 2 h at 4°C, the pellet resuspended in 200–300 μl volumes of PBSα and layered on top of a 35% (w/w) CsCl solution. After centrifugation to equilibrium at 155 000 × *g* for 17 h at 4°C, 0.5 ml fractions were collected by gravity using a top unloading method. A 15–20 μl volume of each fraction was monitored for refractive index at 25°C. Fractions were examined by SPIEM as described previously but grids were washed with 10 consecutive 150 μl volumes of MilliQ water to remove CsCl. Buoyant densities were calculated from refractive indices corrected for the refractive index of PBSα. Buoyant densities were also determined by weighing. The weight of 3–6 100 μl volumes of each fraction, taken by a calibrated Gilson pipette, was measured to 4 decimal points at 25°C and the average was calculated for each fraction. The accuracy of the procedure was validated by measuring the density of MilliQ water at the same temperature and taking the density at 25°C as 0.99707 g ml^{-1}.

2.5. Western blotting

Two or three fractions from CsCl density gradients around the NA1 buoyant density of 1.34 g cm^{-3} and containing the highest numbers of particles, were pooled and ultracentrifuged at 83 000 × *g* for 2 h. The resultant invisible pellet was resuspended in a 100–150 μl volume of MilliQ water and 15–60 μl volumes were used in Western blotting. With the day 0 faecal sample, an ultracentrifuge pellet was made of two–three pooled CsCl fractions around a buoyant density of 1.34 g cm^{-3}. The FCV antigen was prepared by freon extraction and ultracentrifugation using a 10 ml volume of infected cell culture fluid. The same procedure was applied to uninfected flasks. The resultant pellet was resuspended in 100 μl of MilliQ water and a 3 μl volume used in Western blotting. A 0.1 μl volume of the Lordsdale recombinant capsid protein was used. Preparations were mixed with an appropriate volume of sample loading buffer (5% SDS, 10% glycerol from BDH, 5% 2-mercaptoethanol, 50 mM Tris–HCl pH 6.8 and 0.1% bromophenol blue) boiled for 4 min and loaded onto Laemmli discontinuous buffer gels with 10% resolving and 5% stacking gels. One μg of bovine immunoglobulin in MilliQ water and Kaleidoscope pre-stained standard marker proteins (Bio-Rad or Sigma Chemical Co.) were used as controls for the electroblotting and immunostaining steps. A mixture of biotinylated protein markers (from 200 to 6.5 kDa; Bio-Rad) was co-run with the disrupted virus proteins for accurate molecular mass determinations. Three percent gelatin was used to block non-specific binding sites and virus-specific antibodies and immunoglobulin conjugates were diluted in 2% gelatin. Virus-specific antibody reactions were performed overnight; immunoglobulin conjugate reactions were performed for 0.5–1 h. The dilutions of antisera and conjugates used were 1:2000 and 1:5000 of the NA1 pre- and

post-inoculation bovine antisera, 1:10 000 of the rabbit FCV antiserum and rabbit Lordsdale recombinant capsid protein antiserum, 1:5000 for the rabbit anti-bovine IgG conjugated to alkaline phosphatase (Sigma Chemical Co.), 1:3000 for the goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co.) and avidin alkaline phosphatase conjugate (Bio-Rad) used to detect the biotinylated standards. Substrates were Ready-to-use Western blue™ (Promega) or BCIP (5-bromo-4-chloro-3-indolylphosphate)/NBT (nitro blue tetrazolium) (Promega).

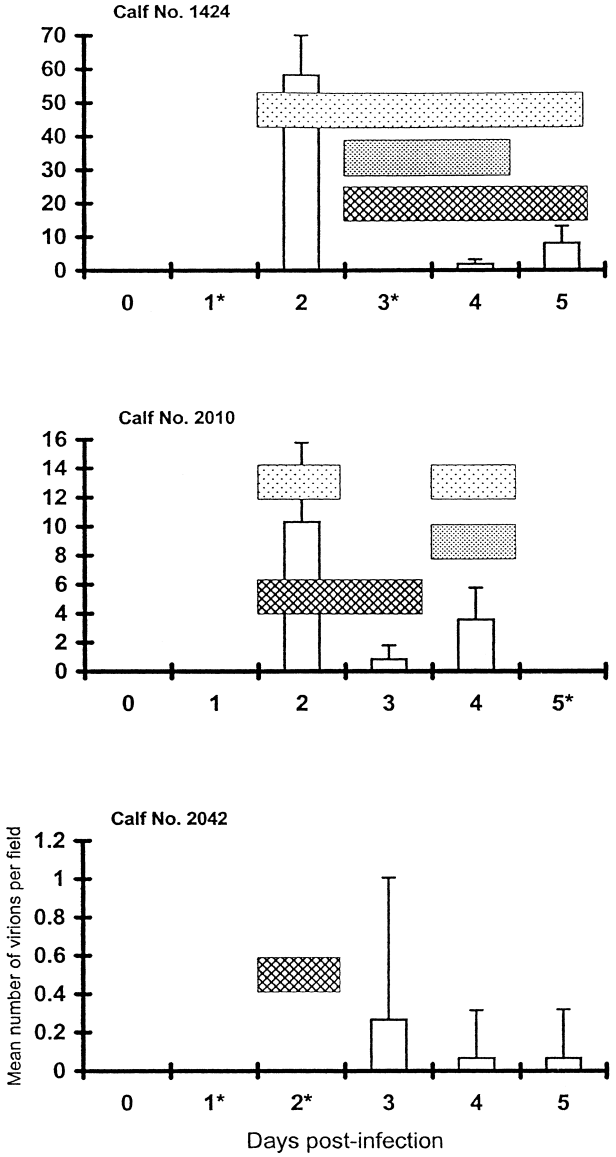

Fig. 1. Clinical signs and faecal NA1 excretion by SPIEM (white box) in three gnotobiotic calves inoculated with NA1. Faecal colour changes (box with dots), increased rectal temperatures (box with dashed lines) and diminished appetite (box with lines) are indicated. Absence of a parameter indicates it was not present. Asterisks indicate that a faecal sample could not be collected on that day. The diminished appetite and abnormal faecal colour lasted till day 7 p.i. for calf 1424 (not shown). Error bars represent standard deviations of the mean particle counts per field.

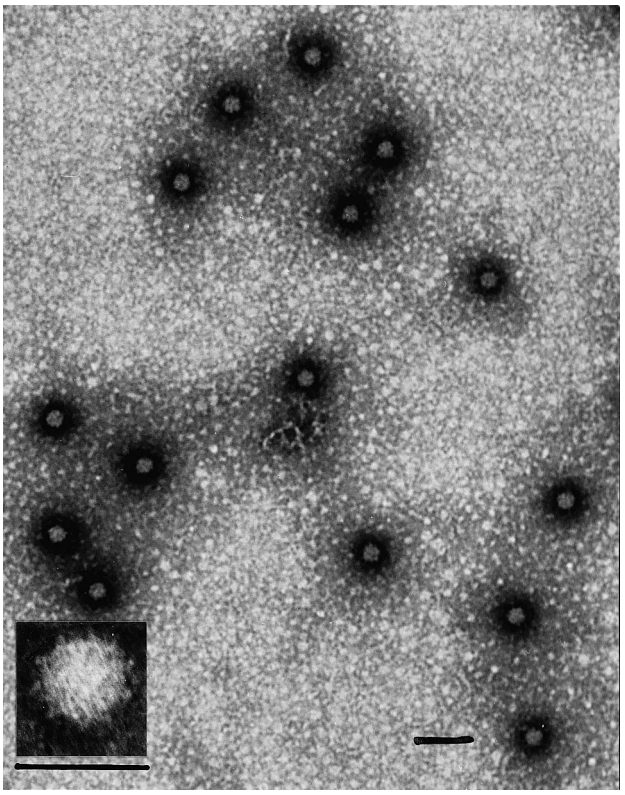

Fig. 2. NA1 virus particles trapped by SPIEM from the day 2 faecal sample of gnotobiotic calf 1424 using a 1:20 dilution of the NA1 immunoglobulin concentrate. Bar indicates 100 nm. Inset shows surface structure of an NA1 particle and spiked outer edge; bar indicates 50 nm. Stained with 2% PTA at pH 6.0.

3. Results

3.1. Source of NA1 virions

Adequate levels of virus for further study were identified by SPIEM in one faecal sample from one (calf no. 1424) of three experimentally inoculated gnotobiotic calves (Fig. 1). Virus was first detected on the second day after inoculation in two calves (calf nos. 1424 and 2010) as clinical signs commenced. Particles were excreted for at least 2 or 3 more days but numbers were low. Particles were not observed in pre-inoculation samples. Only occasional particles were detected in the faecal samples of the third calf, no. 2042, but a day 2 sample was not available. The SPIEM technique allowed accurate quantification of NA1 virions (Fig. 1). Variation in the mean number of particles trapped per field on duplicate grids was small, ranging from 1- to 2.5-fold in four independent experiments. There was little non-specific attachment to negative control grids coated with rotavirus antiserum. Use of immunoglobulin concentrates was essential in SPIEM, in contrast to Western blotting, as 1:10 to 1:160 dilutions of antiserum failed to trap particles above background levels.

Two of the three calves developed multiple clinical

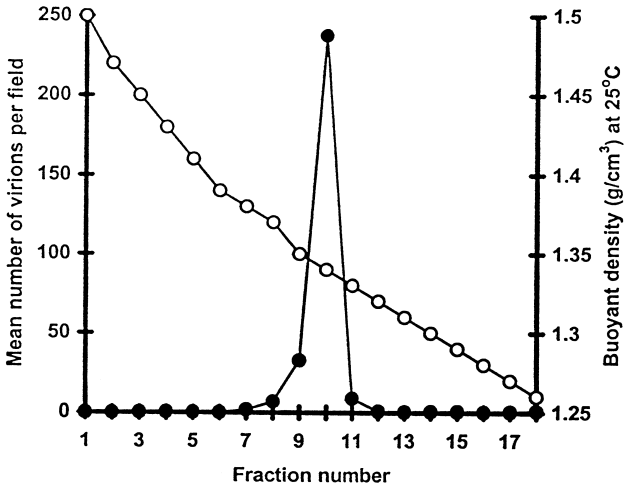

Fig. 3. Mean number of NA1 particles (●) trapped per field by SPIEM in fractions from a representative CsCl density gradient centrifuged to equilibrium. Buoyant density (○) determined by refractometry.

signs. All three developed a diminished appetite which lasted for 1–5 days and began at the time of peak virus excretion (calf 2010) or the day after (calf 1424). Faecal colour changed from brown/yellow to white diarrhoea in calves 1424 and 2010 at day 2 p.i., the time of peak virus excretion. The colour remained abnormal for 2–7 days. Increased temperature (above 39.2°C) was observed in calves 1424 and 2010, on days 3 or day 4 p.i., that is, 1 or 2 days after peak virus excretion.

3.2. Particle morphology, diameter and CsCl buoyant density

In faecal suspensions and purified preparations, NA1 virions appeared as small round viruses with indefinite surface morphology and ragged outer edges (Fig. 2). Some particles displayed projections on their outer edges almost giving a 10-spiked appearance. Their morphology was indistinguishable from particles prepared by direct electron microscopy [9] but clearly differed from the feline calicivirus NADC strain, which showed classical calicivirus morphology (not shown). The average particle diameter was 36.6 ± 1.7 nm determined using an internal calibration standard. A mean CsCl buoyant density of 1.34 ± 0.0044 g cm^{-3} was obtained at 25°C in eight independent experiments by measuring refractive indices (Fig. 3) and in one experiment by weighing fractions.

3.3. Protein composition of purified NA1 virus

Western blotting analysis of NA1 virions purified from faeces by CsCl density gradient centrifugation demonstrated the presence of a single protein band in each of five separate preparations from the day 2 p.i. faecal sample from calf 1424 (Fig. 4a, track 2). The specificity of the protein band was confirmed by examination of day 0 versus day 2 p.i. faecal samples, staining with pre- and post-inoculation antisera and association with the presence of virus particles in CsCl gradients. No signal was seen with the day 0 faecal sample which did not contain virus particles but which was purified in the same way as the day 2 p.i. faecal sample (Fig. 4a, track 1). A comparable band was absent in the day 2 purified fractions when probed

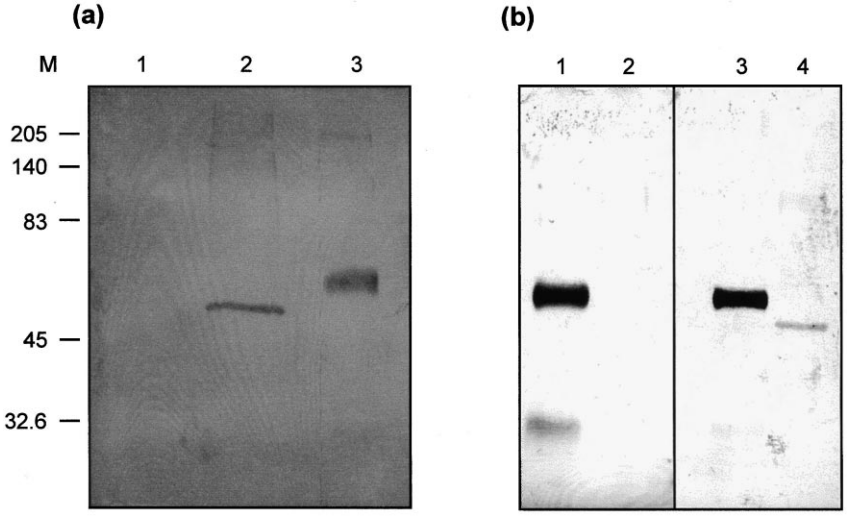

Fig. 4. (a) Western blotting analysis of fractions from day 0 (track 1) and day 2 (track 2) faecal samples of calf 1424 purified by CsCl centrifugation stained with the post-inoculation NA1 serum. A mean of 130.5 NA1 particles per field were present by SPIEM in the fraction from the day 2 sample; no particles were detected in the fraction from the day 0 sample. Track 3: bovine IgG as a control for electroblotting and immunostaining. M, molecular mass markers (kDa). (b) Western blotting analysis of fractions from the day 2 faecal sample of calf 1424 stained with the pre- (track 2) and the post-inoculation NA1 serum (track 4). Tracks 1 and 3: bovine IgG as a control for electroblotting and immunostaining. M, molecular mass markers (kDa).

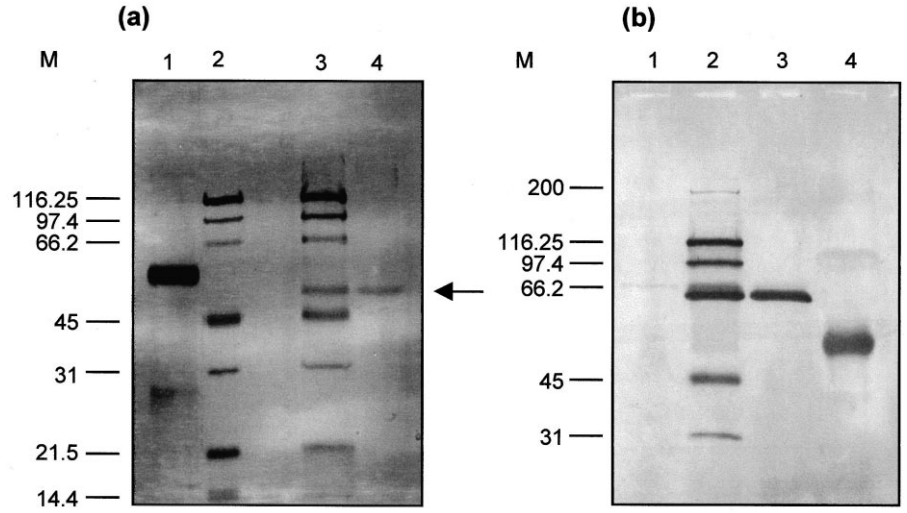

Fig. 5. (a) Molecular mass estimation of the capsid protein of NA1 particles purified by CsCl density gradient centrifugation. M, molecular masses of biotinylated standard proteins (kDa). Track 1: bovine IgG as a control for blotting and staining with the anti-bovine conjugate; track 2: biotinylated standard proteins; track 3: biotinylated standard proteins plus the NA1 capsid protein; track 4: the NA1 capsid protein; arrow indicates the NA1 protein. (b) Molecular mass estimation of the capsid protein of FCV grown in CRFK cells using the same electrophoresis conditions as those used for NA1. Arrow indicates the FCV capsid protein. M, molecular masses of biotinylated standard proteins (kDa). Track 1: preparation from uninfected CRFK cells; track 2: biotinylated standard proteins plus the preparation from FCV-infected cells; track 3: FCV-infected cells; track 4: rabbit IgG (the FCV NADC rabbit antiserum) used as a control for blotting and immunostaining with the anti-rabbit conjugate.

with the pre-inoculation NA1 serum (Fig. 4b, track 2). In CsCl density gradients, the protein band was observed only in the CsCl fraction with the peak number of virus particles and not in fractions with no or low numbers (not shown).

In five independent experiments, the single NA1 band migrated faster than the heavy chain of bovine immunoglobulin indicating a lower molecular mass than expected compared to the capsid molecular masses of known caliciviruses. Molecular masses of 49.8 and 49.0 kDa (mean 49.4 kDa; $r = -0.99$) were obtained by co-electrophoresis with biotinylated molecular mass standards (Fig. 5a). The accuracy of the method was assessed with the established caliciviruses, FCV and Lordsdale virus and found to give the molecular masses expected for these viruses. The FCV capsid protein, with a reported molecular mass of 66 kDa [10], was determined as 64.2 and 65.1 kDa by co-electrophoresis in two independent experiments (mean 64.65 kDa) (Fig. 5b). Co-electrophoresis of disrupted recombinant Lordsdale particles (rLVPs) with the biotinylated molecular mass markers revealed the presence of the expected 58 kDa protein (not shown) [11].

3.4. Antigenic relationships

Reciprocal cross-reactions by SPIEM demonstrated a complete lack of an antigenic relationship between NA1 and the bovine calicivirus NA2. Less than one particle was seen per field (0.0 to 0.4 ± 0.7) with 1:10, 1:20 and 1:40 dilutions of the NA1 and NA2 heterologous immunoglobulin concentrates which trapped an average of 50.4 ± 11.9 to 79.6 ± 20.2 homologous particles per field in every experiment (means are given from two experiments but the lack of heterologous reactions was observed in over four experiments).

Post-inoculation NA2 antisera failed to react with the 49.4 kDa capsid protein of NA1 by Western blotting. However, it was not possible to test the homologous reactivity of the NA2 antiserum in Western blotting as attempts to concentrate and purify NA2 particles in sufficient numbers failed.

4. Discussion

The bovine enteric calici-like virus NA1 had properties consistent with classification within the *Caliciviridae*. The particle diameter of 36.6 nm and CsCl buoyant density of 1.34 g cm^{-3} were within the ranges for established caliciviruses, including the human SRSVs [12,13]. In purified preparations, NA1 morphology was confirmed as being similar to human SRSVs. Purified NA1 virions contained a single capsid protein, a property characteristic of caliciviruses. However, antigenic diversity between bovine enteric caliciviruses was demonstrated in vitro for the first time by the complete lack of cross-reactivity by SPIEM between NA1 and the established bovine enteric calicivirus NA2. This supported earlier experiments in vivo where the two viruses failed to cross-protect [9]. Considerable antigenic diversity is well established amongst human enteric caliciviruses (SRSVs) [14–16]. Further studies will be needed to establish the extent of antigenic diversity amongst bovine enteric caliciviruses and their relationship to the equivalent human viruses.

The capsid molecular mass of 49.4 kDa, was lower than expected from the 57–63 kDa range reported for human enteric caliciviruses with SRSV morphology [12,13,17–20]. It was also lower than the 55 kDa molecular mass of the in vitro translated capsid protein of the Jena virus, the only other bovine enteric calicivirus with a known capsid molecular mass [8]. Considerable care was taken to determine the molecular mass of the NA1 capsid protein by electrophoresis of the capsid protein with molecular mass standard proteins. Capsid proteins with molecular masses less than 55 kDa have been associated with some calicivirus capsids but, in most instances, a higher molecular mass protein has been apparent [21–23]. There was no evidence that the NA1 49.4 kDa band represented a degradation product of the capsid protein. A higher molecular mass band was never seen in the NA1 preparations and the CsCl preparations used contained intact virus particles with typical SRSV size and morphology. Confirmation of the low NA1 capsid molecular mass will have to await elucidation of the deduced amino acid sequence of the NA1 capsid protein from genomic analysis and recombinant capsid expression studies. However, it has not been possible to amplify the NA1 genome from faecal suspensions or purified preparations using a range of PCR primers used successfully with NA2, further supporting the notion that bovine enteric caliciviruses are diverse.

SPIEM greatly improved detection of NA1 from faecal samples. Earlier studies used direct electron microscopy but only low numbers of particles were identified making it impossible to identify samples with adequate numbers of virions for further study [2,9]. The present study showed that virions are not difficult to detect if early samples are examined by SPIEM. SPIEM enabled NA1 to be detected in the absence of a cell culture infectivity assay or knowledge about its genomic composition and, thus, allowed us to characterise the virus. The short period of virus shedding seen in the experimental calves by SPIEM was similar to that found in experimental infections with the previously described bovine enteric calicivirus NA2 [7] and the human Norwalk virus [24,25] but contrasts markedly with bovine rotaviruses, another enteric pathogen, which are excreted at high levels for 4–6 days. The short virus excretion period and the lack of a broadly reactive and sensitive detection method may explain why so few investigators have reported the involvement of caliciviruses in outbreaks of calf diarrhoea. Enteropathogens were not detected in 31% of diarrhoeic faecal samples in a study of the microbiology of calf diarrhoea in the UK [3]. Failure to detect caliciviruses may be the reason for the lack of a diagnosis and further studies are warranted.

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