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High prevalence and diversity of bovine astroviruses in the faeces of healthy and diarrhoeic calves in South West Scotland

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ABSTRACT

Astroviruses (AstV) are single-stranded, positive-sense RNA viruses and one of the major causes of infant diarrhoea worldwide. Diarrhoea is a common and important cause of morbidity and mortality in calves; therefore, we investigated whether the presence of AstV is associated with calf diarrhoea. We identified diverse AstV lineages from faecal samples of both healthy and diarrhoeic calves and healthy adult cattle in South West Scotland. AstV was common in calves (present in 74% (85/115) of samples) but uncommon in adult cattle (present in 15% (3/20) of samples). No association was found between the presence of AstV and calf diarrhoea or the presence of a specific AstV lineage and calf diarrhoea. AstV was strongly associated with the presence of rotavirus Group A (RVA), and a protective effect of age was evident for both AstV and RVA. Co-infections with multiple AstV lineages were detected in several calves and serial infection with different viruses could also be seen by longitudinal sampling of individuals. In summary, our study found genotypically diverse AstV in the faeces of calves in South West Scotland. However, no association was identified between AstV and calf diarrhoea, which suggests the virus does not play a primary role in the aetiology of calf diarrhoea in the group studied.

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1. Introduction

Diarrhoea in dairy and beef calves is very common and causes substantial morbidity and mortality through dehydration, metabolic acidosis and electrolyte depletion. Approximately half of the mortality in dairy calves up to 1 month old has been attributed to diarrhoea (Brickell et al., 2009). Diarrhoea in calves has many causes including infectious agents. The four pathogens most often associated with the disease are the protozoal parasite *Cryptosporidium parvum*, the viruses rotavirus and coronavirus, and enterotoxigenic strains of the bacteria *Escherichia coli* (Cho and Yoon, 2014). Other viral pathogens which have a less well defined association with calf diarrhoea include bovine torovirus, bovine calicivirus and bovine astrovirus.

Astroviruses (AstV) are single stranded, positive-sense, non-enveloped RNA viruses of the family *Astroviridae*. The family is divided into the two genera *Mamastrovirus* and *Avastrovirus* based on their mammalian and avian host species, respectively. Astroviruses have long been recognised as an important cause of paediatric diarrhoea in human infants (reviewed in (Moser and Schultz-Cherry, 2005)); however, their role in enteric disease in other species is less clear. A diverse range of AstVs have been detected in faecal samples from diarrhoeic (Englund et al., 2002; Snodgrass et al., 1979; Toffan et al., 2009; Xiao et al., 2013) and healthy animals (Luo et al., 2011; Ng et al., 2013; Reuter et al., 2011; Tse et al., 2011) from a wide variety of species; however, their presence has only been convincingly linked with enteritis in mink and turkeys (Behling-Kelly et al., 2002; Englund et al., 2002). Further studies are required to define the role of AstV as a causative agent of diarrhoea in other species.

AstV displays a high degree of sequence variability. In humans, for example, there are currently four identified species that can be further subdivided into numerous serotypes and subtypes. These serotypes and subtypes have been found, in some situations, to differ in their virulence (Caballero et al., 2003 Holtz et al., 2011). There have also been reports that particular AstVs differ in their tissue tropism with some being associated specifically with

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neurological disease in humans (Quan et al., 2010), mink (Blomstrom et al., 2010), and cattle (Bouzalas et al., 2014; Li et al., 2013).

Infections of cattle with AstV were first described in 1978 (Woode and Bridger, 1978). Early studies suggested that these viruses were non-pathogenic in calves upon challenge (Bridger et al., 1984) but could exacerbate disease when calves were co-infected with rotavirus (Woode et al., 1984). Recent studies have described detection of AstV at a low prevalence in the faeces of adult cattle in Hong Kong (Tse et al., 2011) and calves in Korea (Oem and An, 2014). The latter study re-raises the potential pathogenic role of these viruses as detection was restricted to diarrhoeic samples. In order to examine the prevalence, diversity and disease associations of bovine AstV we analysed faecal samples from healthy calves, diarrhoeic calves and healthy adult cattle from farms in South West Scotland.

2. Materials and methods

2.1. Sampling design

Seventy faecal samples from calves with a clinical history of diarrhoea were received by SAC Consulting: Veterinary Services for routine diagnostic investigation of neonatal enteritis between 27th November 2012 and 25th January 2013. All calves were under 4 weeks old and from 36 different dairy farms in South West Scotland. Control samples were obtained from 45 healthy calves from 5 dairy farms in South West Scotland with no reported calf diarrhoea at the time of sampling. Faecal samples were collected from 20 adult cattles over 2 years old with no evidence of diarrhoea from 3 farms. All control samples were collected at the time of defaecation and not per rectum.

2.2. Samples and nucleic acid extraction

Faecal samples were collected and suspended in 1 ml of RNAlater (Ambion) and stored at 4 °C for a maximum of 2 days prior to processing. Particulate material was removed by centrifugation for 5 min at 13,000 × *g*. Nucleic acids were extracted from 120 μl of faecal supernatants using an AllPrep DNA/RNA Mini Kit (Qiagen) and recovered in 30 μl of nuclease-free water.

2.3. Reverse transcription (RT) and polymerase chain reaction (PCR)

cDNA was synthesised from 6 μl of recovered RNA using Superscript III reverse transcriptase (Life Technologies) with random hexamer primers. The bovine AstV RNA dependent RNA polymerase (RdRp) PCR was performed using a previously described protocol (Chu et al., 2008) but with the BoAst3561as primer (5′-CCYTTRTTMABRWADGCRAACTCAAA-3′) in place of the

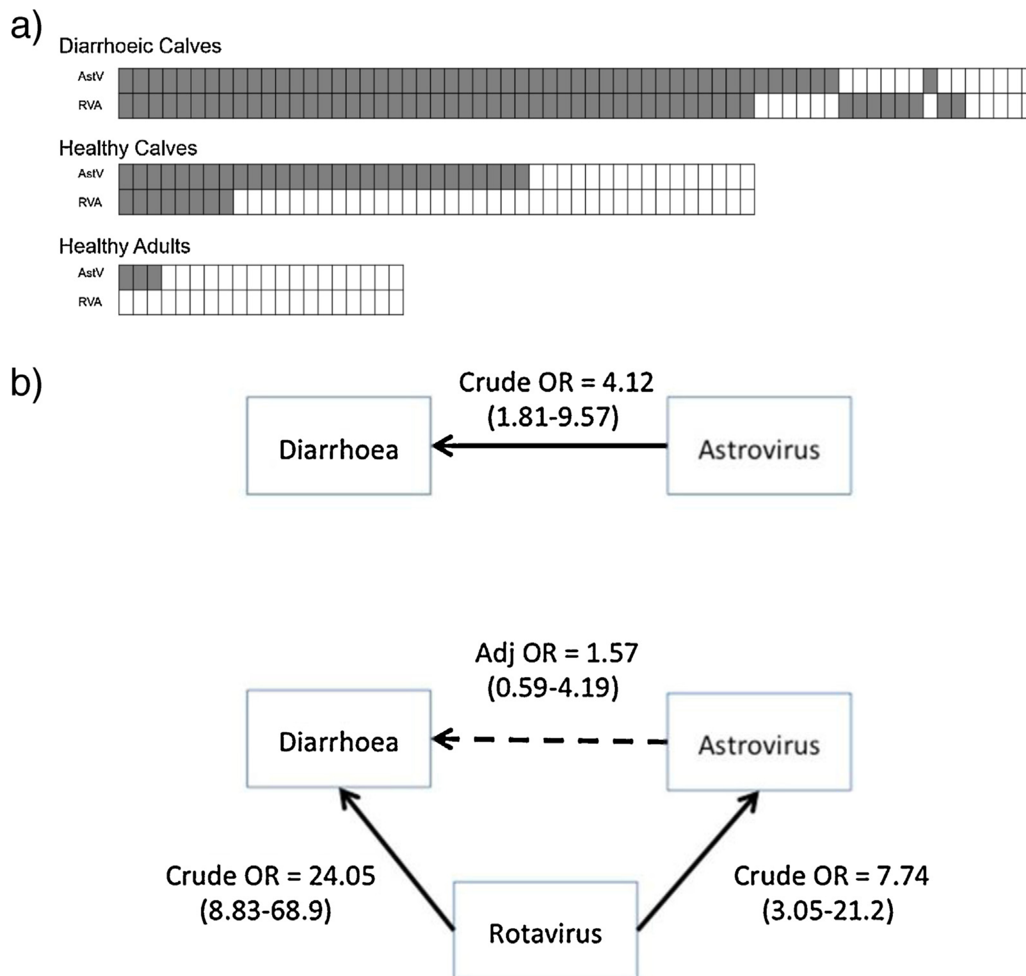

Fig. 1. AstV in faeces of calves is not associated with diarrhoea. (a) The presence of astrovirus (AstV) and rotavirus Group A (RVA) in diarrhoeic calves, healthy calves and healthy adult cattle was determined by PCR. Each column represents an individual animal, coloured squares indicate a positive PCR result and clear squares a negative PCR result (b). Schematic diagram showing the crude odds ratio (with 95% confidence intervals in parentheses) for diarrhoea and the presence of AstV in the study population (top) and the Mantel–Haenszel adjusted odds ratio (bottom) after adjusting for RVA status, showing that the AstV relationship is confounded by RVA status.

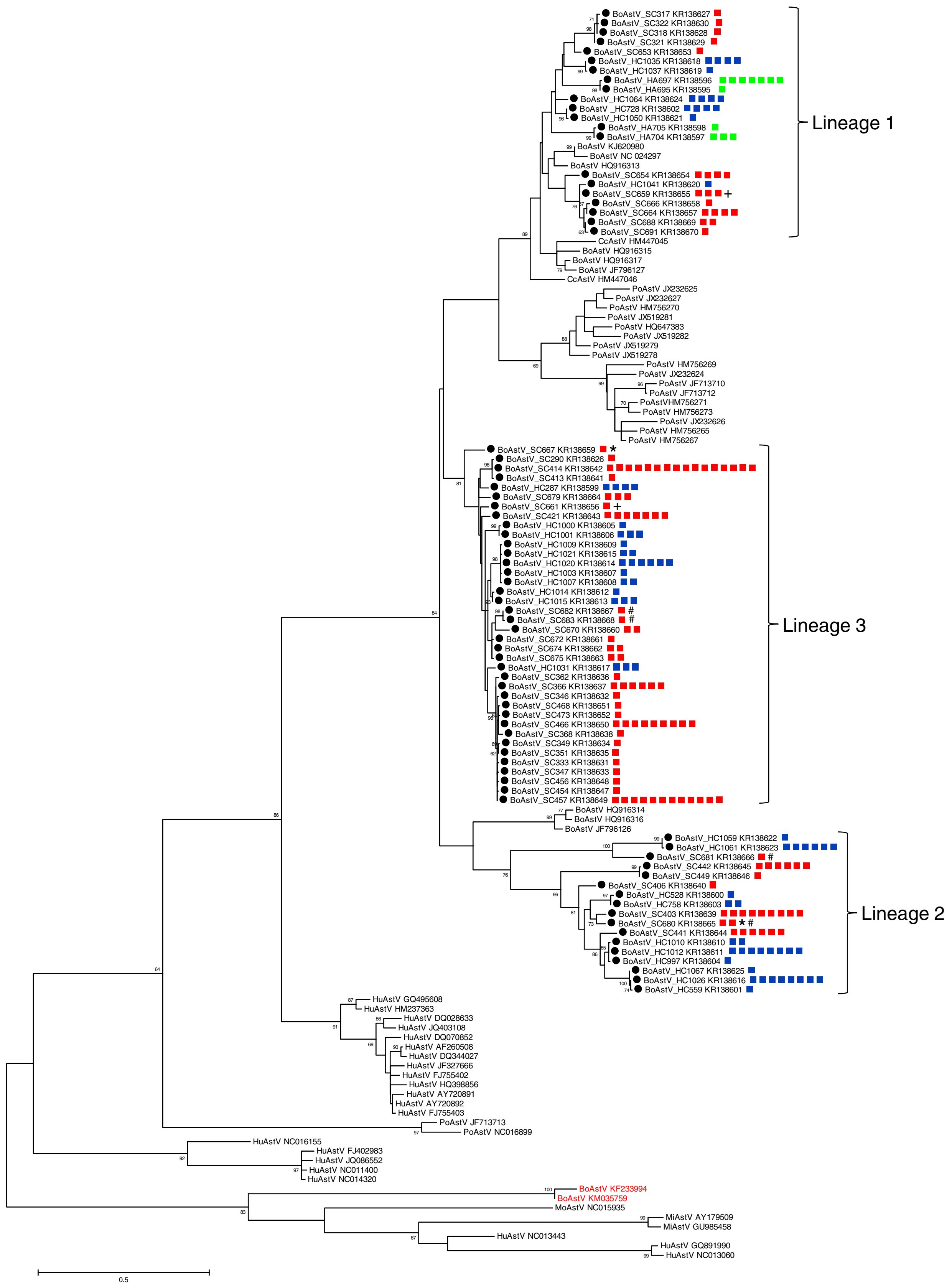

Fig. 2. Phylogeny of unique AstV clones as inferred from partial RdRp nucleotide sequences (equivalent to nucleotides 3316–3535 of the bovine AstV sequence HQ916313). The tree was constructed using representative AstV sequences from humans (HuAstV), pigs (PoAstV), rodents (MoAstV), cattle (BoAstV), mink (MiAstV) and deer (CcAstV). Squares on each node represent the total number of clones matching each unique sequence from scouring calves (red), healthy calves (blue) and healthy adults (green). Nodes representing sequences derived from three individuals determined as having mixed infections are marked *, + and #. The BoAstV sequences with red node labels are from viruses detected in association with neurological disease. The evolutionary history was inferred using maximum likelihood methods. The optimum maximum likelihood models (lowest Bayesian information criterion score and typically greatest maximum likelihood value) for the nucleotide sequence alignment was first determined and used

reverse primer in the second round of amplification. The rotavirus Group A (RVA) nested PCR was performed using primers designed based on an alignment of mammalian RVA segment VP1 sequences from 15 human, 3 equine, 2 porcine, 2 simian, 1 feline, 1 ovine, 1 antilopine and 4 bovine viruses (GenBank accession numbers NC011507,JF990805, JF693169, JF693103, HQ657138, JN013987, HQ657160, JF693158, JF693114, JF693180, JF693125, JF693191, JF693136, JF693147, JF693081, JN903527, JN903528, JN872865, X76486, M32805, EU636924, FJ422131, GU827406, FJ031024, FJ495126, JN831220, JF693059, JF693026 and DQ838640). Reactions were performed using GoTaq DNA polymerase (Promega) and primers RotaA_19_OS (5′-ATGGGGAAGTAYAATCTAATCTTGTCAG-3′) and RotaA_467_OAS (5′- TCYARCCARAACATRACTGCATTTAA-3′) in the first round and RotaA_47_IS (5′- AATATYTRTCATTYRTWTA-TAAYTCRCAATC-3′) and RotaA_368_IAS (5′- TCAGAHGTTA-TYTTRTTRTTYTCATAATC-3′) in the second round. First round reactions were performed using 2 μl of cDNA as template under the following conditions: initial denaturation at 94 °C for 60 s and 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 60 s at 68 °C. Second round reactions were performed using 5 μl of first round template under the following conditions: initial denaturation at 94 °C for 60 s and 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 60 s at 68 °C.

PCR amplicons were ligated into pGEM-T Easy (Promega) according to manufacturer's instructions and these ligation reactions were used to transform DH5α chemically competent *E. coli* which were plated on LB/agar plates supplemented with 100 μg/ml ampicillin, 0.5 μM IPTG and 80 μg/ml X-gal. Positive clones were identified using PCR with primers specific for the M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAA-CAGCTATGAC-3′) sites located on opposite sides of the multiple vector cloning site. Plasmid-containing colonies were picked from LB/agar plates and resuspended in 15 μl of LB broth, 1 μl of which was added to GoTaq (Promega) PCR master mix. Reactions for screening clones were performed under the following conditions: initial bacterial lysis/denaturation for 2 min at 94 °C, 30 cycles of 18 s at 94 °C, 30 s at 50 °C and 60 s at 72 °C and a final extension of 5 min at 72 °C. Direct sequencing of positive colony amplicons was carried out using BigDye Terminator v3.1 (Applied Biosystems) according to manufacturer's instructions with the M13F primer. Sequencing reactions were read by Edinburgh Genomics.

2.4. Sequence analysis

Cloned amplicon sequences, with vector nucleotides removed, were analysed and aligned with published AstV genome sequences using SSE v1.1 (Simmonds, 2012). Phylogenetic trees were constructed using maximum likelihood methods as implemented in the MEGA 6.0 software package (Tamura et al., 2013).

2.5. Statistical analysis

Statistical analysis was carried out in the R statistical environment version 3.1.2 (R Core Team, 2014). Univariable associations were estimated using the *cc* function in the *epicalc* package (Chongsuvivatwong, 2012). A Mantel–Haenszel adjusted odds ratio controlling for the presence of RVA was estimated using the *epi.2by2* function in the *epiR* package (Stevenson et al., 2014). The multivariable model was estimated using a Firth adjusted logistic regression model and the *logistf* function in the package of *logistf* package (Heinze et al., 2013) to deal with low counts for certain covariate patterns.

Table 1
AstV in in the faeces of calves is not associated with diarrhoea. Multivariable logistic regression model to estimate the association between AstV status and diarrhoea adjusted for the presence of RVA and the age of animal.

Variable	Level	Adjusted OR	95% CI	*p*-value
AstV	No	1		0.855
	Yes	0.901	0.316–2.52	
RVA	No	1		<0.001
	Yes	14.6	5.80–41.2	
Adult	Calf	1		0.003
	Adult	0.053	0.004–0.459	

2.6. Nucleotide sequence accession numbers

The nucleotide sequences of AstV amplicons were submitted to GenBank under the accession numbers KR138595–KR138670 and KR187112–KR187175.

3. Results

3.1. Epidemiological investigations do not reveal a statistical association between diarrhoea and AstV in faeces of calves in South West Scotland.

In order to investigate the role of AstV in calf diarrhoea we compared the presence of AstV in faecal samples from healthy calves and diarrhoeic calves. The presence of AstV was tested for using a nested RT-PCR which amplifies the AstV RNA dependent RNA polymerase (RdRp) gene. Faecal samples were collected from 45 healthy calves from 5 dairy farms with no reported calf diarrhoea at the time of sampling and 70 calves with clinical signs of diarrhoea from 36 dairy farms. Faecal samples were also collected from 20 adult cattles with no recent history of diarrhoea. AstV was detected in a high proportion of both healthy and diarrhoeic calves (29 out of 45 (64.4%) and 56 out of 70 (80.0%), respectively). In contrast to the results from calves, AstV was uncommon in older cattles from the herds examined, being detected in only 3 out of 20 adult cattles (15.0%) none of which had signs of diarrhoea (Fig. 1(a)). We also investigated the presence of rotavirus Group A (RVA), a common cause of calf diarrhoea (Dhama et al., 2009), by analysing the faecal samples from healthy and diarrhoeic calves for RVA using PCR directed against highly conserved sequences in the RVA segment VP1. RVA RNA was detected in 8 out of 45 healthy calves (17.8%) and 54 out of 70 diarrhoeic calves (77.1%) (Fig 1(a)) revealing, as expected for this well characterised enteric pathogen (Castrucci et al., 1994; Holland, 1990), an association between RVA and diarrhoea.

A simple univariable analysis looking for statistical associations between variables of interest and the presence of diarrhoea initially suggested that AstV was also strongly associated with diarrhoea in the study samples (Fig. 1(b)). However, AstV was also found to be strongly associated with RVA (Fig. 1(b)), which could therefore be acting as a confounding factor. Indeed, after adjusting for the presence of RVA using a Mantel–Haenszel adjusted odds ratio the relationship between AstV and diarrhoea was no longer statistically significant (Fig. 1(b)). To examine this further a multivariable model was developed to estimate the association between AstV and diarrhoea after adjusting for both RVA and animal age using a Firth logistic regression model (Table 1). The model suggests that adult animals have a lower odds of having diarrhoea (OR = 0.53; 95% CI 0.004–0.459) and that animals with RVA presence have ∼15 (OR = 14.6: 95% CI 5.80–41.2) times the odds of having diarrhoea compared to those without. After

for phylogenetic reconstruction. This was the Tamura–Nei model with a gamma (γ) distribution. Bootstrap support of branches (500 replications) is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adjusting for age and the presence of RVA there was no statistically significant association with the presence of AstV (p-value = 0.855). We also tested for any evidence of a statistical interaction/effect modification between the two viruses but this was not significant and not included for simplicity. Importantly, the multivariable model is in agreement with the Mantel–Haenszel adjusted odds ratio, revealing that after adjusting for RVA and age, there is no statistically significant association between diarrhoea and AstV.

3.2. Variation in the genotype of astrovirus in the faeces of calves

There is evidence that certain lineages of human AstV exhibit greater virulence (Caballero et al., 2003 Holtz et al., 2011). In order to determine if particular bovine AstVs are differentially associated with calf diarrhoea, amplicons from AstV PCRs selected to represent all positive farms and a variety of amplicon intensities were cloned and sequenced. A total of 209 individual clones from 48 amplicons (1–8 clones per amplicon from 28 diarrhoeic calves, 17 healthy calves and 3 healthy adults) were sequenced. Primer sequences were trimmed and a 220 nucleotide region equivalent to nucleotides 3316–3535 in the bovine AstV sequence HQ916313 was used for further analysis. 76 unique sequences were identified from the 209 clone sequences analysed. Phylogenetic analysis of these sequences revealed the presence of three distinct viral lineages (Fig. 2) with varying degrees of relatedness to previously described AstV from cattle, deer and pigs. The overall diversity of clones from healthy and diarrhoeic calves was similar with comparable numbers of clones from these two sample sets in the three viral lineages identified. The sequences in each of the three groups were diverse, with those classified as lineage 2 showing the highest variability with a mean pairwise nucleotide divergence across the analysed region of 18.1% compared to 11.9% and 6.2% in lineages 1 and 3, respectively. Pairwise distances between lineages were 30.0% (lineage 1 vs 2), 23.3% (lineage 1 vs 3) and 28.8% (lineage 2 vs 3). While the majority of AstV clones from a single individual contained identical or closely related sequences, there were three cases (Fig. 2, nodes marked *, + and #) where sequences of multiple lineages were cloned from a single individual's PCR amplicon indicating concurrent infection with multiple, diverse AstVs. Overall, many different AstVs were found in the faeces of calves; however, no association of a particular lineage with calf diarrhoea was identified.

In the 21 clones that were sequenced from 5 RVA PCR amplicons, a pairwise nucleotide divergence of <1.2% over the 261 nucleotides amplified (322 nucleotide amplicon size minus primers) was found, as is to be expected with a highly conserved target region. All sequenced amplicons clustered phylogenetically with R2 genotypes (data not shown).

3.3. Astrovirus persistence

The temporal dynamics of AstV infection were examined by sequential sampling of three calves with diarrhoea from a single farm to determine if a change in AstV lineage correlated with clinical deterioration or improvement. Two calves (calves A and B) died from diarrhoea during the study while the third calf (calf C) survived. All 3 individuals remained AstV PCR positive over the sampling time. The relationship between the most prominent viruses detected at each time point was determined by phylogenetic analysis of 4–15 clones per sample (Fig. 3). In calf B, viral sequences detected at initial sampling and at time of death (15 days after initial sampling) were highly similar differing in, at most, 1 out of 223 nucleotides. Calf A, however, showed a complete change from detecting lineage 3 sequences on initial sampling (15 out of 15 clones) to detection of lineage 1 sequences at time of death (8 out of 8 clones). Calf C survived throughout the sampling period. No significant change was seen in viral sequences detected from samples on the 29th November, 17th December and 20th December with all being within lineage 3 and differing at most by 5 out of 223 nucleotides. However, from the sample taken on the 28th of December, lineage 1, 2 and 3 sequences were detected, demonstrating a clear mixed infection. From the sample collected on the 10th of January only lineage 2 sequences were detected. Overall these results show marked variation in the AstVs in the faeces of a single animal over time, with different AstVs present at different times and presence of multiple AstV lineages at the same time. In the three animals examined no temporal link was found between a change in AstV and the outcome of disease.

4. Discussion

Experimental studies have shown that inoculation of gnotobiotic calves with AstV does not result in clinical disease (Woode and Bridger, 1978; Woode et al., 1984); however, the situation on the farm where multiple pathogens are invariably present is less clear. Here we report a high prevalence of genetically varied astroviruses in faecal samples from both healthy and diarrhoeic calves in South West Scotland. No association between diarrhoea and presence of AstV was detected, neither was there an association between diarrhoea and specific AstV genotypes. This suggests that AstV were not an important cause of diarrhoea in our case animals. The finding is in contrast to the well-characterised enteric pathogen RVA which was found to be significantly associated with diarrhoeic calves in our study.

Our findings are similar to recent studies of porcine AstV which found a high prevalence of AstV in swine faeces (Xiao et al., 2013), and no association of AstV presence with diarrhoea (Zhang et al., 2014). However, our findings are in contrast to a recently reported screen of bovine faeces from Korean cattle where bovine AstVs were detected at a much lower prevalence and only in samples from diarrhoeic calves (9 out of 91 diarrhoeal calves and none of the 24 non-diarrhoeal samples tested were positive by PCR (Oem and An, 2014)). It is possible that different PCR design may explain the difference since during optimisation of the AstV PCR in our study we found that the use of primers designed specifically for bovine AstV strains resulted in more sensitive and reproducible detection of AstV from bovine faeces compared to use of pan-AstV primers (data not shown).

We examined age stratification of bovine AstV and found that AstVs were significantly more likely to be found in calves than adults, suggesting the younger animals are more susceptible to infection. A low level of detection in adult cattle was also reported by Tse et al. (2011) where only 5 of 209 samples from adult cattle were found to be positive for AstV. This age stratification is similar to reports from human medicine where AstV diarrhoea occurs predominantly in children (Afrad et al., 2013) and reported disease outbreak tend to be in children (Dalton et al., 2002; Li et al., 2010) and the elderly (Lewis et al., 1989), suggesting that protection against AstV is acquired after an initial infection and later wanes. However, the specific immune responses activated upon AstV infection and the level of protection they afford are not yet completely understood in either humans or veterinary species.

Sequencing of the RNA dependent RNA polymerase gene of the bovine AstV species found in Scottish cattle revealed several highly divergent lineages within the *Mamastrovirus* genus. By sequencing sequential samples from the same animals, we have shown AstV infection to be highly dynamic with different AstVs present in the same animal concurrently and sequentially. It is unclear whether this absence of genotypic consistency is due to constant re-infection with viruses from different lineages, differential shedding of certain lineages over time or detection of non-infecting viruses acquired through dietary contamination. Our ability to classify the

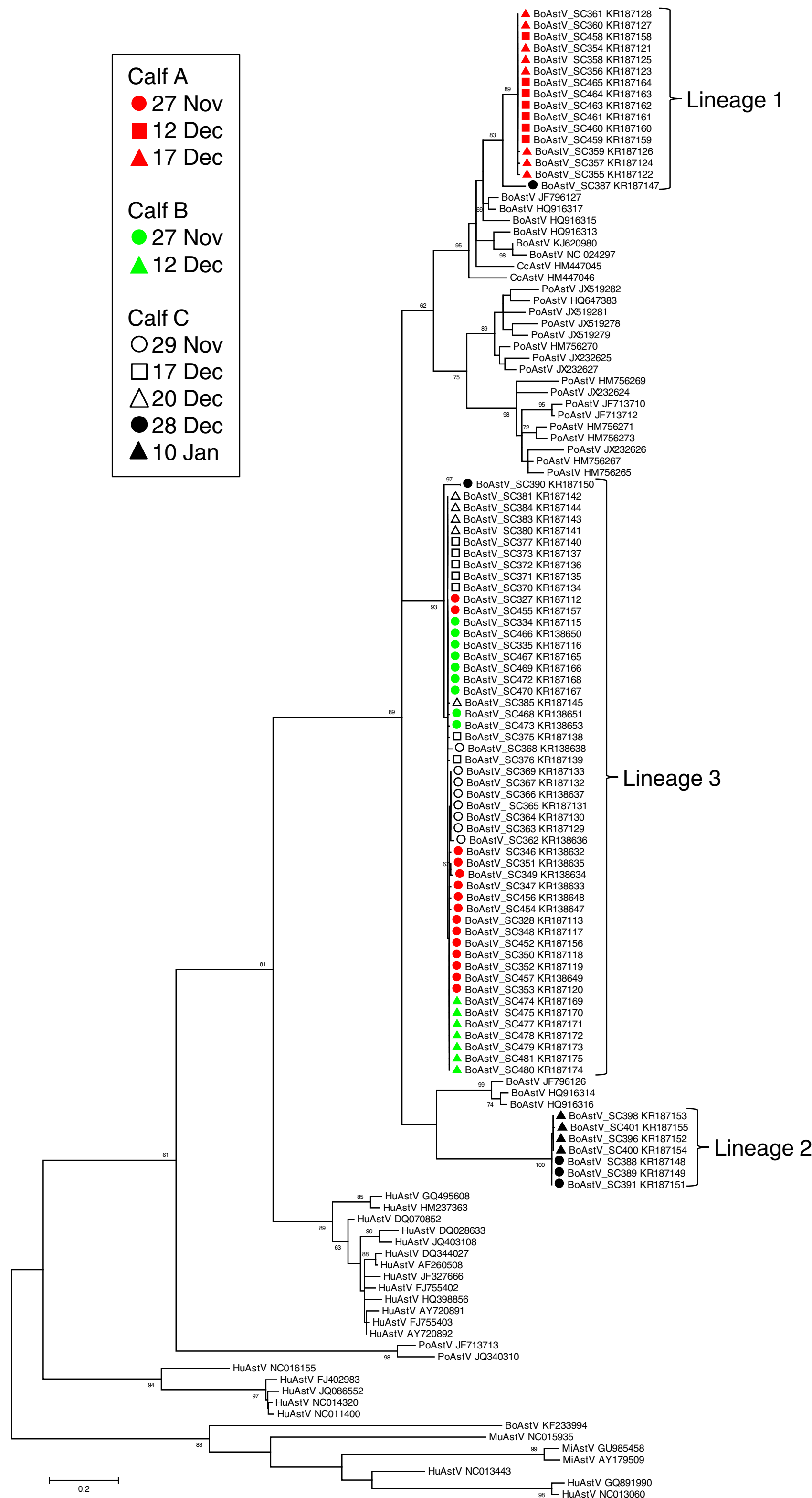

Fig. 3. Phylogeny of unique AstV clones as inferred from partial RdRp sequences (equivalent to nucleotides 3316–3535 of the bovine AstV sequence HQ916313). The tree was constructed using representative AstV sequences from humans (HuAstV), pigs (PoAstV), rodents (MuAstV), cattle (BoAstV), mink (MiAstV) and deer (CcAstV). Nodes with different symbols represent clones collected on different dates from four calves as indicated by the key. The evolutionary history was inferred using maximum likelihood methods. The optimum maximum likelihood models (lowest Bayesian information criterion score and typically greatest maximum likelihood value) for the nucleotide sequence alignment was first determined and used for phylogenetic reconstruction. This was the Tamura 3-parameter model with a gamma (γ) distribution. Bootstrap support of branches (500 replications) is indicated.

viral sequences detected was restricted by the limited size of the screening amplicons; however, further characterisation of complete genomes from these viruses will better inform classification and investigate the potential for recombination given the detection of concurrent infection with multiple AstV lineages. The use of the more specific PCR in this study compared to the published pan AstV assay (Chu et al., 2008) may have resulted in a restriction of viral lineages detected although the diversity of sequences reported here and obtained in an initial screen of these samples with the published assay was comparable (data not shown). Importantly, no sequences identified in the initial screen were shown to cluster with the recently described bovine neurotropic AstV (marked with red node labels in Fig. 2) (Bouzalas et al., 2014; Li et al., 2013) indicating that this lineage of viruses may have a more restricted prevalence or different tissue tropism.

We investigated co-infection of AstV with a common viral enteric pathogen—RVA. Co-infection with AstV and other enteric pathogens has been reported previously in calves (Oem and An, 2014; Woode et al., 1984) and humans (Afrad et al., 2013; Li et al., 2010), and our study found that presence of AstV and RVA in calf faeces were strongly associated. We were unable to determine if this association is causal, for example if AstV presence predisposes calves to RVA infection or vice versa, or the clinical significance of the association for example if AstV potentiates the severity of diarrhoea caused by RVA, as has been reported experimentally (Woode et al., 1984). Definitive determination of bovine AstV as an enteric pathogen, either singly or in combination with another pathogen such as RVA, would require viral isolation and experimental infections under natural conditions. However, our study, with the important inclusion of age and geographically matched controls, found no evidence that detection of AstV, or even specific AstV lineages, in the faeces of calves is associated with diarrhoea.

Conflicts of interest

None

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