Natural interspecies recombinant bovine/porcine enterovirus in sheep

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INTRODUCTION

The genus Enterovirus of the family Picornaviridae currently consists of 10 species: Human enterovirus A to D (HEV-A to -D) including polioviruses in HEV-C, Simian enterovirus A (SEV-A), Bovine enterovirus (BEV), Porcine enterovirus B (PEV-B), Human rhinovirus A to C (HRV-A to -C) and a number of unclassified enteroviruses (Knowles et al., 2012). The species Porcine enterovirus B consists of four types: PEV-9, PEV-10 and two recently described types which we propose to name PEV-14 and PEV-15 (Knowles et al., 1979; Boros et al., 2011a, 2012).

Most enterovirus infections are subclinical, but occasionally they can cause severe and potentially fatal diseases in humans and also in animal species (Pallansch & Roos, 2007; Blas-Machado et al., 2007). Some enteroviruses can infect more than one animal species, e.g. BEV-like viruses have been detected in bottlenose dolphins and bush-tail possums [bottlenose dolphin enterovirus (BDEV) and possum EV, respectively], and partial BEV-like sequences were also detected in faecal samples from goats, sheep, deer and horses (Nollens et al., 2009; Zheng, 2007; Ley et al., 2002; Jiménez-Clavero et al., 2005). Knowledge of the host range of PEVs is more limited, with only a few non-pig partial PEV-B-like sequences being available in the public sequence databases. These examples are from humans (GenBank accession numbers GU262993–GU262994) and rhesus monkeys (GQ250050–GQ250074).

Enteroviruses are small, non-enveloped viruses with an ssRNA genome of positive polarity that is approximately 7400–7500 nt in length. The single ORF, which encodes the viral polyprotein, is flanked by 5′ and 3′ UTRs (Pallansch & Roos, 2007). The length of the 5′ UTR varies between 700 and 825 nt and contains highly structured genetic elements (Zell et al., 1999; Lukashev, 2010). The viral polyprotein contains four structural proteins (VP4, VP2, VP3 and VP1 in order of genomic position) encoded in the P1 region and the non-structural viral proteins of the P2 (2A to 2C) and P3 (3A to 3D) regions (Pallansch & Roos, 2007; Knowles et al., 2012).

In picornaviruses, continuous genomic change is the result of a lack of proofreading by the viral 3D (three-dimensional)
RNA-dependent RNA polymerase (RdRp) (Wimmer et al., 1993; Lukashev, 2010), resulting in point mutations which may be selected during viral adaptation (reviewed by Savolainen-Kopra & Blomqvist, 2010). Large-scale changes may also arise as the result of recombination events between viruses. These exchanges of genome parts from different parental sequences may lead to higher genetic diversity and sometimes could contribute to the crossing of the viral host species barrier, as has been postulated for swine vesicular disease virus, which emerged from coxsackievirus B5 (Lukashev, 2010; Zhang et al., 1999). Intratypic, intertypic and interspecies recombinants have been described within and between the four human enterovirus species (Ledinko, 1963; Sergiescu et al., 1966; Lukashev, 2005; Lukashev et al., 2003; Savolainen-Kopra & Blomqvist, 2010). There are only a few studies reporting possible intraspecies recombination events within either simian or bovine enteroviruses (Oberste et al., 2003; Savolainen-Kopra & Blomqvist, 2010). There are only a few interspecies recombinants (Lukashev, 2005, 2010; Kyriakopoulou et al., 2010) and only a few interspecies examples have been reported (Yozwiak et al., 2010; Smura et al., 2007; Bolanaki et al. 2007). To our current knowledge none of them were detected from animal sources, although simian enterovirus (EV) SA5 and the recently identified simian EV-B110 suggest recombination between human and (unidentified) simian enteroviruses (Harvala et al., 2011). However, much less attention has been dedicated to the study of recombination among non-primate enteroviruses. It has been shown that all human EV-D70 isolates have a common ancestor that emerged in one place (probably west Africa) in 1967 (+/-15 months) (Takeda et al., 1994). The recent origin of EV-D70 and wide host range in cells of both EV-D70 (Yoshii et al., 1977) and EV-D94 (Smura et al., 2007) have led some workers to suggest that these serotypes may have originated from animal enteroviruses (Smura et al., 2007), possibly acquiring HEV-C-like 5’ UTRs by recombination.

In this study, we report for the first time the discovery and complete genome characterization of a novel interspecies (BEV/PEV-B) recombinant enterovirus from sheep faecal samples, provisionally named ovine enterovirus type 1 (OEV-1). Complete sequence analysis of OEV-1 revealed a close relationship to BEVs in the 5’ UTR region but considerable divergence of the other genome regions, which in turn showed a closer relationship to PEV-Bs. We also report the endemic presence of OEV-1 in faecal samples collected from the same sheep farm in two consecutive years.

RESULTS

Detection and complete genome acquisition of OEV-1

Faecal samples were collected from eight 3-week-old clinically healthy sheep in 2009 and 2010. None of the samples tested positive by applying the generic UnivEntero-5’ UTR primer pair. However, one of the eight (from 2009) and six of the eight (from 2010) were RT-PCR-positive for enteroviruses using the generic Non-HumanEntero-5’ UTR primers (Table S1, available in JGV Online). Three samples (one sample from 2009 and two samples from 2010) were sequenced and identified as bovine-like enterovirus, with the highest nucleotide sequence identity (85–90 %) to uncultured bovine-like enterovirus isolate Ov2.3 partial 5’ UTR (GenBank accession no. AY831715) and BEV type 2 strain Wye 8844 partial 5’ UTR (GenBank accession no. AY724744) with a relatively low E-value using BLASTN search (data not shown). The 220 bp long partial 5’ UTR sequences showed >93–100 % nucleotide identity to each other. To determine the complete genome of this enterovirus originating from sheep, two series of long-range RT-PCR and 5’ RACE PCRs were performed on one of the selected enterovirus-positive samples (TB4). The acquired complete genome was analysed and provisionally named as a novel candidate enterovirus, OEV-1 (GenBank accession no. JQ277724).

Genome characterization and phylogenetic analysis of OEV-1

The complete genome length of OEV-1 was 7408 nt excluding the poly(A) tail. A large ORF of 6519 nt, which encoded a 2172 aa long polyprotein precursor, was flanked by an 815 nt long 5’ UTR and a 74 nt long 3’ UTR. The P1, P2 and P3 regions were 2517 nt (839 aa), 1734 nt (578 aa) and 2265 nt (755 aa) long, respectively. The base composition of the complete genome was 26.6 % A, 24.0 % C, 24.7 % G and 24.7 % U.

Pairwise sequence identity comparisons using SimPlot analysis based on the complete genome of OEV-1 and representative members of PEV-B and BEV revealed the close relationship between OEV-1 and PEVs in every genome region except the 5’ UTR, which was most similar to BEVs (Fig. 1). The pairwise nucleotide/amino acid sequence identities of OEV-1 compared with PEV-10 (LP54/UK/75, GenBank accession no. AF363455) were 65/75 % (P1), 73/83 % (P2) and 78/89 % (P3), while compared with the closest bovine enterovirus, BEV-2 (BJ001, GenBank accession no. HQ663846), they were 61/61 % (P1), 61/64 % (P2) and 65/74 % (P3).

Recombination analysis of OEV-1

The position of OEV-1 in the nucleotide and deduced amino acid phylogenetic trees constructed from different genome regions (5’ UTR, P1, P2,P3) shows results consistent with the SimPlot analysis. OEV-1 clustered together with PEV-B, closest to PEV-10 (LP54/UK/75) in every genome region except the 5’ UTR, where it grouped with the BEVs (Figs 2, 3). Bootscanning analysis was also performed on the complete genome of OEV-1 for investigation of the interspecies recombination using PEV-10 (LP54/UK/75, GenBank accession no. AF363455) and BEV-2 (BJ001, GenBank accession no. HQ663846). Throughout the complete length of the 5’ UTR, the close
sequence relationship to BEV-2 was supported with high bootstrap values (>95%), while the remainder of the genome of OEV-1 showed a closer relationship to PEV-10 with high bootstrap support (>70%) except in parts of the VP2 coding region (Fig. 4). Analysis of the similarity plot, bootscanning and nucleotide alignment suggested that the recombination breakpoint was at the border of the 5' UTR and VP4 adjacent to the double adenine of the possible parental sequences at nucleotide position 814 (Fig. 5).

5' UTR structure analysis of OEV-1

The 5' UTR sequence length (815 nt) of OEV-1 was similar to those of both the PEVs (814 nt) and BEV-2 (824 nt). The predicted secondary RNA structure of the OEV-1 5' UTR shows a high degree of similarity to BEVs (Fig. 6). The distribution analysis of dissimilar nucleotides in the 5' UTR compared with BEV-2 (BJ001, GenBank accession no. HQ663846) revealed two highly conservative domains (III and V), where only one out of 16 (6.3%) and one out of 120 (0.9%) different nucleotides were present and none of them participated in the formation of the secondary structure caused by base pairing. Meanwhile, the pairwise comparison between OEV-1 and PEV-10 (LP54/UK/75) shows 31 out of 120 (25.8%) different nucleotides in domain V. Domain III in OEV-1 and domain III in PEV-10 could not be compared because they had a different number of nucleotides and different secondary structure (Fig. 6).

Identification of additional OEV-1 sequences

A specific primer pair was designed to amplify the 3’ part of VP1 of OEV-1 and PEV-B to screen the 5’ UTR-positive samples (Table S1). All of the 5’ UTR-positive samples also showed positive results using the VP1-specific primer pair. Two VP1-positive samples (from 2010) were sequenced. The pairwise comparisons of the partial VP1 sequences from the same collection date show 99% nucleotide and 98% amino acid sequence identity, while the comparisons of sequences from different dates show only 90% nucleotide and 96% amino acid identity. These nucleotide and amino acid differences were also noticeable in the phylogenetic trees, where all OEV sequences grouped together with the known PEVs and formed a distinct cluster among other enteroviruses (Fig. S1). The VP1 of OEV-1 (ovine/TB4-OEV/2009/HUN) showed 64% nucleotide and 71% amino acid identity compared with its closest relative, PEV-10 (LP54/UK/75).

DISCUSSION

In this study, we report for the first time the presence and complete genome sequence of a novel natural interspecies recombinant bovine/porcine enterovirus, provisionally named ovine enterovirus type 1 (OEV-1), from faecal samples of young, apparently healthy, sheep using generic 5’ UTR and VP4 primer pairs. The presence of OEVs in two consecutive years with significant nucleotide and amino acid similarity in the VP1 region and relatively high incidence among the second-year group suggest at least local endemic circulation of OEV among sheep on the farm.

Taxonomic classification of OEV-1

The results of the pairwise nucleotide and amino acid identity analysis of OEV-1 compared with the closest known relative, PEV-10 (LP54/UK/75), fulfil the current species demarcation criteria for the genus Enterovirus (Knowles et al., 2012); therefore, OEV-1 ovine/TB4-OEV/2009/HUN belongs to the species Porcine enterovirus B. The current criterion for the sequence-based sero/genotype determination is that VP1 nucleotide and amino acid sequence divergence should be ≥25% and 12%, respectively (Oberste et al., 1999; Pallansch & Roos, 2007). The VP1 of OEV-1 shows 36% nucleotide and 29% amino acid difference from the closest relative, PEV-10 (LP54/UK/75).
These results indicate that ovine/TB4-OEV/2009/HUN is a new genotype/serotype in the PEV-B group.

**Recombination analysis and mapping of the recombination breakpoint**

The results of the similarity plot and phylogenetic trees strongly suggest an interspecies recombination between PEV and BEV at the border of the 5’ UTR and VP4 of OEV. Bootscanning analysis on the complete genome of OEV-1 as a possible recombinant, with the closest available parental sequences PEV-10 (LP54/UK/75, GenBank accession no. AF363455) and BEV-2 (BJ001, GenBank accession no. HQ663846), confirmed the recombination event of OEV-1. The recombination process has been well-studied among human polioviruses due to the emergence of recombinant vaccine-derived viruses (reviewed by Savolainen-Kopra & Blomqvist 2010; Lukashev, 2005).

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**Fig. 2.** Phylogenetic relationships among enteroviruses based on alignment of P1 (a) and P2P3 (b) deduced amino acid sequences. Labels are as follows: sero/genotype name, strain name in parentheses and accession numbers in square brackets. Every major enterovirus group contained homologous and heterologous serotypes. Study sequence OEV-1 (ovine/TB4-OEV/2009/HUN, GenBank accession no. JQ277724) is indicated by an arrow. Numbers above branches indicate bootstrap values (based on 1000 replicates; only values >50% are shown). Bars indicate amino acid substitutions per site.
Fig. 3. An unrooted phylogenetic tree constructed from the 5’ UTR sequences of representative strains of human (HEV), simian (SEV), bovine (BEV) and porcine (PEV-B) enteroviruses and human rhinoviruses (HRV). Labels are the same as Fig. 2. The BEV and PEV clusters are depicted by grey shading; study sequence OEV-1 (ovine/TB4-OEV/2009/HUN, GenBank accession no. JQ277724) is indicated by an arrow. Numbers above branches indicate bootstrap values (based on 1000 replicates; only values >50% are shown). Bar indicates 0.05 nucleotide substitutions per site.
According to our current knowledge, there are two types of recombination model: the non-replicative and the strand-switching (or copy-choice) models, which have been demonstrated in several studies (Agol, 2010; Gmyl et al., 2003; King, 1988; Pilipenko et al., 1995; Cooper et al., 1974; Jarvis & Kirkegaard, 1992). There is no experimental evidence regarding the exact process of recombination during the emergence of OEV-1, although some properties could suggest the applicability of the strand-switching model to describe the recombination process between PEV and BEV. These properties were: (i) the presence of a stable hairpin loop (domain VII) before the AUG start codon in PEV-10, which could cause slowdown and termination of the negative-strand RNA synthesis; (ii) high sequence conservation (5′ end of VP4) near to the possible recombination breakpoint among the two presumable parental sequences, which could serve as a target of the heteroduplex formation; and (iii) the presence of adenosine dinucleotides (AA) at the very 5′ end of VP4 in the parental sequences, which could be the position of the template switching of the RdRp during the negative-strand synthesis (King, 1988). The considerable role of sequence conservation and secondary RNA structure in the recombination process among polioviruses was also reported by others (Romanova et al., 1986; Pilipenko et al., 1995). Considering the above-mentioned properties, and the results of SimPlot and bootscanning analyses, the presumed recombination breakpoint was mapped at nucleotide position 814 in OEV-1. However, due to the lack of sequence information of the exact parental sequences, there is a possibility that the recombination occurred at a different nucleotide position around the 5′ UTR/VP4 junction. To answer this question, future sequences (especially more PEV-like sequences) and in vitro recombination studies are necessary. According to the recombinant nature of OEV-1, there is a possibility that BEVs and PEVs (along with OEV-1) are related more closely than had been suspected previously. The natural recombinant ovine enterovirus is a further example demonstrating that picornaviruses consist of modular coding and non-coding elements (genomic regions) that can exchange and evolve independently.

The recombinant nature of OEV-1 was most unlikely to be an artificial product caused by an error in the RT-PCR amplification of distinct genome parts from a PEV-BEV co-infected sample, because: (i) no PEV-like 5′ UTR sequences were detected in any of the samples using UnivEntero-5′ UTR-R/F primers, which suggest that there were no PEVs in the examined samples; and (ii) the applied primers (BEV 5′ UTR specific forward and generic 2C reverse; see Table S1) and the chosen genome acquisition strategy (long-range RT-PCR followed by complete sequencing of the resulting long PCR products) also demonstrated the presence of a BEV-like 5′ UTR and PEV-like downstream sequences in OEV-1. In addition, specific amplicons that were designed to flank the putative recombination site were seen by RT-PCR in all of the seven sheep samples that contained OEV-1. All of these PCR-products show a similar nucleotide sequence and recombination pattern to OEV-1 by direct sequencing at the 5′ UTR/VP4 junction including the AA site. On the other hand, RT-PCR amplicons were not found using a forward primer specific for BEV 5′ UTR and a reverse primer specific for PEV VP4 in an artificial mixture of BEV and PEV.

**Analysis of the 5′ UTR secondary structure of OEV-1**

The close relationship between OEV-1 and BEVs at the 5′ UTR region was also supported by the strong similarities of the predicted 5′ UTR secondary structures. OEV-1 5′ UTR contains all of the unique features of BEV 5′ UTRs, i.e. the size and shape of the six previously identified domains (I–VI), the presence of a second putative cloverleaf-like structure (domain I*) and a short stem–loop (domain I**) connected to domain I (Zell & Stelzner, 1997). The nucleotides that differed from BEV-2 (BJ001) were mapped
in all six domains to analyse the nucleotide variability of the different domains during the possible adaptation process to the new host species. Most of the nucleotide substitutions did not affect the predicted structures. A relatively low number of point mutations were observed in domains II and V, domains that were required for translation and RNA synthesis of polioviruses (Borman et al., 1994; Minor, 1992; Hellen et al., 1994). The strong conservation of these two domains, and the structure conservation of the other internal ribosome entry site (IRES) domains, could suggest both the compatibility of ovine ribosomes and the PEV-like viral proteins (which interact with tertiary genome structures in this region) to the BEV-like IRES. (Note: the hosts cattle and sheep, both in the family Bovidae, are phylogenetically related more closely to each other than to pigs, which are in the family Suidae.)

There are no experimental results regarding the possible advantage of this recombinant virus in the infection–replication process in sheep compared with PEVs in pig and BEVs in cattle; however, the continuous presence of OEV-1 in sheep in two consecutive years suggests that this recombinant may have evolutionary advantage in this host species. The precise recombination source origin (sheep, pig, cattle or other animal) of the novel bovine/porcine interspecies recombinant OEV-1 should also be investigated. Recently it has been reported that PEVs (PEV-9 strain UKG/410/73 and PEV-10 strain W47H) and BEVs (strains BF-1 and C-121E) can be cultivated in the fetal lamb kidney cell line FLK-N3 (Matsuura et al., 2011). This suggests the possibility of natural BEV/PEV co-infections in sheep, which could serve as a possible recombination source.

**Possible misidentification of the presence of BEVs in different samples in the past**

BEV-like viruses in sheep were reported previously from Spain and India based upon only the partial 5′ UTR sequences (Jiménez-Clavero et al., 2005) and serological results [32.8% (92/281) seropositivity to BEV-1 in sheep] (Gür et al., 2008). However, our report provides the first complete insight into the genetic nature of enteroviruses in sheep. In the light of the interspecies recombinant nature of OEV-1, we cannot exclude the possibility of misidentification, misclassification or misinterpretation of BEVs in some animals (e.g. sheep, deer, horses) and environmental samples (water, oysters, etc.) in the past due to the generally applied RT-PCR primers targeting only the conservative BEV 5′ UTR region (Ley et al., 2002; Fong et al., 2005; Jiménez-Clavero et al., 2005). Thus future studies searching for enteroviruses in animals or the environment should seek to examine other genome regions in addition to the 5′ UTR.

As (re)emerging infectious diseases pose a continuous health threat to wild and domestic animals as well as to humans, more attention should be paid to combating newly emerging viral diseases and the continuous genetic changes of viruses. The discovery of a natural interspecies recombinant bovine/porcine enterovirus in domestic sheep is an example of the necessary expansion of our knowledge of the virus diversity and variability present in domestic and wildlife species.
Fig. 6. The secondary structure of the OEV-1 (ovine/TB4-OEV/2009/HUN, GenBank accession no. JQ277724) 5′ UTR based on the results of the Mfold program and the previously predicted secondary structure of BEV 5′ UTR (Zell & Stelzner, 1997; Zell et al., 1999). Light-grey circles represent ‘silent mutations’ (maintained base-base connections regardless of the Watson–Crick or wobble nature of base pairing) and dark-grey circles show point mutations causing the gain or loss of base pairing of the OEV-1 5′ UTR compared with the 5′ UTR nucleotide sequence of the closest available BEV-2 strain (BJ001, GenBank accession no. HQ663846). The predicted structures of the PEV and BEV 5′ UTRs are shown in frames for comparison. Grey boxes of the BEV 5′ UTR show the most conservative domains compared with the study sequence.
METHODS

Sample collection. Faecal samples from approximately 3-week-old domestic sheep (*Ovis aries*) were collected from a farm located in central Hungary in March 2009 (*n* = 8; sample identification: TB1-8) and April 2010 (*n* = 8; TB9-16). At this farm, merino ewes were mated with blackhead meat rams from Germany. None of the sampled animals showed any signs of clinical symptoms when the samples were collected. Sheep were kept in captive breeding, and had no contact with domestic pigs or cattle.

RNA extraction, RT-PCR and complete genome determination. RNA was extracted from 150 μl faecal suspension (35–40% v/v in 0.1 M PBS) using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were tested for enterovirus by RT-PCR using two generic primer pairs targeting the conservative 5′ UTRs of either the known human, simian and porcine enteroviruses (UniEntero-5′ UTR-R/F) or the bovine, ovine, possum and bottlenose dolphin enteroviruses (Non-HumanEntero-5′ UTR-R/F) in separate reactions (Table S1). The RNA extraction and the screening RT-PCR method were the same as described previously (Boros *et al.*, 2011a, b).

One of the enterovirus-positive samples (TB4) was selected for complete genome amplification using the long-range RT-PCR method and the primer-walking technique. All long-range RT-PCR reagents were purchased from Promega unless otherwise specified. The cDNA synthesis was carried out in 20 μl final volume containing 8 μl RNA, 10 mM dNTP, 4 μl 5 × MAXIMA RT buffer (Fermentas), 10 pmol of the antisense primer, 40 U RnaseH and 200 U MAXIMA Reverse Transcriptase (Fermentas). Reverse transcription was performed at 50 °C for 1 h. The RNA template was degraded with RNaseH. The PCR was conducted in 50 μl final volume using 5 μl of the RT reaction mixture as a template. The PCR mix contained 5 μl 10 × Long PCR buffer, 10 mM dNTP, 10-10 pmol sense and antisense primers and 2.5 U of Long PCR Enzyme mix (Fermentas). The PCR was conducted under the following conditions: one cycle at 94 °C for 1 min, 40 cycles of 94 °C for 30 s, (primer T<sub>m</sub>−5) °C for 30 s, 68 °C for 60 s and 68 °C for 10 min.

To determine the 5′ and 3′ ends of the genome, a series of 5′ and 3′ RACE reactions were conducted using the 3′/5′ RACE system (Roche Diagnostics) using the protocol described previously (Boros *et al.*, 2011b). A generic VP1/2A primer pair was designed using the sequences from the genome acquisition of OEV-1 and all available porcine enterovirus sequences were collected from GenBank to screen the EV 5′ UTR-positive samples. Primer sequences and localizations are presented in Table S1. PCR products were sequenced directly with a BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) using sequence-specific primers and run on an automated sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems).

Sequence and phylogenetic analysis. Enterovirus sequences were obtained from GenBank and the study sequences were aligned using CLUSTAL_X software v. 2.0.3 (Thompson *et al.*, 1997); similarity calculations were performed using GeneDoc v. 2.7 (Nicholas & Nicholas, 1997). Phylogenetic trees were constructed using MEGA 5 (Tamura *et al.*, 2011). The maximum-likelihood method using the Jones–Taylor–Thornton matrix-based model was used to construct trees for P1, P2P3 and VP1 using amino acid sequences, while the Jukes–Cantor model was used for the 5′ UTR. Bootstrap values (based on 1000 replicates) for each node are given if >50%. Similarity plot analysis was performed with the SimPlot software v. 3.5.1 (Lole *et al.*, 1999) using the Jukes–Cantor model with a window size of 200 nt and a step size of 20 nt. Recombination analysis was performed with RDP software v. 4.14 using the Jukes–Cantor model using a window size of 400 nt, a step size of 20 nt and 1000 bootstrap replicates (Martin *et al.*, 2010). The secondary structure of the 5′ UTR was predicted using the Mfold program (Zuker, 2003) and a two-dimensional model was drawn using the CorelDraw Graphics Suite v. 12. The complete genome sequence of ovine/TB4-OEV/2009/HUN and partial VP1 sequences of ovine/TB9-OEV/2010/HUN and ovine/TB16-OEV/2010/HUN were submitted to GenBank and assigned the accession numbers JQ277724–JQ277726.

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