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# Identification and genetic characterization of bovine enterovirus by combination of two next generation sequencing platforms

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ABSTRACT

Prompt and accurate diagnosis is warranted for infectious diseases of domestic animals which may have a significant impact on animal production or clinical practice. In this study, the identification and genetic characterization of a bovine enterovirus (BEV) strain isolated from a calf with diarrhea, are described. Two different next generation sequencing platforms were employed. Shotgun metagenomic accomplished by MinION sequencing (Oxford Nanopore Technologies) allowed the identification of BEV RNA from a cell-culture isolate. BEV was then confirmed by a specific real time RT-PCR assay. To achieve the whole genome of this isolate, sequence reads obtained by MinION were coupled with those originating from NextSeq500 (Illumina). Genomic relatedness and phylogeny with extant BEV strains is also reported. Overall, this manuscript highlights the use of the portable MinION sequence technology as a tool for support diagnostics in veterinary practice.

## 1. Introduction

Available diagnostic techniques for suspected infectious, either human or animal, diseases are based upon prior knowledge of the likely causative pathogen. Clinical presentation, epidemiological data, or simply, a specific request of the physician or veterinary practitioner, direct the choice for specific and targeted diagnostic assays. Polymerase chain reaction (PCR)-based and/or serologic diagnostic methods are the most frequently used laboratory assays. However, these approaches have fundamental limitations and contribute to the relatively high proportion of cases that remain undiagnosed. First, the difficulties of testing for the plethora of rare pathogens that might be expected to cause a given pathology, second, new or unexpected pathogens (included those originating from cross-species jumps) cannot be identified as for the existence of standardized, validated, and normally accredited, diagnostic algorithms. This scenario is further emphasized in laboratories with a relatively high daily routine which are regularly demanded to give a fast, accurate and reliable response.

In this context, the application of innovative molecular technology in clinical diagnosis is a rapidly developing area and is predicted to greatly improve the speed, efficiency and accuracy of diagnostic medicine. One of these novel and attractive technologies is certainly represented by next generation sequencing (NGS). NGS and bioinformatic analyses, indeed, allow the detection and identification of nucleic acids of pathogens from either cell isolates or clinical samples. Until recently, this methodology was hampered by the limited laboratory infrastructure and computing resources. However, the release of the MinION (Oxford Nanopore Technologies, Cambridge, UK), a novel portable real-time NGS sequencer which produces long reads, enables the application of metagenomic for rapid diagnosis even in low-throughput laboratories (Wang et al., 2015; Batovska et al., 2017).

Bovine enteroviruses (BEVs) are small, non-enveloped single positive-stranded RNA viruses, belonging to the genus *Enterovirus* within the family *Picornaviridae*. According to the ICTV (https://talk.ictvonline.org/taxonomy/) the genus *Enterovirus* is divided into 13 species defined as Enterovirus (EV) A-I and J and Rhinovirus (RV) A-C. BEVs belong to EV-E and EV-F, formerly classified as BEV-A and BEV-B, respectively (Knowles et al., 2012; Tsuchiaka et al., 2017). EV-E and EV-F are further sorted in genotypes, with EV-E comprising four recognized types (E1-E4) and one novel candidate (Mitra et al., 2016) and EV-F containing six recognized types (F1-F6) and one novel candidate (He et al., 2017). The genome contains a single open reading frame (ORF), flanked

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by untranslated regions (UTRs) at the 5' and 3' ends, that encodes a single long polyprotein, comprising structural proteins (VP1-4, encoded in P1) and non-structural proteins (2A-C, encoded in P2, and 3A-D, encoded in P3) (Knowles et al., 2012). BEVs have been isolated worldwide from cattle with a wide range of clinical signs including abortion, stillbirths, infertility, neonatal deaths but also diarrhea and pyrexia, dehydration and weight loss. BEV is also detected in asymptomatic animals; thus, BEV virulence in hosts remains to be elucidated and further investigated (Zell et al., 2006). In this manuscript, we describe the identification and genetic characterization of a BEV strain isolated in cell-culture from a calf with diarrhea. This calf tested negative for the most common viral and bacterial pathogens affecting the enteric tract of ruminants. BEV was identified by MinION and confirmed by a specific real time RT-PCR test. The whole genome sequence of the occurring strain, BEV Italy/17DIAPD2208/15/2017 was then obtained by the integration of reads originated from MinION and Illumina NGS technologies.

## 2. Case study and preliminary laboratory analyses

Post-weaning calves from a free-range farm of about 70 beef cattle located in Tuscany region, central Italy, showed signs of severe aqueous diarrhea and drooling. In addition, few animals also displayed cough and dyspnea. This farm reported undiagnosed clinical problems over the last three years, registering losses during the weaning process. Bacteria and parasites were detected in calves but in a discontinuous manner, and all treatments carried out resulted unsuccessful. Fecal samples from diarrheic calves, which tested negative for the most common viral and bovine enteric pathogens (data not shown) were used for virus isolation attempts. Fifteen fecal samples were suspended with a 1:5 dilution ratio in minimum essential medium (MEM) (Sigma Aldrich) with 1% antibiotics and 1% albumin, incubated overnight at 4 °C and then centrifuged at 4,200 X *g* for 5 min. Supernatants were filtered through 0.45-µm-pore-size filters and used as an inoculum for virus isolation on Vero cells (ATCCCCL-81) as previously described (Chen et al., 2014).

Out of 15 fecal samples, one infected cell culture showed cytophatic effect (CPE) after a first blind passage on Vero cells. Electron microscopy demonstrated the presence of icosahedral shaped viral particles of about 28-30 nm; shape and size were compatible with viruses of the *Picornaviridae* family (data not shown).

## 3. MATERIALS AND METHODS

### 3.1. Shotgun metagenomics by MinION

Cell culture supernatant was recovered for nucleic acid purification (High Pure Viral Nucleic Acid Kit, Roche) and used for metagenomic analysis. Nucleic acid elution was divided in two aliquots to perform RNA and DNA sequencing in two different runs. RNA sample was processed by means of the Sequence Independent Single Primer Amplification (SISPA) method (Allander et al., 2005) with some modifications to obtain cDNA (Marcacci et al., 2016; Marcacci et al., 2018). DNA and amplified cDNA were quantified by Qubit dsDNA HS assay (Thermo Fisher Scientific, Waltham, MA) and used for library preparation (SQK-LSK108 1D ligation genomic DNA, Oxford Nanopore Technologies, Cambridge UK) following manufacturer's guidelines. Briefly, DNA ends were end-repaired and dA-tailed using the NEBNext End Repair/dA tailing module (New England Biolabs, Hitchin, UK). Subsequently, sequencing adapters, supplied in the kit, were ligated onto the prepared ends using the Blunt/TA Ligase Master Mix (New England Biolabs, Hitchin, UK). All purification steps were carried out using AMPure XP beads (Agencourt, Beckman) according to the SQK-LSK108 sequencing protocol. The two libraries were quantified using the Qubit 2.0 fluorimeter and finalized by adding a mix composed of 35 µl of Running Buffer with fuel (RBF), 25.5 µl of Library Loading Beads (LLB) and 3.5 µl of Nuclease-free water to 11 µl of adapted DNA. Each library was loaded onto a primed MinION Spot-ON Flow Cell Mk I, R9 version (Oxford Nanopore Technologies,) and sequenced by running the program NC_48hr_sequencing_FLO-MIN107_SQK-LSK108_plus_Basecaller on MinKNOW Software. During the sequencing run, "What is in my pot" (WIMP) workflow (Juul et al., 2015) was initiated in the EPI2MEAgent Software v2.47.537208 for analyzing the raw FASTQ files produced by MinKNOW. Briefly, WIMP tool enables the comparison in real time of the raw reads against the NCBI Organism Database; this tool classifies and identifies the species by looking at the k-mers produced. Statistical data analysis of MinION sequencing run was performed by using NanoPlot software (De Coster et al., 2018).

### 3.2. Specific Real Time RT-PCR for BEV

A real time RT-PCR using specific primers for BEV (Jiménez-Clavero et al., 2005) was performed using RNA purified from cell culture supernatant and RNA purified (Viral RNA Mini Kit, Qiagen) from the fecal sample. PCR was run on the HT7900 fast Real Time PCR System (Applied Biosystems, USA). The 20 µL reaction volume contained 5 µL of total purified RNA, 10 µL of KAPA SYBR FAST qPCR Master Mix (2X) Universal, 0.4 µL 50X KAPA RT Mix, 200 nM of BEV primers forward and reverse, 0.4 µL of 50X ROX High and ultrapure DNase-RNase-free water to volume. RNA was denatured 5 min at 95 °C and then incubated 5 min on ice. The thermal profile consisted of a single cycle of reverse transcription for 5 min at 42 °C and 3 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation. The amplification of cDNA was performed by 40 cycles including denaturation at 95 °C for 3 sec, and annealing/extension/data acquisition for 20 sec at 60 °C.

### 3.3. Shotgun metagenomic by Illumina

Sequencing by NextSeq 500 platform (Illumina) was performed following procedures recently described by our group (Marcacci et al., 2016; Savini et al., 2017; Lorusso et al., 2018) to obtain the whole genome of the viral isolate. *De novo* assembly was performed using SPADES (version 3.8.0; Nurk et al., 2013; Bankevich et al., 2012) based on multiple kmer length. To obtain higher coverage and accuracy of the final consensus sequence, the obtained *de novo* sequence was used as reference for mapping by using Bowtie2 (version 2.3.4.1).

### 3.4. Genome characterization and phylogeny

Nucleotide (nt) sequences of representative BEVs and EVs were retrieved from GenBank database and aligned with that obtained in this study using MAFFT version 7.017 (Katoh and Standley, 2013). Phylogenetic analyses of the whole genome and VP1 were carried out using the Maximum Likelihood (ML) method implemented in MEGA version 6 (Tamura et al., 2013). The best-fit model of nucleotide substitution was identified by the Find Best DNA/protein Model available in Mega 6 (GTR + G). To assess the robustness of individual nodes on the phylogenetic trees, a bootstrap resampling analysis with 500 replications was performed using default procedures available in MEGA6.

## 4. RESULTS

### 4.1. MinION sequence data

In about 4 hours, a total number of 304,432 reads were obtained from the cDNA sequencing run. By assessing a quality cut-off (> Q7), a total number of 225,789 reads of sequencing reads was selected. The average of the reads length was 1,047 nt (Table 1). Of these, 19,117 were classified as eukaryote. Nearly 620 reads were assigned to viruses, specifically viruses of the Picornaviridae family, genus *Enterovirus*. Remaining reads were unclassified.