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Host range of mammalian orthoreovirus type 3 widening to alpine chamois

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ABSTRACT

Mammalian orthoreoviruses (MRV) type 3 have been recently identified in human and several animal hosts, highlighting the apparent lack of species barriers. Here we report the identification and genetic characterization of MRVs strains in alpine chamois, one of the most abundant wild ungulate in the Alps. Serological survey was also performed by MRV neutralization test in chamois population during five consecutive years (2008-2012). Three novel MRVs were isolated on cell culture from chamois lung tissues. No respiratory or other clinical symptoms neither lung macroscopic lesions were observed in the chamois population. MRV strains were classified as MRV-3 within the lineage III, based on S1 phylogeny, and were closely related to Italian strains identified in dog, bat and diarrheic pig. The full genome sequence was obtained by next-generation sequencing and phylogenetic analyses showed that other segments were more similar to MRVs of different geographic locations, serotypes and hosts, including human, highlighting genome reassortment and lack of host specific barriers. By using serum neutralization test, a high prevalence of MRV-3 antibodies was observed in chamois population throughout the monitored period, showing an endemic level of infection and suggesting a self-maintenance of MRV and/or a continuous spill-over of infection from other animal species.

1. Introduction

Mammalian Orthoreoviruseses (MRV) have a worldwide distribution with a wide host range including humans, livestock, companion animals and wildlife (Qin et al., 2017 and references therein).

MRV species includes three serotypes, according to the capacity of type-specific antisera to neutralize virus infectivity, inhibit hemagglutination and based on molecular analysis of S1 gene (Day, 2009). A putative MRV-4 has been also proposed (Attoui et al., 2001).

MRVs were traditionally associated to asymptomatic or mild respiratory and enteric infections (Tyler, 2001). Nevertheless, in recent years, a variety of clinical contexts caused by MRVs have been reported both in human and mammalian hosts. To date, MRV-2 and 3 have been identified in human cases of respiratory infections, encephalitis and diarrhea (Tyler et al., 2004; Chua et al., 2008; Ouattara et al., 2011; Steyer et al., 2013). Moreover, MRV-3, alone or in association with other pathogens, contributed to enteric disease in pigs in North America, Asia and Europe (Zhang et al., 2011; Thimmasandra Narayanappa et al., 2015; Lelli et al., 2016; Qin et al., 2017).

Concerning wildlife, several investigations highlighted MRV infections in bats, with MRV-3 as the most prevalent type in Europe (Kohl et al., 2012; Lelli et al., 2013; Naglič et al., 2018). MRVs are known to be variable viruses and genome reassortment have been detected in MRV-1 and MRV-2 strains from bats with reassortment involving human and animal species strains (Lelli et al., 2015; Wang et al., 2015). More recently, MRV-3 isolated in pigs also showed reassortment among different bat MRV types (Qin et al., 2017).

Because of the apparent lack of species barriers (Steyer et al., 2013; Li et al., 2016), there is an increasing interest and concern about the spread of MRVs in human and animal hosts and a zoonotic transmission could likely occur (Chua et al., 2011; Lelli et al., 2016). Aims of this study were: i) to identify and genetically characterize MRVs strains in chamois (*Rupicapra r. rupicapra*) by virus isolation, electron microscopy

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and molecular biology techniques and ii) to define seroprevalence and clarify the epidemiology of MRV-3 infection in chamois in Italian Alps by a serological survey performed during five consecutive years.

2. Materials and methods

2.1. Study area and sampling

The study was performed in north-western Italian Alps (Verbano-Cusio-Ossola province) where wild ruminants such as chamois, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and ibex (*Capra ibex*) are present. Furthermore, domestic ruminant herds graze alpine pastures in this area during summer season. The characteristics of the area are particularly favourable for chamois, which is the most abundant among wild ungulates with 6.6 individuals/100 ha, whereas roe deer are present with 2.6 individuals/100 ha, red deer with1.8 individuals/100 ha and ibex with 114 individuals in the whole area, as estimated by census activity performed during the investigation period.

Chamois are legally selective-hunted during the period September-October and in accordance with Italian Law (N. 157 of 11/02/1992), hunters must carry culled wild ungulates to the control centres, where age, sex and morpho-biometric measurements are registered. Gross lesions inspection of carcass and organs are also performed.

Blood samples, collected from yearling and adult animals from major blood vessels or heart clot by hunters during the bleeding of carcasses, were centrifuged and serum was stored at −20 °C until further processing. Lung samples were collected from yearlings, more susceptible to infectious agents, during post-mortem macroscopic inspection and stored at −20 °C for 1–2 weeks in the control center facility and subsequently transferred at −80 °C to the laboratory until further processing. Blood (n = 102) and lung samples (n = 87) were collected during five (2008–2012) and four (2009–2012) hunting seasons, respectively. Samples were obtained only from legally hunted animals and animals were not culled expressly for this study.

2.2. Viral isolation

Lung samples were homogenized, centrifuged and the supernatant was inoculated in 24 well plates in subconfluent monolayers of Madin Darby Bovine Kidney (MDBK ATCC CCL-22) cells, maintained in minimal essential medium (MEM) with 1% L-glutamine 200 mM, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 10% of fetal bovine serum (FBS), free of antibodies to bovine herpes virus^{-1}, bovine respiratory syncytial virus, parainfluenza 3 virus and free of both virus and antibodies to bovine viral diarrhea virus. The inoculated plates were incubated at 37 °C in 5% CO$_2$ and after a 1–2 hours adsorption period the cell cultures were rinsed and maintenance medium was added. The cell cultures were observed daily for cytopathic effect (CPE) for 6 days. Two blind passages were made if no CPE was observed, the cell cultures were scraped and vigorously mixed with culture medium and used for inoculation of fresh monolayers.

2.3. Negative staining electron microscopy

The supernatant fluids from cell cultures showing CPE were submitted to negative staining electron microscopy (nsEM) using the Airfuge method (Lavazza et al., 1990). Grids were stained with 2% NaPT, pH 6.8 for 1.5 min and examined at 19-30000x by using a Tecnai G2 Spirit TEM (FEI, Eindhoven, The Nederland) operating at 100 kV. Viral particles were identified based on their morphological characteristics.

2.4. RT-PCR, sequence analysis and phylogeny

Viral RNA was extracted from lysates of cell cultures showing CPE using TRIZOL® LS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was resuspended in 10 μl of DPEC water. The eluted RNA was used for retrotranscription using QIAamp One-For-All Nucleic Acid kit (Qiagen, Mississauga, Ont) and a PCR assay was performed for the detection of MRV using primer pairs L1-rv5/L1-rv6 (Leary et al., 2002) and S1-R1F/S1-R1R, S1-R2F/S1-R2R, S1-R3F/S1-R3R (Decaro et al., 2005).

Amplicons of the expected sizes were purified and sent for outsource sequencing (Primm). Sequences were aligned with MRV representative reference strains and other sequences retrieved from GenBank and used to build the phylogenetic trees. Sequences were aligned using Clustal X; manual editing was performed with Bioedit software version 7.0 (freely available at http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogeny was estimated by the neighbor-joining algorithm (NJ) using MEGA7 (Kumar et al., 2016). The robustness of the tree topologies was evaluated after 1000 bootstrap replicates.

The full genome sequence of one representative sequence of MRV from Italian chamois (id. 84407) was determined starting from fetal monkey kidney cell culture (MARC 145 - BS CL 127). Libraries were constructed from total RNA sample using the TruSeq RNA Library Prep Kit v.2 (Illumina, San Diego, CA, USA) according the manufacturer's protocol with the exception that the initial poly A enrichment step was skipped. Libraries were sequenced on an Illumina MiSeq Instrument using Miseq reagent nano kit v.2 (300 cycles) (Illumina, San Diego, CA, USA). To obtain consensus viral sequences reads were *de novo* assembled and mapped to reference genome segments retrieved from Genbank using CLC genomic Workbench v.11.0.1 (Qiagen, Milan, Italy).

The nucleotide and deduced amino acid sequences of all genome segments were compared for similarity against all sequences available from GenBank using BLAST analysis. The phylogenetic trees, generated by the neighbor-joining method, were constructed for each genome segment. The nucleotide sequences of all 10 genome segments L1-L3, M1-M3 and S1-S4 were deposited in GenBank under Accession Nos. MK408599-MK408608

The deduced amino acid (aa) sequence of the S1 gene of chamois MRV was aligned with the reference strain T3/human/Ohio/Dearing/1955 and selected Italian MRV-3 strains.

2.5. Serological screening

Sera were tested by serum neutralization test (SNT) against MRV Type 3 strain Abney (ATCC VR-232). Moreover, a subset of sera was

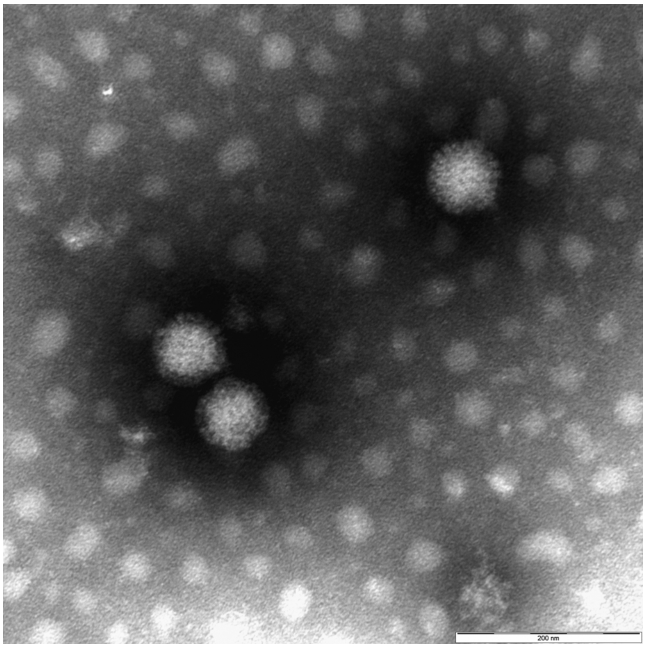

Fig. 1. Electron micrograph of reovirus particles in the supernatant of MARC 145 cells. Negative staining (2% sodium phosphotungstate). TEM FEI Tecnai G2 Spirit, 85 kV. Bar = 200 nm.

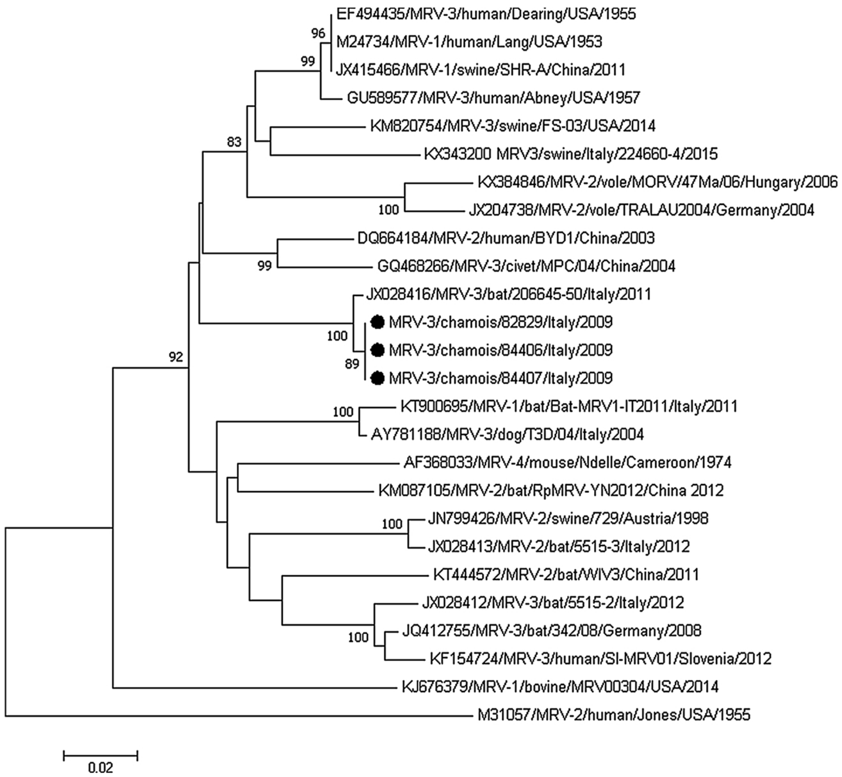

Fig. 2. Phylogenetic tree of the partial L1 genome segments of MRV chamois sequences (348 nts), reference strains and most related sequences from GenBank. Phylogenetic analyses were performed with MEGA7 using the NJ method. Bootstrap values > 75% are shown. Published sequences and references are identified by GenBank accession numbers. The symbol ● indicates sequences obtained in this study.

tested against chamois MRV field strains. Two-fold serial dilutions of heat inactivated serum in duplicate were mixed with equal volumes of virus containing 100 $TCID_{50}$. SNT was performed onto MDBK cells, maintained in MEM supplemented as previously described and with 10% of FBS. The plates were incubated at 37 °C with 5% of CO_2 for 72 h. SN titre was defined as the highest serum dilution or proportionate

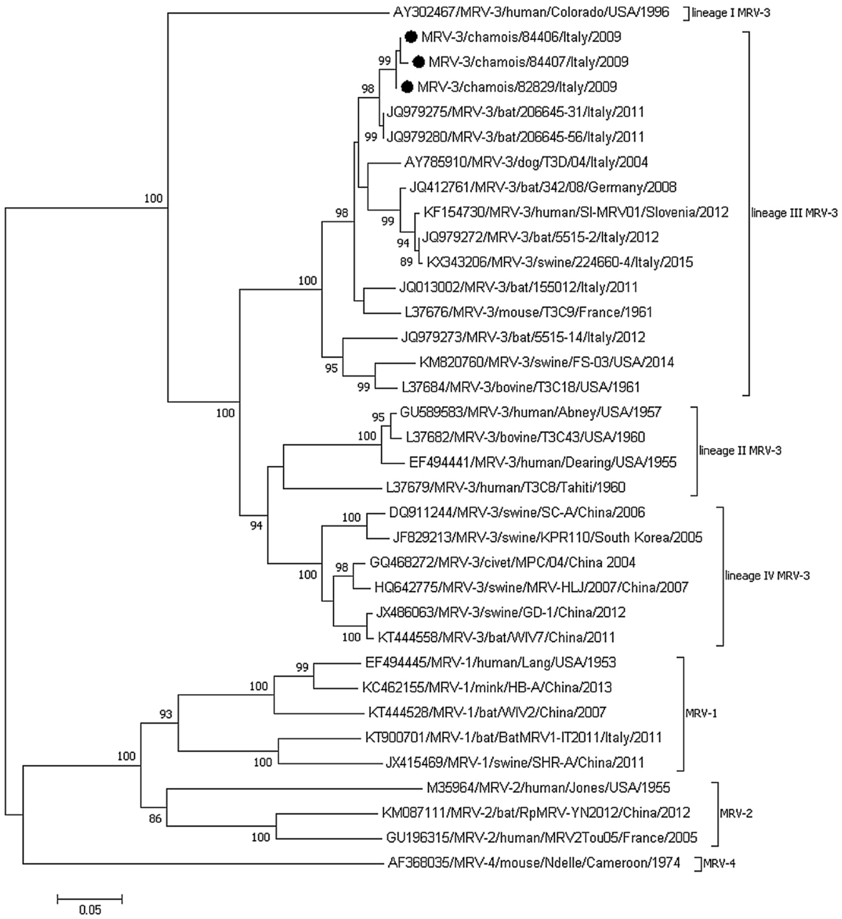

Fig. 3. Phylogenetic tree of the partial S1 genome segments of MRV chamois sequences (326 nts), reference strains and most related sequences from GenBank. Phylogenetic analyses were performed with MEGA7 using the NJ method. Bootstrap values > 75% are shown. Published sequences and references are identified by GenBank accession numbers. The symbol ● indicates sequences obtained in this study.

Table 1
Highest nucleotide and amino acid identities for each gene segment of the novel MRV sequence from Italian chamois compared to MRV isolates from GenBank.

MRV-3 chamois 84407	Identity (%)		MRV strain	Serotype[a]	Lineage	Host	Country	Year	GenBank Accession No.
	nt	aa							
L1	99.4	100	206645-50/2011	3	III	Bat (*Tadarida teniotis*)	Italy	2011	JX028416
L2	95	98.7	SI-MRV06	1	–	Bat (*Myotis emarginatus*)	Slovenia	2009	MG457119
L3	91.4	98.6	MPC/04	3	IV	Masked palm civet	China	2004	GQ468270
M1	98.3	99.2	SI-MRV02	3	III	Bat (*Eptesicus serotinus*)	Slovenia	2010	MG457081
M2	98.1	99.7	MRV2Tou05	2	–	Human	France	2005	GU196310
M3	96.5	98.4	MRV2Tou05	2	–	Human	France	2005	GU196314
S1	99.8	99.8	206645-31/2011	3	III	Bat (*Vespertillio murinus*)	Italy	2011	JQ979275
S2	97.8	99.3	MRV2Tou05	2	–	Human	France	2005	GU196311
S3	97.5	99.5	T3C9/61	3	III	Murine	France	1961	U35352
S4	97.5	98.6	MRV-HLJ/2007	3	IV	Pig	China	2007	HQ642778

[a] Serotype is determined by genetic characterization of S1 genome segment.

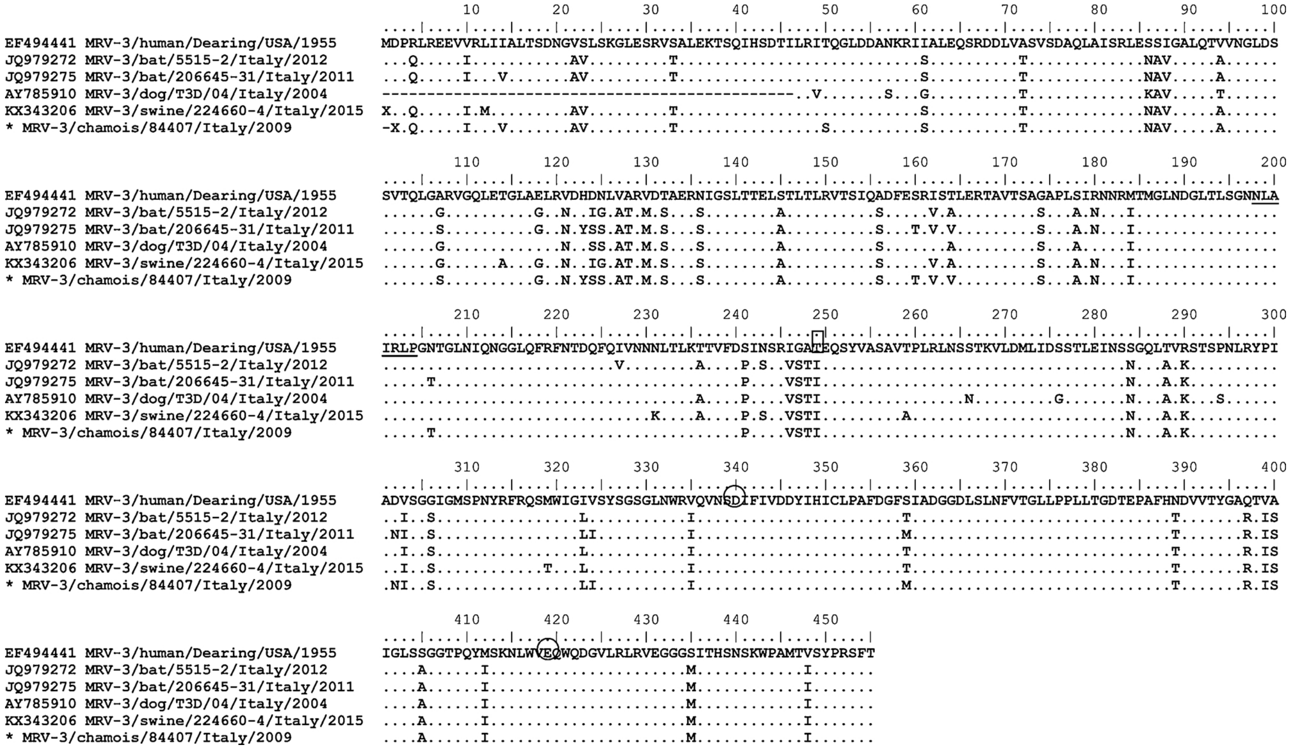

Fig. 4. Alignment of deduced amino acid sequences of the σ 1 protein of the Italian chamois MRV-3 strains, selected Italian MRV-3 strains and the Dearing strain. The predicted sialic acid-binding domain is underlined; the sequence associated with sensitivity to cleavage by intestinal proteases is boxed; the sequences associated with neuronal tropism are circled. The symbol * indicates the sequence obtained in this study.

distance between two dilutions able to inhibit virus replication, and was calculated according to Reed and Muench method (Reed and Muench, 1938). A titre > 4 was defined as positive.

2.6. Statistical analysis

Serological data were analysed with statistical software (IBM® SPSS®, Version 20). For statistical purposes, the distribution of the frequencies of different variables (age and sex classes, date and place of culling) were compared with One-Way ANOVA test. Significance was accepted for p values < 0.05.

3. Results

3.1. Virological tests

Three CPE positive samples were detected on MDBK cells from a total of 87 lungs tested. CPE, giving evidence of virus growth, was observed at the second blind passage for two samples and at the third passage for the remaining one.

NsEM performed on supernatants of the infected cell cultures revealed the presence of typical inner and outer icosahedral, non-enveloped capsids of approximately 70 nm in diameter characteristic of reovirus (Fig. 1). MRV identification was confirmed for all CPE positive samples by RT-PCR for L1 and S1 fragments.

The three MRV positive lungs were detected among 19 samples collected in 2009. MRV positive yearlings were two female (id. 83829, 84407) and one male (id. 84406) culled in September 2009 from different localities. No macroscopic lesions were observed in MRV positive and negative lung tissues and no clinical respiratory symptoms were reported in chamois population.

3.2. Sequence analysis and phylogeny

The phylogenetic analysis based on L1 confirmed MRV identification (Fig. 2). Based on S1 phylogeny, the novel chamois MRV strains were classified as MRV-3 within the lineage III, closely related to Italian strains identified in dog (Decaro et al., 2005), bat (Lelli et al., 2013) and

Table 2
Seroprevalence of MRV-3 and serum neutralization titres in chamois.

Year	N. animal tested	N. positive	Prevalence (%) (C.I. 95%)	Mean geometric titre	range of positive titres
2008	18	9	50.00 (29.03–70.97)	26	11-45
2009	37	23	62.16 (46.10–75.94)	33	11-91
2010	20	9	45.00 (25.82–65.79)	36	11-64
2011	12	9	75.00 (46.77–91.11)	24	11-45
2012	19	10	52.63 (31.71–72.67)	30	11-64

Table 3
Serum neutralization titers against MRV-3 reference strain and chamois field strains on a subset of chamois sera.

MRV strain/ chamois sera id.	Type 3 Abney	chamois 84407	chamois 84406
84406[a]	64	91	91
cm4	< 4	< 4	< 4
83650	91	23	n.t.[b]
84353	64	32	n.t.
84442	45	23	n.t.
83823	32	45	n.t.
83966	32	32	n.t.

[a] MRV positive chamois.
[b] not tested.

swine (Lelli et al., 2016) (Fig. 3). Pair-wise nucleotide comparisons of the three chamois MRV strains showed a 100% and 99% identity of L1 and S1 segments, respectively.

The results analysis showing the highest nucleotide and amino acid identities for each genome segment against publicly available sequences from GenBank are reported in Table 1. The Italian chamois strain was highly similar to MRV-3 strains not only according to S1 segment but also based on L1, L3, M1, S3 and S4 segments (97.5–99.4 % nucleotide and 98.6–100% amino acid identities). These MRV-3 similar strains were identified from different bat species in Italy and Slovenia, masked palm civet and pig in China, and mouse in France. The L2 segment was more similar to MRV-1 strains identified in a bat in Slovenia whereas M2, M3 and S2 were similar to a MRV-2 strain identified in human in France. Phylogenetic trees on segments other than L1 and S1 confirmed these results (figure S1).

The comparison of the deduced amino acid sequences of the σ1 protein encoded by S1 gene of our chamois MRV strain with that of the reference strain T3/human/Ohio/Dearing/1955, revealed that the sequence NLAIRLP, representing amino acids198-204 and constituting a binding site for sialic acid, was conserved (Fig. 4). Polymorphisms at amino acid 249 have been found to affect the susceptibility of MRV-3 σ1 protein to cleavage by intestinal proteases. Our representative Italian chamois orthoreovirus encoded an isoleucine residue at amino acid 249, which is characteristic of all MRV-3 strains with protease-resistant σ1 proteins and is required for efficient viral growth as well as migration to secondary replication sites, including the CNSs (Chappell et al., 1998). Two amino acid residues (340D and 419E) have been implicated in the neurotropism of orthoreoviruses (Bassel-Duby et al., 1986; Kaye et al., 1986) and both residues were conserved in the Italian chamois orthoreovirus.

3.3. Serology

The serological investigation showed an overall MRV-3 prevalence of 56.60% (C.I. 95% 47.10–65.64) in chamois population during 2008–2012 period, with a prevalence of 47.36% and 61.19% in yearlings and adult chamois respectively. Positive sera were detected throughout the whole monitoring period and both in yearlings and adult animals (Table 2). SN antibody titres, ranging from 1:11 to 1:91, showed no significant differences among years and between yearlings and adult chamois. Sera tested against MRV chamois strains showed similar SNT titers compared to the type 3 reference strain (Table 3).

4. Discussion

In the present study, we report the identification and genetic characterization of MRV strains in Alpine chamois, one of the most abundant wild ungulate in Italian Alps (Carnevali et al., 2009). MRV strains were isolated from lung tissues of three apparently healthy hunted animals in north-western Italian Alps and were classified as MRV-3, according to S1 phylogeny.

The pathogen role of MRV-3 is still controversial, as the virus is considered responsible for either asymptomatic or symptomatic infections, alone or in association with other pathogens in human (Lewandowska et al., 2018) and animals (Lelli et al., 2016; Li et al., 2016). Concerning our investigation, no respiratory or other clinical symptoms neither lung macroscopic lesions were reported in the chamois population. Nevertheless, we cannot exclude mild subclinical symptoms or sporadic severe infections, because comprehensive observations of population were performed only during annual census.

Based on S1 phylogeny, the chamois MRV-3 strains belonged to lineage III, prevalent in Europe, clustering with strains isolated in Italy from dog, bat and pig (Decaro et al., 2005; Lelli et al., 2013, 2016), in Germany from bats (Kohl et al., 2012) and with a human strain identified in Slovenia (Steyer et al., 2013). In addition, to molecularly characterize the chamois MRV strain, the full genome sequence was obtained by next-generation sequencing, highlighting that other segments were more similar to MRVs of different geographic locations, serotypes and hosts, including human. These results confirmed the reassortment capacity of this segmented RNA virus and contributed to our understanding of molecular evolution of mammalian orthoreovirus in wildlife. Moreover, molecular analysis of the S1 gene revealed that the amino acid residues associated with neurotropism (198-204NLAIRLP, 249I, 340D and 419E) were conserved in the representative Italian chamois strain, suggesting that potentially neurotropic MRV-3 strains are present in the chamois population, as previously reported for Italian bats (Lelli et al., 2013).

Our investigation gives a contribution to MRV epidemiology, widening the host range of MRV to wildlife. This result arises questions on the epidemiological link between chamois and other animal species. With this regard, it has to be considered that a high prevalence of MRV-3 antibodies was observed in chamois during the five years survey, showing an endemic level of infection in the population and suggesting a self-maintenance of MRV and/or a continuous spill-over of infection from, or even to, other animal species. The transmission of MRV is not limited to close contacts, but can occur due to indirect contamination, since infective particles have been found in environmental samples (Lodder et al., 2010). At this regard, it has to be noted that most of MRV strains that showed the highest similarity with the ones from chamois were obtained from intestine or fecal samples, supporting a role of environmental contamination in the spreading of this infection.

In conclusion, current knowledge on MRVs epidemiology has been extended to alpine chamois. This result increases interest on the widespread nature of these viruses, also for their reassortment ability that likely contributes to their genetic evolution and adaptation to animal and human hosts.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.01.012.

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