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Short communication

Detection of Toggenburg Orbivirus by a segment 2-specific quantitative RT-PCR

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A B S T R A C T

Toggenburg Orbivirus (TOV) has been detected recently in healthy goats in Switzerland. The virus is related closely to bluetongue virus (BTV) and is considered tentatively as a 25th serotype of BTV. Upon detection of additional TOV-positive goats in Switzerland, Germany, and Italy, these TOV isolates were characterized genetically by partial sequencing of the viral genome segment 2 which encodes VP2, the major outer capsid protein of orbiviruses. A TOV-specific RT-qPCR was developed, targeting conserved areas within segment 2. Since TOV cannot be propagated up to now outside its natural host, a synthetic positive control for the RT-qPCR was constructed by cloning the entire coding region of segment 2 and subsequent *in vitro* transcription of RNA from both ends to obtain double-stranded RNA. The TOV-specific RT-qPCR was able to detect as few as 30 dsRNA copies and proved to be equally sensitive as a pan BTV assay that was shown previously to have a detection limit of 0.001 $TCID_{50}$.

Toggenburg Orbivirus (TOV) has been detected recently in healthy animals from two epidemiologically unrelated goat flocks in Switzerland (Chaignat et al., 2009; Hofmann et al., 2008b). Based on its genome organization and nucleotide sequence, TOV is considered currently as a putative 25th serotype of bluetongue virus (BTV), which belongs to the genus Orbivirus within the *Reoviridae* family. Several serotypes of BTV have been introduced recently into Europe in the past 3 years (Hateley, 2009), among which serotype 8 (BTV-8) has caused a large-scale epidemic with thousands of disease outbreaks, primarily in France, The Netherlands, Belgium, Germany, and Switzerland. However, other BTV serotypes have also been detected recently in France (BTV-1), The Netherlands (BTV-6), Belgium (BTV-6, BTV-11) and Germany (BTV-6), stressing the need for serotype-specific diagnostic tools for virus and antibody detection.

TOV was detected initially by accident in samples derived from healthy goats in two epidemiologically unrelated flocks that were tested for BTV by a pan BTV-specific quantitative RT-PCR (RT-qPCR) targeting segment (S) 10 of the viral genome (Hofmann et al., 2008a; Orru et al., 2006). However, when these samples were tested with additional RT-qPCR protocols which are able to detect all 24 known serotypes of BTV (Shaw et al., 2007; Toussaint et al., 2007), results were always negative, due most likely to several mismatches between the primer and probe sequences and the corresponding target regions of the TOV genome (Hofmann et al., 2008b). All other ruminant species (cattle, alpacas) kept on the same farm yielded negative results with the S10 RT-qPCR protocol.

It was shown by experimental inoculation of goats that TOV replicates in the inoculated animals, triggers a specific immune response, and can be transmitted to other goats and sheep by inoculation of blood collected from the goats infected previously (Chaignat et al., 2009). However, neither goats nor sheep showed any BTV-specific disease signs upon experimental infection. TOV cannot be propagated outside its natural animal host, either in cell culture or in embryonated chicken eggs, in contrast to all other BTV serotypes.

Since its initial detection (Chaignat et al., 2009) the presence of TOV has been confirmed repeatedly in different regions within Switzerland as well as in southern Germany and northern Italy. By employing the S10-specific RT-qPCR protocol TOV- or BTV-specific viral RNA could be detected in each region in several animals. Since TOV interferes with the specific laboratory diagnosis of all BTV serotypes identified previously, it was decided to design a TOV-specific RT-qPCR targeting S2 of the viral genome, which encodes VP2, the major outer shell protein of orbiviruses. The antigenic properties of VP2 are used to distinguish BTV serotypes.

Since full-length cDNA amplification of the entire S2 by RT-PCR was possible only for a few TOV-positive samples previously identified by S10 RT-qPCR, S2-specific internal primers TOV_S2_981_F (5′-TCAGAGAGGCAGCAAGGTAA-3′) and TOV_S2_1302_R (5′-TGGCGCTACTTCTTCATAACA-3′) were designed. These are located in two areas within the VP2 coding region of S2 that are relatively conserved among the different BTV serotypes (data not shown). The primers yielded the expected PCR DNA prod-

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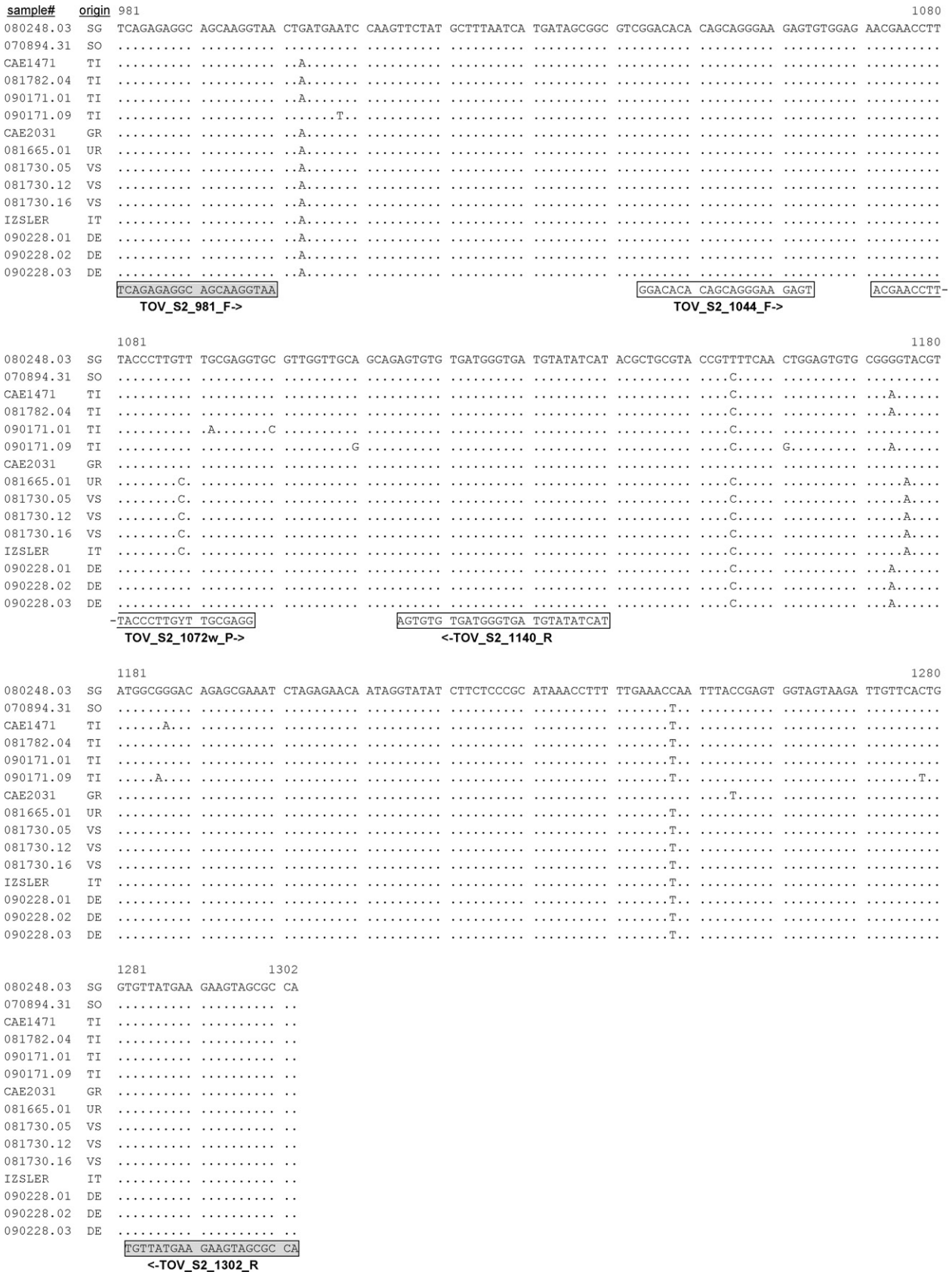

Fig. 1. Sequence alignment of the S2 RT-qPCR target region amplified with PCR primers TOV_S2_981_F and TOV_S2_1302_R (grey boxes). RT-qPCR primers and probe are indicated (white boxes). Origin, geographic origin of the analyzed samples, i.e. Swiss canton (SG, SO, TI, GR, UR, VS), or country (DE, IT). Nucleotide numbers refer to the S2 ORF (GenBank accession number EU839840).

Table 1
Geographic and temporal origin of TOV-positive field samples used to determine the partial S2 sequence shown in Fig. 1. na: not applicable.

Sample number	Collection date (month/year)	Geographic origin	
		Village (canton)	Country
070894.31	12/2007	Nunningen (SO)	CH
080248.03	02/2008	Oberhelfenschwil (SG)	CH
081665.01	11/2008	Altdorf (UR)	CH
081730.05	12/2008	Zermatt (VS)	CH
081730.12	12/2008	Zermatt (VS)	CH
081730.16	12/2008	Zermatt (VS)	CH
081782.04	12/2008	Muggio (TI)	CH
090171.01	02/2009	Cavergno (TI)	CH
090171.09	02/2009	Brontallo (TI)	CH
CAE1471	07/2008	Casima (TI)	CH
CAE2031	10/2008	Seewis (GR)	CH
090228.01	03/2009	Heidelberg (na)	DE
090228.02	03/2009	Heidelberg (na)	DE
090228.03	03/2009	Heidelberg (na)	DE
IZSLER	02/2008	Como (na)	IT

uct of 322 bp in each of the 15 RT-qPCR positive samples (Table 1). Amplified DNA was then ligated into the cloning vector pCR4-TOPO (Invitrogen). At least three clones from each sample were sequenced using M13 primers to determine the respective consensus sequence. The TOV sequences were aligned by ClustalW alignment using MEGA version 4 software (Tamura et al., 2007). As shown in Fig. 1, all analyzed sequences were highly similar to each other, illustrated by a 97.5% identity score. Nevertheless, phylogenetic analysis of these sequences allowed to identify several branches in the dendrogram (Fig. 2A). However, no correlation

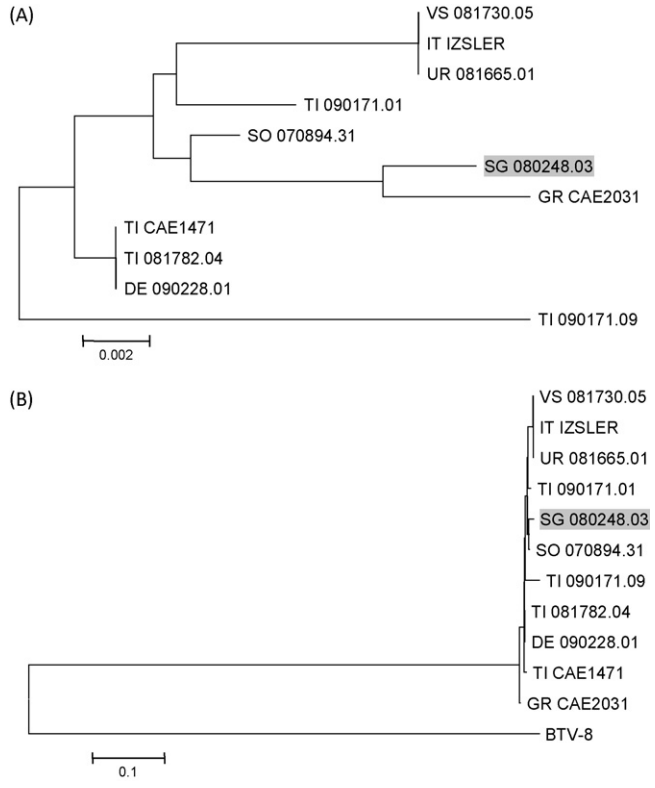

Fig. 2. Phylogenetic analysis by neighbor-joining tree construction of TOV isolates based on the partial S2 sequence alignment shown in Fig. 1. Only one sequence is displayed from those TOV isolates where more than one sample per flock were analyzed (found to be identical to each other). The first TOV case described (Hofmann et al., 2008b) is shaded in grey. (A) Dendrogram representing TOV sequences only; (B) dendrogram obtained after aligning the TOV sequences to the respective BTV-8 S2 fragment. Scale bar indicates number of nucleotide substitutions per site.

was found between the genetic differences of the TOV strains, and their geographical or temporal origin. Whenever multiple samples from individual goat flocks were compared, though (e.g. the German or Valais samples) they proved to be identical, suggesting that the TOV strain circulating within one flock was stable genetically. When the TOV S2 sequences were compared to the respective sequence from a BTV-8 strain isolated in 2006 in The Netherlands (GenBank accession number AM498052), it was evident that TOV forms a closely related clade which is vastly different from BTV-8 (Fig. 2B).

Based on this sequence alignment, primers TOV_S2_1044_F (5′-GGACACACAGCAGGGAAGAGT-3′) and TOV_S2_1140_R (5′-ATGATATACATCACCCATCACACACT-3′), and TaqMan probe TOV_S2_1072w_P (5′-FAM-ACGAACCTTTACCCTTGYTTGCGAGG-BHQ1-3′) were designed (Fig. 1). Using these primers and probe and the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA), an RT-qPCR protocol was established on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal profile: 48 °C/30 min → 95 °C/2 min → 50× [95 °C/15 s → 56 °C/30 s → 72 °C/30 s]. In order to monitor RNA extraction, reverse transcription and PCR amplification, *in vitro* transcribed RNA encoding enhanced green fluorescent protein (EGFP) (Hoffmann et al., 2006) was added into the lysis buffer before RNA extraction as an internal positive control (IPC). Newly designed forward primer EGFP2_F (5′-GGGCACAAGCTGGAGTACAAC-3′), reverse primer EGFP2_R (5′-CACCTTGATGCCGTTCTTCTG-3′), and probe EGFP2_P (5′-HEX-ACAACAGCCACAACGTCTATATCATGGCC-BHQ1-3′) were used to detect the EGFP IPC. Fourteen of the 15 TOV samples that were identified initially by the pan BTV S10 RT-qPCR and verified by partial S2 sequencing (Fig. 1) scored also positive in the TOV-specific S2 RT-qPCR, yielding comparable Ct values (data not shown).

Since TOV cannot be propagated to date in any cell culture system, a synthetic positive control RNA was constructed. To this end, a 2914 bp near full-length cDNA of the S2 segment containing the entire VP2 coding region was amplified by using two separate RT-PCRs for the 5′ and the 3′ half, respectively, which were then fused by assembly PCR. This PCR DNA was inserted into the pCR4-TOPO plasmid vector (Invitrogen). Ten clones were sequenced using M13 forward and reverse primers. Since none of the clones represented the TOV VP2 ORF consensus sequence which had been deposited on GenBank under the accession number EU839840 (Hofmann et al., 2008b), two clones harboring 1 and 2 silent mutations in their 5′ and 3′ part, respectively, were cut with BsrG1, and the desired fragments from each restriction were re-ligated. The resulting clone was termed pTOPO TOV S2. In order to obtain a positive control for the TOV S2 RT-qPCR as similar as possible to the authentic viral S2 dsRNA, 2 ssRNA were transcribed in both orientations from Pst1- or Not1-linearized pTOPO TOV S2, using the MEGAscript® T7 or MEGAscript® T3 *in vitro* RNA transcription system, respectively (Ambion, Austin, TX, USA). The resulting ssRNA strands were annealed to each other by denaturation at 95 °C for 5 min followed by slowly reducing the temperature to <30 °C.

To assess analytical sensitivity, amplification efficiency and linearity of the TOV S2 RT-qPCR, a serial 10-fold dilution ranging from 10e−3 through 10e−11 of the *in vitro* transcribed and annealed dsRNA was tested. As shown in Fig. 3, the assay yielded positive results, i.e. Ct values <50, up to the 10e−10 dilution. This corresponded to a detection limit of 30 dsRNA copies. An amplification efficiency of 90.3% and a linearity, expressed as the correlation coefficient R^2, of 0.9975 (based on the 10e−3 through 10e−10 dilution) were calculated. This demonstrates that the assay is sensitive, and has a large linear dynamic range spanning at least 8 $\log_{10}$. Specificity of the TOV RT-qPCR was confirmed by yielding always

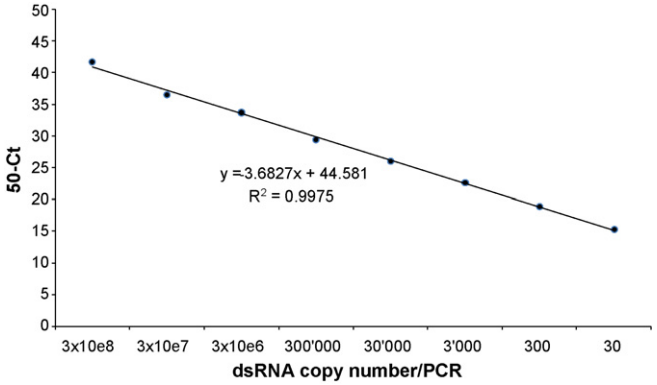

Fig. 3. Sensitivity, amplification efficiency and correlation coefficient of the TOV RT-qPCR determined by a serial 10-fold dilution of the *in vitro* transcribed S2 dsRNA used as synthetic positive control for the assay.

negative results when RNA extracted from undiluted cell culture-derived stocks of all 24 BTV serotypes were tested (data not shown).

When a total of 82 samples for diagnosis from healthy goats which scored mostly weak positive in the pan BTV S10 were retested with the TOV S2 RT-qPCR, 48 (59%) were found positive by the TOV assay, yielding similar Ct values in both assays (Fig. 4). However, the remaining 34 samples (showing slightly higher average Ct values in the pan BTV assay) were negative in the TOV-specific assay. Taken together, the results obtained with the field samples suggest that the two methods are equally sensitive for samples yielding Ct values of up to 30, whereas the TOV-specific protocol is slightly less sensitive for pan BTV weak positive (Ct >32) samples. This might be due to slightly different amplification efficiencies of the two assays (data not shown).

Furthermore, samples collected from a goat infected with TOV experimentally (Chaignat et al., 2009) revealed similar Ct values by both RT-qPCR protocols (Table 2), and both assays detected TOV on the same day p.i., demonstrating that the diagnostic sensitivity of the new TOV-specific RT-qPCR was equal to the sensitivity of the S10 pan BTV RT-qPCR, which had earlier been determined as 0.001 $TCID_{50}$/PCR (data not shown).

The TOV S2 RT-qPCR reported here will allow a specific detection of TOV and its differentiation from BTV serotypes 1–24. This can either be achieved by using a pan BTV-specific assay such as the S10 RT-qPCR described above as a screening test, followed by determination of the BTV serotype by using serotype-specific RT-qPCRs

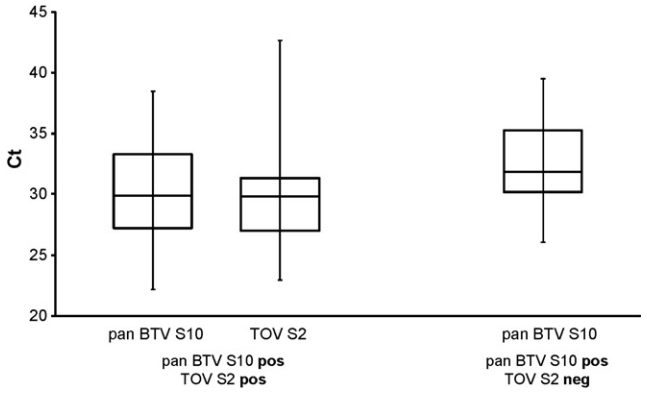

Fig. 4. Box plot showing median Ct values and 25–75% percentiles as well as maximal and minimal values obtained with field samples of goats that scored either positive in both the pan BTV S10 and the TOV S2 RT-qPCR ($n = 48$) or were positive in pan BTV S10 but negative in the TOV S2 assay ($n = 34$).

Table 2
Comparative assessment of the diagnostic sensitivity of the two RT-qPCR protocols. Ct values obtained by pan BTV S10 and TOV S2 RT-qPCR using blood cells prepared from sequentially collected EDTA blood samples of experimentally TOV-infected goat #5131 (Chaignat et al., 2009).

Day p.i.	Ct values RT-qPCR	
	pan BTV S10	TOV S2
0	No Ct	No Ct
1	No Ct	No Ct
3	No Ct	No Ct
4	No Ct	No Ct
5	No Ct	No Ct
6	No Ct	No Ct
8	29.4	28.1
10	29.5	26.8
12	30.2	26.5
14	27.8	24.3
16	29.3	27.4
21	30.4	27.1
24	30.3	27.8
29	32.2	30.2

which have been established for several serotypes (Vandenbussche et al., 2009) or by conventional RT-PCR using serotype-specific primers (Mertens et al., 2007). However, the TOV S2-specific RT-qPCR could also be used as a screening test, if the emphasis is on TOV detection only, based on its specificity for detecting only TOV but not any other BTV serotype. One potential disadvantage of using the S2 of orbiviruses as an RT-qPCR target is the high sequence variability even within one serotype (Maan et al., 2007). Sequencing of numerous additional TOV S2 segments upon detection of the virus by, e.g. the S10 RT-qPCR will allow to determine whether all TOV isolates share a high degree of sequence conservation in the TOV S2 RT-qPCR primer and probe binding region, as has been observed among the sequences analyzed so far (Fig. 1). The new assay provides a valuable tool for future studies for determining the worldwide prevalence of TOV in goats and its host range.

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