Detection of Peste des petits ruminants virus RNA in Culicoides imicola *(Diptera: Ceratopogonidae) in Turkey*

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Keywords
Peste des petits ruminants, Transmission, *Culicoides imicola*, Molecular characterization.

Summary
An outbreak of Peste des petits ruminants (PPR) occurred in the Antalya Province in Turkey during October 2015. The Antalya Province has suitable habitats for vectors. There is no information available on the role of *Culicoides* spp. in the transmission of Peste des petits ruminants virus (PPRV). In this study we investigated the potential role of the *Culicoides* spp. in the transmission of PPRV. *Culicoides* were trapped throughout middle of October and middle of December, 2015. A total of 12 pools of non-engorged females were analysed with real-time RT-PCR targeting the nucleocapsid (N) gene of the PPRV. PPRV RNA was detected in 7 of 12 *Culicoides* pools. These pools were negative for the bovine/ovine beta-actin mRNA. *Culicoides* spp. were identified to the species level by sequence analysis of the mitochondrial cytochrome oxidase subunit I gene. The species of *Culicoides* found PPRV positive was *Culicoides imicola*. Molecular characterization of field isolates from recent outbreaks and pools of midges that tested positive for PPRV suggests that PPRV replication might occur in *Culicoides imicola*, and it may have played a role in transmitting PPRV.

Virus della Peste dei piccoli ruminanti in Culicoides imicola *(Diptera: Ceratopogonidae) in Turchia*

Parole chiave
Caratterizzazione molecolare, *Culicoides imicola*, Peste dei piccoli ruminanti, Trasmissione.

Riassunto
Ad ottobre 2015 un focolaio di Peste dei piccoli ruminanti (PPR) ha colpito Antalya, una provincia della Turchia caratterizzata da condizioni climatico ambientali ottimali per gli artropodi vettori. Dal momento che non ci sono dati su un possibile ruolo svolto dalle specie di *Culicoides* nel trasmettere il virus della PPR (PPRV), questo studio si è posto come obiettivo quello di valutare se *Culicoides* spp. possano effettivamente agire come vettori del virus. Numerosi *Culicoides* sono stati catturati da metà ottobre a metà dicembre 2015. Dodici pool di femmine non ingorgate sono stati predisposti ed esaminati per rilevare la presenza del virus attraverso una RT-PCR real time specifica per il gene nucleocapside del PPRV. Dei 12 testati, 7 pool sono risultati positivi. L'analisi della sequenza del gene della subunità I della citocromo ossidasi mitocondriale, effettuata su *Culicoides* spp. per identificare la specie implicata, ha individuato *C. imicola* nei pool positivi. Il confronto tra le sequenze genomiche dei ceppi di PPRV responsabili dei focolai del 2015 e quelli rilevati in *C. imicola* suggerisce che *C. imicola* potrebbe aver avuto un ruolo nella trasmissione del PPRV nei focolai di Antalya.

Peste des petits ruminants (PPR) is a transboundary small ruminant disease characterised by high fever, nasal and ocular discharges, erosive stomatitis, conjunctivitis, pneumonia, and severe diarrhoea (Gibbs *et al.* 1979, Lefevre and Diallo 1990). Peste des petits ruminants virus (PPRV), classified within the genus *Morbillivirus* in the family *Paramyxoviridae*, is the causative agent of the disease (Gibbs *et al.* 1979). Although PPRV has also been reported in cattle and pigs, sheep and goats are considered the natural hosts of PPRV (Anderson and Mckay 1994).

Vaccination of lambs and kids has been used to control PPR in Turkey, however, sporadic outbreaks have been observed. In October 2015, an outbreak of PPR occurred in the Antalya Province, which is located in the Mediterranean region of Turkey. It is known that PPRV needs close contact between infected and susceptible animals for transmission (Lefevre and Diallo 1990). The Antalya Province has suitable climatic circumstances for the growth and distribution of *Culicoides*. Several species of biting midges of the genus *Culicoides* play a role in the transmission of viral diseases of ruminant livestock, including Bluetongue (BT), Epizootic hemorrhagic disease (EHD) and Schmallenberg virus (SBV) (Paweska *et al.* 2002, Ruder *et al.* 2012, Balenghien *et al.* 2014). However, there are no evidence of vectorial transmission of PPR in affected (endemic) regions of Africa, Middle East and India. The purpose of this study therefore was to investigate the potential role of the *Culicoides* spp. in the transmission of PPRV.

This study was conducted in the Antalya Province (29°20′-32°35′ E, 36°07′-37°29′ N) in South-West Anatolia. The Antalya Province has Mediterranean climate characterised by mild and rainy winters and hot, dry summers. During 1981-2014, the annual mean temperature was 18.6 °C and annual precipitation was 783.8 mm (Turkish State Meteorological Service). Its elevation is 30 metres, and the average temperature during the study was 17.8 °C. Three sheep farms within a radius of 2 km from the PPRV-infected flocks were selected for the collection of *Culicoides*. Two of the three farms were located in Manavgat district and one in Döşemealti district where the highest number of PPR cases occurred. *Culicoides* were trapped at biweekly intervals between middle of October and middle of December 2015 by using Onderstepoort-type blacklight traps. *Culicoides* spp. were separated from other insects using a stereomicroscope, followed by species identification (Dyce 1969). Subsequently, they were grouped into males, non-engorged and blood-fed females (engorged). Non-engorged females were used for detection of PPRV RNA.

Twelve pools of non-engorged females, 10 midges per pool, were homogenized in nuclease-free water (400 µl) by using a tissueruptor (Qiagen, Hilden, Germany). RNA was extracted from the supernatant by using a QIAamp viral RNA Mini Kit (Qiagen) on a QIAcube (Qiagen). The real-time RT-PCR was performed to detect N gene of the PPRV (Batten *et al.* 2011). Furthermore, *Culicoides* midges were tested for the recent intake of a blood meal by a quantitative real-time RT-PCR specific for beta-actin mRNA (Toussaint *et al.* 2007). Nuclease free water (Qiagen, Hilden, Germany) was used as the negative control in all real-time RT-PCR assays. DNA was extracted from the supernatant of the *Culicoides* pools by using a QIAamp DNA Mini Kit (Qiagen). The species of *Culicoides* spp. of the PPRV positive pools was identified to the species level by sequence analysis of the mitochondrial cytochrome oxidase subunit I gene (COI) using the primers C1-J-1718 and C1-N-2191 (Dallas *et al.* 2003).

PPRV RNA was detected in 7 of the 12 pools assayed. The Ct-values of the real-time RT-PCR ranged from 29.8 to 34.1. Reported Ct values generated by using the same assay from blood of experimental infected goats with a Moroccan strain of PPRV were 25.7-34.5 (Hammouchi *et al.* 2012). Generally, 100-200 µl of sheep or goat blood are used for detection of PPRV, whereas less than 1 µl of blood remains in a midge after a blood meal. Therefore, Ct values should have been 6-7 units higher when the biting midge is tested by real-time RT-PCR (Hoffmann *et al.* 2009). Additionally, bovine/ovine beta-actin mRNA was not determined in positive pools. This result showed that the detection of PPRV RNA within the *Culicoides* midges didn't result from recent blood meals from infected animals. It has been reported that sub-transmissible infections are common in *Culicoides* and Ct values can be used to define transmissible and sub-transmissible infections (Mellor 2000, Veronesi *et al.* 2013). Comparison of the Ct-values (29.8 to 34.1) obtained from the PPRV positive *Culicoides* pools in this study with predicted quantities (25.7-34.5) of PPRV in the experimental infection study (Hammouchi *et al.* 2012) demonstrated that PPRV replication may occur in the biting midges.

PPRV-positive *Culicoides* pools (n = 7) and one positive sheep blood sample from each district (Aksu, Alanya, Döşemealtı, Korkuteli, Manavgat and Muratpaşa), where PPR outbreaks (n = 6) have been recently reported, were selected for sequence analysis. The primers described by Forsyth and Barrett (Forsyth and Barrett 1995) were used to amplify the fusion (F) gene of the PPRV, whereas N gene was amplified with the primer pairs N1/N2 (Kerur *et al.* 2008).

Nucleotide sequences of the F and N genes were obtained from 2 of the 7 PPRV positive *Culicoides* pools (pool-c1 and pool-c4). The two positive nucleotide sequences obtained from F and N genes

in *Culicoides* were from the same samples (pool-c1 and pool-c4). Furthermore, nucleotide sequences of the F and N genes were obtained from PPRV positive field samples from the Alanya, Döşemealtı, Manavgat and Muratpaşa districts. Nucleotide sequences of the F gene were not obtained from PPRV positive field samples from the Aksu and Korkuteli districts, only nucleotide sequences of the N gene were obtained from these two field samples. The analysis of the F gene sequences revealed that the homology between the four field isolates in the present study ranged between 99.3% and 100%, whereas the similarity between two isolates from *Culicoides* midges (KU175170 and KU1751702) and four field isolates ranged from 98.9-99.6%. The two sequences from *Culicoides* midges showed 98.9% nucleotide homology with each other. The deduced amino acid homology of the isolates of the present study was 100%.

The analysis of the N gene sequences revealed that the homology between the six field isolates in the present study ranged between 97.2% and 100%, whereas the similarity between two isolates from *Culicoides* midges (KU175171 and KU325486) and six field isolates ranged from 97.6-99.4%. The two sequences from *Culicoides* midges showed 99.8% nucleotide homology with each other. The deduced amino acid homology of the isolates of the present study ranged from 96.4-100%.

The network analysis based on F gene sequences showed that isolates from this study and previously characterised Turkish isolates clustered in the same taxon (Figure 1). The isolates *Culicoides*-PPRV F 01 and *Culicoides*-PPRV F 02 differentiated into two nodes with field isolates of the present study and previously characterised Turkish isolates. These two nodes were separated from each other with a single mutation. A previously characterized Turkish isolate (KF478918) from the Antalya Province in 2012 was placed on the same node with the isolate *Culicoides*-PPRV F 02.

The network analysis based on N gene sequences showed that eight isolates of the present study clustered in two taxa (Figure 2). The isolates *Culicoides*-PPRV N 01 and *Culicoides*-PPRV N 02 formed a node with isolate of Alanya district (KU325487). The field isolates of Döşemealti district (KU325485 and KU325492) were the closest to this node with a single mutation. A Turkish isolate characterised in 2009 (KF478928) was the most distant previously characterised Turkish isolate from this node. A second node with six mutations was formed by three field isolates (KU325489, KU325491 and KU325493) of the present study. Network analyses showed that the number of mutations, among the isolates of the present study, was higher in N genes compared with F genes of corresponding isolates. This is expected because PPRV is more

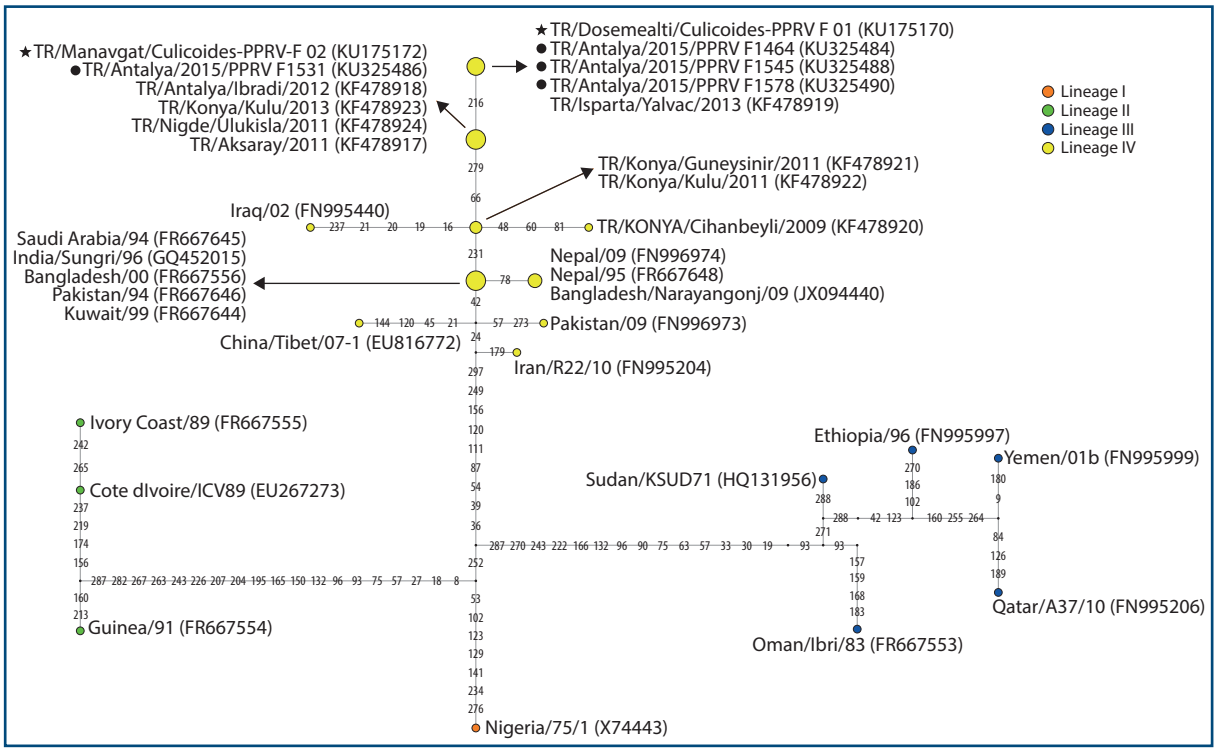

Figure 1. *Phylogenetic network analysis based on the F gene of the field isolates with corresponding sequences published in the GenBank.* Numbers along the branches represent the nucleotide changes separating the nodes. Field isolates and isolates from *Culicoides* midges in this study are marked with round black spot (●) and black star (★), respectively.

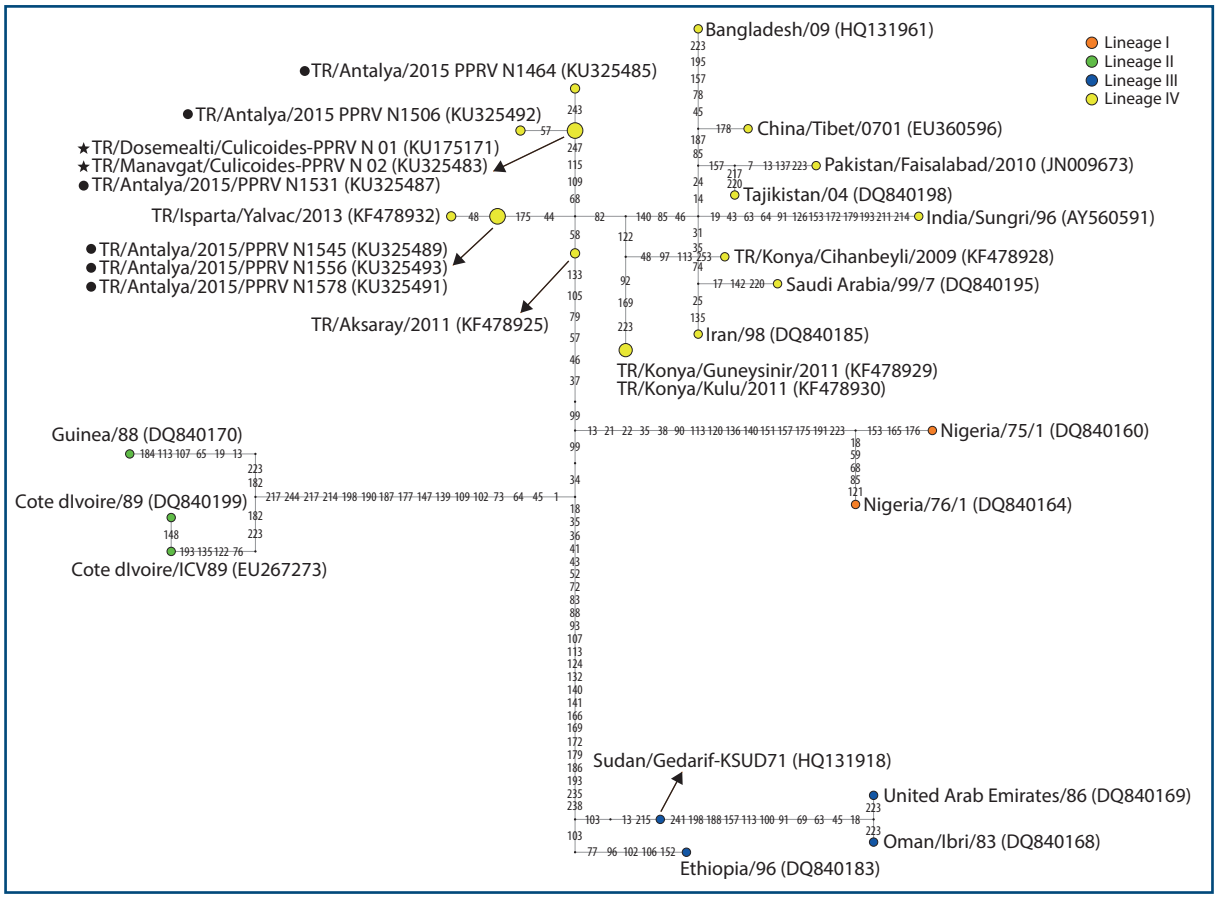

Figure 2. *Phylogenetic network analysis based on the N gene of the field isolates with corresponding sequences published in the GenBank.* Numbers along the branches represent the nucleotide changes separating the nodes. Field isolates and isolates from *Culicoides* midges in this study are marked with round black spot (●) and black star (★), respectively.

prone to mutations on the N gene compared to the F gene (Munir *et al.* 2015).

Sequence comparisons and network analyses (based on F and N genes) showed that circulating PPRV isolates and isolates from *Culicoides* midges are very closely related and PPRV isolates from *Culicoides* midges seem to have diverged from previously characterised Turkish isolates (Figure 1 and Figure 2). These findings suggest that virus replication occurred in the biting midges.

The sequence analysis of COI gene showed that the species of *Culicoides* in PPRV positive pools was *C. imicola*. Previous studies have reported that *C. imicola* plays a role in transmitting the Schmallenberg, Bluetongue and Epizootic hemorrhagic disease viruses (Paweska *et al.* 2002, Ruder *et al.* 2012, Balenghien *et al.* 2014). However, to our knowledge, its role in the transmission of PPRV has not been previously reported.

In conclusion, the absence of bovine/ovine beta-actin mRNA in PPRV positive pools and the results of sequence and phylogenetic network analyses suggest that PPRV might replicate in *Culicoides* midges and *C. imicola* may have played a role in the transmission of PPRV. However, further experimental studies are needed to confirm PPRV field isolates within *C. imicola*.

References

Anderson J. & Mckay J.A. 1994. The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implication to rinderpest control programme. *Epidemiol Infect*, **112**, 225-231.

Balenghien T., Pagès N., Goffredo M., Carpenter S., Augot D., Jacquier E., Talavera S., Monaco F., Depaquit J., Grillet C., Pujols J., Satta G., Kasbari M., Setier-Rio M.L., Izzo F., Alkan C., Delécolle J.C., Quaglia M., Charrel R., Polci A., Bréard E., Federici V., Cêtre-Sossah C. & Garros C. 2014. The emergence of Schmallenberg virus across *Culicoides* communities and ecosystems in Europe. *Prev Vet Med*, **116**, 360-369.

Batten C.A., Banyard A.C., King D.P., Henstock M.R., Edwards L., Sanders A., Buczkowski H., Oura C.C. & Barrett T. 2011. A real time RT-PCR assay for the specific detection of Peste des petits ruminants virus. *J Virol Methods*, **171**, 401-404.

Dallas J.F., Cruickshank R.H., Linton Y.M., Nolan D.V., Patakakis M., Braverman Y., Capela R., Capela M., Pena I., Meiswinkel R., Ortega M.D., Baylis M., Mellor P.S. & Mordue Luntz A.J. 2003. Phylogenetic status and matrilineal structure of the biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. *Med Vet Entomol*, **17**, 379-387.

Dyce A.L. 1969. The recognition of nulliparous and parous *Culicoides* (Diptera: Ceratopogonidae) without dissection. *J Austr Entomol Soc*, **1**, 11-15.

Forsyth M.A. & Barrett T. 1995. Evaluation of polymerase chain reaction for the detection and characterisation of rinderpest and petse des petits ruminants viruses for epidemiological studies. *Virus Res*, **39**, 151-163.

Gibbs E.P.J., Taylor W.P., Lawman M.P.J. & Bryant J. 1979. Classification of peste des petits ruminants virus as a fourth member of the genus Morbillivirus. *Intervirology*, **11**, 268-274.

Hammouchi M., Loutfi C., Sebbar G., Touil N., Chaffai N., Batten C., Harif B., Oura C. & El Harrak M. 2012. Experimental infection of alpine goats with a Moroccan strain of peste des petits ruminants virus (PPRV). *Vet Microbiol*, **160**, 240-244.

Hoffmann B., Bauer B., Bauer C., Bätza H.J., Beer M., Clausen P.H., Geier M., Gethmann J.M., Kiel E., Liebisch G., Liebisch A., Mehlhorn H., Schaub G.A., Werner D. & Conraths F.J. 2009. Monitoring of putative vectors of Bluetongue virus serotype 8, Germany. *Emerg Infect Dis*, **15**, 1481-1484.

Kerur N., Jhala M.K. & Joshi C.G. 2008. Genetic characterization of Indian peste des petits ruminants virus (PPRV) by sequencing and phylogenetic analysis of fusion protein and nucleoprotein gene segments. *Res Vet Sci*, **85**, 176-183.

Lefevre P.C. & Diallo A. 1990. Peste des petits ruminants virus. *Rev Sci Tech*, **9**, 951-965.

Mellor P.S. 2000. Replication of arboviruses in insect vectors. *J Comp Pathol*, **123**, 231-247.

Munir M., Saeed A., Abubakar M., Kanwal S. & Berg M. 2015. Molecular characterization of Peste des petits ruminants viruses from outbreaks caused by unrestricted movements of small ruminants in Pakistan. *Transbound Emerg Dis*, **62**, 108-114.

Paweska J.T., Venter G.J. & Mellor P.S. 2002. Vector competence of South African *Culicoides* species for bluetongue virus serotype 1 (BTV-1) with special reference to the effect of temperature on the rate of virus replication in *C. imicola* and *C. bolitinos*. *Med Vet Entomol*, **16**, 10-21.

Ruder M.G., Howerth E.W., Stallknecht D.E., Allison A.B., Carter D.L., Drolet B.S., Klement E. & Mead D.G. 2012. Vector competence of *Culicoides sonorensis* (Diptera: Ceratopogonidae) to epizootic hemorrhagic disease virus serotype 7. *Parasit Vectors*, **5**, 236.

Toussaint J.F., Sailleau C., Breard E., Zientara S. & De Clercq K. 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Methods*, **140**, 115-123.

Veronesi E., Antony F., Gubbins S., Golding N., Blackwell A., Mertens P.P., Brownlie J., Darpel K.E., Mellor P.S. & Carpenter S. 2013. Measurement of the infection and dissemination of Bluetongue virus in *Culicoides* biting midges using a semi-quantitative rt-PCR assay and isolation of infectious virus. *PLoS One*, **8**, e70800.