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Short communication

Transmission Routes of Oropouche Virus: Potential Role of European Biting Midges and First Oral Infection Attempt in Wild-Caught Culicoides (Subgenus Avaritia)

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Abstract

Oropouche virus (OROV) is an emerging arbovirus primarily endemic to South America, capable of infecting humans, diverse animals, and multiple vector species. Although its ecology remains poorly understood, increased globalisation and human mobility raise concerns regarding potential introduction into Europe. To evaluate European risk, vector competence trials were conducted using Italian Culicoides obsoletus/scoticus and C. imicola, major vectors of livestock orbiviruses, orally exposed to a 2024 Cuban reassortant OROV strain. Of 260 surviving C. obsoletus/scoticus and 65 C. imicola midges, all samples tested negative for OROV RNA, suggesting limited vector competence. These findings indicate that tested European Culicoides populations are unlikely to support OROV transmission. Nevertheless, the virus's broad host and vector range, reassortment potential, and presence of anthropophilic midges in Europe warrant continued surveillance and vector competence studies. Improved understanding of transmission dynamics, reservoir hosts, and potential vectors is critical for preparedness against Oropouche virus introduction and spread in non-endemic regions.

Keywords

Oropouche, Vector competence, Culicoides obsoletus/scoticus, Culicoides paraensis

Oropouche is a vector-borne disease affecting humans, caused by an arbovirus (OROV; *Peribunyaviridae* family, *Orthobunyavirus* genus, *Orthobunyavirus oropoucheense* species) that also infects various animal species and vector groups. The disease is present primarily in South America and its ecology is still unclear (Jurado-Cobena, 2024). However, globalisation and increased intercontinental human movement raise concerns regarding its introduction and potential spread beyond its native range, including Europe. In 2024, Oropouche was reported for the first time in EU countries, with imported cases in Spain, Germany, and Italy (ECDC, 2024). As described in Anderson et al. (1961), Oropouche was first identified in 1955 in Trinidad (Vega de Oropouche) in a recently deforested area converted to cocoa, coffee, and citrus cultivation. The first case involved a young man who had worked in the forest a few miles away, sleeping outdoors. Over 500 mosquitoes were collected in the area, belonging to 30 species with *Aedes scapularis* being the most abundant. The OROV strain was inoculated in mosquitoes, and then isolated from *Aedes scapularis*, *Aedes serratus*, *Culex fatigans*, *Psorophora ferox*, although no subsequent transmission to mice was observed. In 1960, OROV was isolated from a pool of *Mansonia venezuelensis* (later referred to as *Coquillettidia venezuelensis*).

As with other members of the genus *Orthobunyavirus*, the OROV genome comprises three single-stranded, negative-

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sense RNA segments: small (S), medium (M), and large (L). Genetic exchange through reassortment is a well-documented and naturally occurring process in segmented RNA viruses (Ladner et al., 2014). Indeed, reassortment of the three OROV segments has been observed following co-infection with distinct OROV strains, driving viral evolution by altering vector competence or virulence (Navarro et al., 2016). Phylogenetic analyses based on the limited number of complete S-segment coding sequences available in GenBank have thus far identified four distinct OROV genotypes (Vasconcelos et al., 2011). However, as previously emphasised, recent outbreaks have involved OROV strains whose genomic segments appear to originate from different lineages, indicating a reassortment event that may have contributed to altered viral characteristics and increased transmissibility (Wesselmann et al., 2024).

Like many arboviroses, Oropouche has a sylvatic cycle and an urban cycle, linked by spillover events.

The sylvatic cycle involves mammals and wild birds as natural reservoirs, with mosquitoes and possibly biting midges as vectors. In the urban settlement, humans are the primary hosts, with *Culicoides* biting midges acting as pivotal vectors and mosquitoes potentially playing a secondary role (Jurado-Cobena, 2024; Wesselmann et al., 2024).

The sylvatic cycle is less understood than the urban one, since definitive reservoir and amplifying hosts have yet to be identified. Sporadic virus isolation from sloths (*Bradypus tridactilus*) and non-human primates, alongside antibody detection in wild birds, sloths, non-human primates, and rodents, indicates a broad host range. Among non-human primates OROV has been detected in black-tufted marmosets (*Callithrix penicillata*), black howlers (*Alouatta caraya*), and tuffed capuchins (*Sapajus alloata*). Among avian hosts, antibodies have been detected across various families (e.g. Formicariidae, Fringillidae, Thraupidae), though their role in virus amplification or dissemination remains unclear, warranting further investigation given the extensive geographic ranges of implicated species (LeDuc et al., 1981; Pinheiro et al., 1976, 1981; Romero-Alvarez et al., 2023).

Humans likely serve as a bridge between sylvatic and urban cycles, acquiring the infection via exposure in the forest and subsequently introducing the virus into urbanised areas. Outbreaks often coincide with human-driven environmental changes such as deforestation and agricultural expansion, which increase human-vector contact and alter vector habitats, leading to new potential breeding sites (Romero-Alvarez et al., 2023).

The urban cycle involves humans as the sole vertebrate host, with viraemic human movement facilitating the disease spread between villages. However, antibodies have also been detected in domestic animals as water buffaloes, sheep, horses, cattle, dogs, and domestic birds in urban and rural settlements (Dias et al., 2024; Jurado-Cobena, 2024; Pinheiro et al., 1976).

Besides *Coquillettidia venezuelensis*, OROV has been isolated from *Aedes serratus*, *Culex quinquefasciatus*, and from the biting midge *Culicoides paraensis* (LeDuc et al., 1981; Pinheiro et al., 1962, 1976; Roberts et al., 1981), or its RNA has been detected in *Culex quinquefasciatus*, *Aedes serratus*, *Psorophora cingulata*, and *Haemagogus tropicalis* (Cardoso et al., 2015; Pereira-Silva et al., 2021).

Studies under laboratory conditions have suggested a midgut infection barrier in major urban mosquito species (*Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus*), with viral replication only occurring upon injection but not oral ingestion (De Mendonça et al., 2021). Other infection trials showed no dissemination in *Culex tarsalis*, limited dissemination in *C ulex quinquefasciatus*, but high infection and dissemination in *Culicoides sonorensis* (a North American vector species), although with low transmission rates (McGregor et al., 2021).

Culex quinquefasciatus is considered a potential secondary vector in the OROV urban cycle. It has demonstrated some ability to transmit OROV from viraemic hamsters to susceptible ones in laboratory settings, though its transmission efficiency appears low (Hoch et al., 1987). Although this mosquito is unlikely to be the principal urban vector, high population densities could potentially compensate for its low vector competence.

Culicoides paraensis is a confirmed vector both in the field and under experimental conditions, capable of transmitting OROV from infected hamsters to susceptible ones, and from viraemic humans to hamsters (Pinheiro et al., 1981, 1982). *Culicoides paraensis* (Goeldi, 1905; subgenus *Haematomyidium*, *C. paraensis* group) is widespread throughout tropical and subtropical regions of the Americas (Felippe-Bauer et al., 2003). This species exhibits clear anthropophilic behaviour and host-seeking midges are found predominantly near houses, however, it also displays opportunistic feeding patterns. *Culicoides paraensis* thrives in moist, decaying vegetation (such as that from recently harvested farms). In the forest, this midge can thrive in tree-holes, leaf debris, and damp soil, whereas in man-modified landscapes and cultivated lands it finds man-made breeding sites, such as banana and cacao remains with high organic matter content and high water retention. *Culicoides paraensis* has a diurnal activity, with peak biting activity around 17.00, and is both endophagic and exophagic, showing higher indoor abundance during rainfall and preference for shaded outdoor areas (Hoch et al 1986, 1990; Pinheiro et al., 1976).

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Neither the mosquito nor the *Culicoides* species reported above occur in Europe. Consequently, the risk for OROV circulation in Europe, following introduction via viraemic travellers, depends on the presence of potential local vectors, specifically European mosquito and biting midge species that are anthropophilic, abundant, and competent for OROV.

Recent studies on Italian populations of *Culex pipiens* and *Aedes albopictus*, pivotal vectors of West Nile and Dengue/Chikungunya viruses, respectively, have shown absence of capability for OROV transmission. (Mancuso et al., 2025).

Within European biting midges, *Culicoides impunctatus* (common in Northern Europe, mainly known as the aggressive midge of the Highlands in Scotland) and *Culicoides riethi* (recently implicated in diurnal human attacks on people in Italy) show a clear anthropophilic behaviour. The majority of *Culicoides* species are primarily livestock pests, however, these biting midges demonstrate opportunistic feeding behaviour rather than specialised. Numerous species are also able to feed also on humans, such as *Culicoides imicola*, *Culicoides obsoletus*, *Culicoides scoticus*, *Culicoides dewulfi*, *Culicoides chiopterus*, *Culicoides pulicaris*, *Culicoides punctatus* (Carpenter et al., 2013; Romiti et al., 2022; Snyman et al., 2021).

Here, we report on vector competence trials assessing oral infection of Italian *Culicoides obsoletus / scoticus* and a preliminary attempt with *C. imicola*, both belonging to the subgenus *Avaritia* and major vectors of livestock orbiviruses (e.g. Bluetongue virus, BTV) in Europe and the Mediterranean Basin. In Italy, both these taxa have been involved in BTV and Epizootic Haemorrhagic Disease (EHDV) virus (*Orbivirus*), and Schmallenberg virus (*Orthobunyavirus*) transmission (Balenghien et al., 2014; Goffredo et al., 2013, 2015; Quaglia et al., 2023).

Wild midges were collected alive on a sheep farm in Sardinia (*C. imicola*, November 2024) and a cattle farm in Central Italy, specifically in Abruzzo (*C. obsoletus*/*scoticus*, May 2025). Previous studies have demonstrated the competence of these populations for orbiviruses under laboratory conditions (Federici et al., 2016, 2019). Due to their morphological indistinguishability, *C. obsoletus* and *C. scoticus* were collectively referred to as *C. obsoletus / scoticus*, however, the studied population was already known to include both species, identified on a molecular basis (Federici et al., 2019; Goffredo et al., 2016).

Wild midges were collected using UV blacklight suction traps operating from sunset, with stretched paper placed in the collection beakers. Early in the morning, the collection beakers were placed in a dark box, allowing the living midges to fly into 64 mm card boxes through funnels. After collection, insects were maintained with sucrose solution 10%, offered via cotton pledgets, and transferred to a BSL3 laboratory. Midges were acclimatized at 25±1°C and 70%>HR>40% and, prior to oral infection, they were starved for 24 hours.

The OROV strain used in this study is a reassortant virus isolated in Italy from a patient who had contracted the infection in Cuba in 2024 (Castilletti et al., 2024; Deiana et al., 2024), representing the reassortant strain that caused the spread of the epidemic in 2024, and still circulating in Brazil and Cuba (WHO, 2025). After primary isolation, the virus underwent a third passage on Vero E6 cells (ATCC CRL-1586), and was titrated using the 50% tissue culture infectious dose assay ($TCID_{50}$). This strain was provided by the Department of Infectious, Tropical Diseases and Microbiology at the IRCCS Ospedale Sacro Cuore Don Calabria (Negrar di Valpolicella, Verona, Italy). As reported by Deiana et al. (2024), whole-genome sequencing was performed using next-generation sequencing (NGS), followed by phylogenetic analysis and genetic variability studies. Consensus sequences were deposited in GenBank under the following identifiers: Strain name: OROV-IRCCS-SCDC_1/2024, S segment ID: PP952117, M segment ID: PP952118; L segment ID: PP952119.

For oral infection, the virus was diluted 1:10 in defibrinated sheep blood (heated at 37°C), and offered to *Culicoides* via cotton pledgets for 60 minutes (Venter et al., 2005). After feeding trials an aliquot of the infected blood meal was retained and titrated (Table I).

Species	Virus Titre ($TCID_{50}$ /ml)		11 dpi (positive/tested)		
	OROV strain	Blood meal	heads	bodies	FTA
Culicoides obsoletus/scoticus	10^ 7.21	10^ 6.65	0/260	0/260	0/11
Culicoides imicola	10^ 6.80	10^5.46 – 10^5.80	0/65	0/65	0/13

Table I. Oral infection of wild-caught Culicoides biting midges with Oropouche virus (OROV) and results at 11 days post infection (dpi).

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Fed *Culicoides* were immobilized on dry ice, and only fully engorged females were sorted out under the stereomicroscope, on a refrigerated chill-table. Engorged midges were placed into new card boxes with *ad libitum* access to cotton pledgets soaked with sucrose solution. Some engorged midges were kept immediately after feeding (0 days post infection, dpi) to determine a baseline of cycle threshold (Ct) values to be compared with those eventually found at the end of the incubation period (Veronesi et al., 2013). At 7 dpi, the pledgets were replaced with FTA cards, aiming to collect saliva.

At 11 dpi, the surviving midges were killed on dry ice and each midge was dissected into head and body, then stored separately at -80°C. In the meanwhile, species identification was confirmed based on morphology under a stereomicroscope (Delécolle, 1985; Goffredo & Meiswinkel, 2004; Goffredo et al., 2016).

Viral detection was performed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) to target a conserved region at the 5′ end of the OROV S segment (Rojas et al., 2020). A cut-off point of 40 was used for the Ct value, above which a sample was considered negative. In addition, C. imicola midges were also tested for BTV and EHDV using qRT-PCR (Portanti et al., 2025) to detect potential prior natural infections with orbiviruses, since they were collected on the island of Sardinia, where both viruses had recently been circulating (Lorusso et al., 2023; Quaglia et al., 2023).

The infected blood was offered to more than 7,000 *C. obsoletus / scoticus*, yielding nearly 500 fully engorged females. Of these, 260 survived until 11 dpi and were dissected and tested. The Ct values at 0 dpi were determined on 20 midges and averaged 38.2 (range 37.1 - 39.7). Conversely, only 65 midges of *C. imicola* survived at 11 dpi, due to an unexpected high mortality during acclimatation and incubation.

All samples (heads, bodies, and FTA cards) tested negative for OROV RNA (Table I). In addition, all heads and bodies of *C. imicola* midges that survived the incubation period were negative for both BTV and EHDV.

The results support the conclusion that *C. obsoletus / scoticus*, a species complex widely distributed across Europe and known as an opportunistic human feeder, is unlikely to serve as a competent vector for the tested OROV strain. The consistent number of midges surviving to the end of the incubation period (n. 260) lends robustness to these findings. Of course, the reported results refer specifically to the combination of the vector population and the OROV strain tested in this study, and it cannot be excluded that other genotypes or reassortant strains of OROV may replicate and be transmitted, or that other European *Culicoides* populations may be susceptible.

Regarding *C. imicola*, the limited sample size prevents firm conclusions about its vector competence. Although these preliminary findings provide some insight, particularly in the absence of data on the subgenus *Avarita* and OROV, they do not rule out potential competence, warranting further studies.

Compared to membrane-based feeding techniques, the feeding method used (soaked cotton pledgets) may underestimate vector competence, yielding higher feeding but lower infection rates. Specifically, *C. obsoletus / scoticus* midges are generally reluctant to feed on membranes, whereas cotton pledgets have proven effective in obtaining substantial numbers of fully engorged females (Federici et al., 2019). Notably, the same midge populations involved in this trial (*C. obsoletus / scoticus* Abruzzo and *C. imicola* Sardinia) have been successfully infected with orbiviruses under the same laboratory conditions and feeding method. As reported by Federici et al. (2019), both taxa were susceptible to BTV (serotypes 2, 4, and 8) when orally infected via blood-soaked cotton pledgets, with significantly higher recovery rates observed in *C. imicola*. This indirectly underscores the appropriateness of the chosen target population and the current experimental design, especially given the considerable challenges associated with simultaneously providing an internal benchmark as a positive control. Further vector competence studies targeting other *Culicoides* species, as well as other OROV strains, are required to better assess the risk of OROV spreading in Europe through local biting midges.

This study underscores the need for continued research into the transmission dynamics of Oropouche virus, taking into account its broad host and vector range, and including investigations of potential reservoirs and amplifying hosts as well as clarification of the potential role of birds.

Ethical approval

The study does not require any ethical approval.

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Conflict of interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: MG, CC, FGG, MQ; Methodology: MQ, CP; Formal analysis: MQ, CP, FV, MDA, AT; Investigation: MQ, SF, SGDA, CF, PC ;Writing original draft preparation: MQ, MG; Writing, review and editing: MG, NDA, MQ, CP, CC, GSatta; Supervision: GSavini, NDA, MG; Project administration: GSavini, GM; Funding acquisition: GSavini, GM.

All authors have read and agreed to the published version of the manuscript.

Data availability

The datasets generated for this study can be found in the manuscript.

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