PAHO Pan American Health Organization World Health Organization Americas

Laboratory Guidelines for the Detection and Diagnosis of Usutu Virus Infection

1 September 2023

Context and general considerations

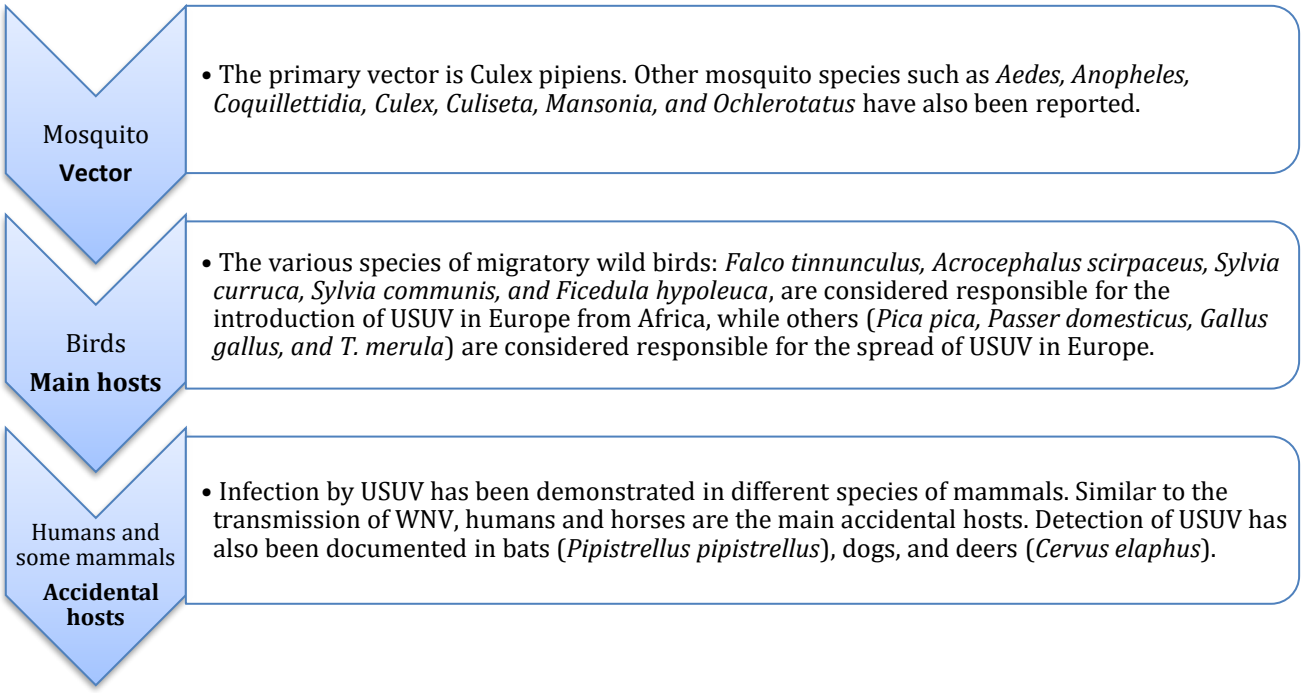
Usutu virus (USUV) is a mosquito-borne virus that belongs to the Japanese encephalitis virus serocomplex, genus *Orthoflavivirus* (formerly *Flavivirus*), *Flaviviridae* family, and group IV of the Baltimore classification (viral genome: single-stranded, positive sense RNA). It is postulated that various species of the serocomplex evolved from an ancestral flavivirus with a natural cycle that included birds and mosquitoes. These species currently include West Nile virus (WNV) and USUV in Africa, Asia, and Europe, Kunjin virus and Murray encephalitis virus in Australia, Japanese encephalitis virus (JEV) in Asia, and St. Louis encephalitis virus (SLEV) in the Americas.

USUV was first isolated in 1959 in a *Culex spp.* mosquito in South Africa near the Usutu River. Since then, the virus has been detected in several African countries such as Senegal, Nigeria, Uganda, Burkina Faso, Côte d'Ivoire, and Morocco. The first human infection was described in the Central African Republic in 1981 in a patient with fever and rash. Until the early 2000s, the virus had not been associated with severe/fatal disease in animals or humans and was considered restricted to tropical and subtropical Africa.

In Europe, USUV was first detected retrospectively in an outbreak among blackbirds (*Turdus merula*) in the Italian region of Tuscany in 1996, causing high mortality. Five years later, in 2001, USUV was again responsible for mortality in blackbirds, this time in the vicinity of Vienna, Austria. Currently, USUV is found recurrently in several European countries, suggesting a persistence of the transmission cycle in the affected areas. Likewise, co-circulation of USUV and WNV has been frequently observed in Europe. Seroprevalence studies in healthy blood donors in Germany and Italy report a prevalence of anti-USUV antibodies of 0.02% and 1.1%, respectively. To date, no infections have been detected outside Africa and Europe in either animals or humans.

Host, vector, and life cycle

The life cycle of USUV is similar to that of other flaviviruses belonging to the JEV serocomplex, such as WNV. The virus circulates in an enzootic cycle involving mosquitoes as vectors (mainly *Culex pipiens*) and birds as the main amplifying host. Through vectors, USUV can also infect humans, equines, and other mammals, which are considered accidental hosts.



Clinical Presentation

To date, about one hundred cases of USUV infection in humans have been reported, the vast majority in Europe. Signs and symptoms reported can range from fever, rash, and mild headache to severe neurological manifestations, particularly in immunosuppressed patients. The most common neurological manifestations are meningoencephalitis, encephalitis, and meningitis, although cases of flaccid paralysis have also been identified. USUV infection can be asymptomatic and has been reported in healthy blood donors in Germany, Austria, and Italy.

Laboratory diagnosis

The diagnosis of USUV requires confirmation by laboratory techniques since the clinical presentation is unspecific. In countries where USUV is circulating, human infections should be suspected in patients with fever and neurological symptoms of unknown etiology. Laboratory methods include virological (direct) diagnostic methods through genome amplification or cell culture, and serological (indirect) methods that detect antibodies produced against the virus. In general, diagnostic samples are serum and cerebrospinal fluid (CSF). CSF is only used in cases with neurological symptoms and by clinical indication.

Biosafety

Fresh biological samples of any type should be considered potentially infectious. Samples should be processed and handled only by trained professionals after a local risk assessment considering all

biosafety indications and appropriate personal protective equipment. Any procedure involving sample manipulation should be carried out in certified class II biosafety cabinets. This includes the lysis step performed during RNA extraction. Lysed samples are considered non-infectious. Handling of extracted RNA does not require a biosafety cabinet. Also, all necessary precautions to avoid percutaneous exposure should be taken.

Virological methods

Viral RNA detection can be performed in serum and CSF by real-time or endpoint **RT-PCR** using USUV-specific primers (and probes). Generic protocols (pan-flavivirus) followed by nucleotide sequencing can also be used. In some cases, the presence of viral RNA in urine has been described.

Viral isolation is performed with the same sample types as RT-PCR. Mammalian cell lines (e.g., Vero cells) as well as mosquito cells (e.g., C6/36 cells) are used. In general, viral isolation is not routinely applied nor is it a requirement for diagnostic confirmation. Technical complexity, costs, as well as the need to identify virus isolates by RT-PCR or immunofluorescence, limit the use and timeliness of virus isolation for diagnosis.

A positive RT-PCR result (or viral isolation) **confirms** an infection. Although the dynamics of viremia in USUV infections have not been fully described, by analogy with similar viruses such as WNV, viremia is likely to be low and short-lived (Figure 1). In particular, if the case is detected in the neurological phase, it is likely that the virus is no longer present in the blood. Therefore, a negative result **does not rule out** infection and, upon clinical and epidemiological suspicion, serological methods should be used (Figure 2).

Serological methods

The detection of IgM or IgG antibodies is conducted using *in-house* **ELISA** or **immunofluorescence**. Detection can be performed in both serum and CSF. The kinetics of antibody production has not been fully described. However, by analogy with WNV, it is likely that antibody detection can be performed early after the onset of symptoms, particularly if neurological symptoms are present (Figure 1).

Due to the potential cross-reactivity between USUV and other flaviviruses (in particular SLEV, WNV and other viruses of the JEV serocomplex), a positive IgM result must be confirmed using neutralization assays such as the **plaque reduction neutralization test (PRNT)** or **microneutralization**. At a minimum and depending on the epidemiological situation of the area where the case was likely infected, it is recommended to detect neutralizing antibodies against USUV, SLEV and WNV in parallel (Figure 2).

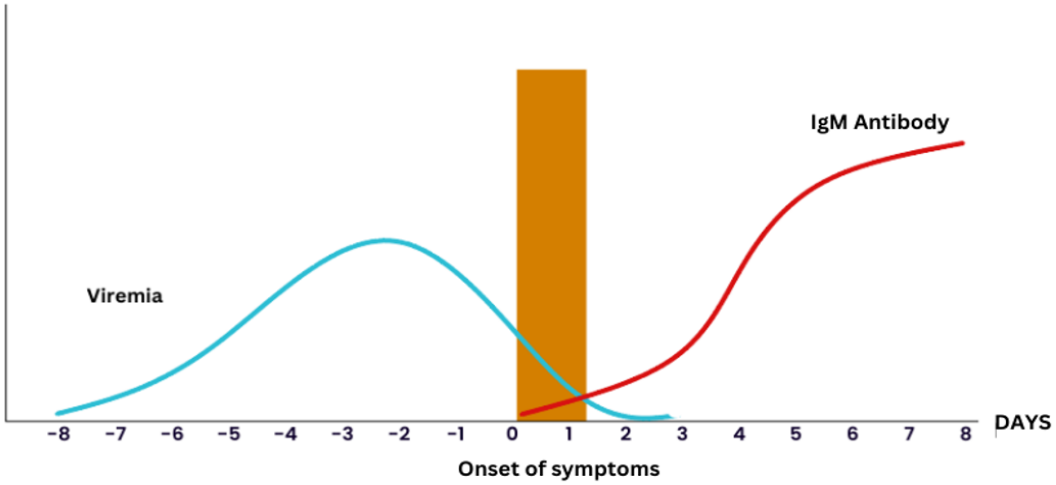
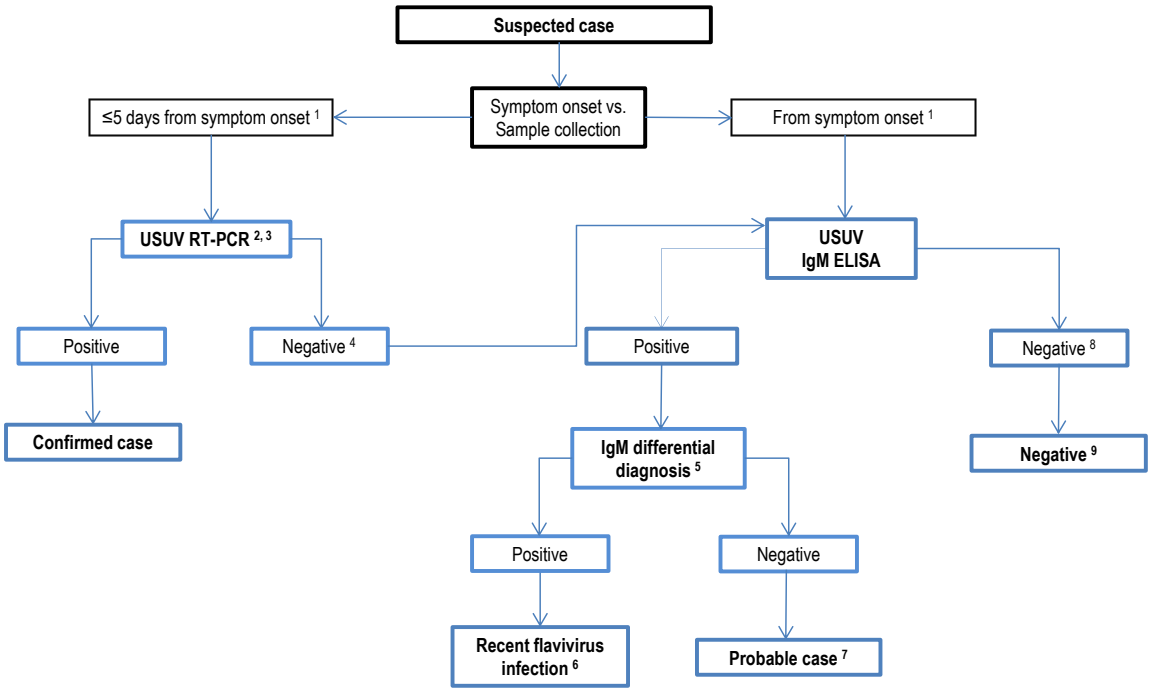


Figure 1. Dynamics of Usutu virus infection based on available information and behavior of similar viruses such as West Nile virus.

Taking these elements into account, in the absence of confirmation by virological methods, the analysis of serological results should be conducted in the light of clinical and epidemiological information. Generally, the detection of specific antibodies in CSF (confirmed by neutralization) is considered as **confirmatory** of a USUV infection in a case with neurological manifestations. Cases with clinical and epidemiological suspicion, with detection of specific IgM antibodies (considering the differential diagnosis by IgM) but without confirmation by neutralization can be considered **probable cases.**

Laboratory algorithm



[1] The number of days is indicative since the dynamics of viral RNA and antibody production have not been described in detail.
[2] Perform RT-PCR in serum and, in cases with neurological presentation, in CSF. The presence of the virus in CSF may be longer than in serum.
[3] Consider other pathogens depending on the epidemiological situation, e.g., SLEV and WNV.
[4] Given clinical and epidemiological suspicion and considering that the viremia may be low and short-lived, use serological methods.
[5] Consider in particular WNV and SLEV, as well as other flaviviruses depending on the epidemiological situation of the area/country.
[6] In the presence of cross-reactivity, IgM ELISA results do not allow confirmation of the etiological agent. However, this result does not rule out USUV infection. Additional clinical and epidemiological criteria should be used for the final interpretation of the case. Neutralization in a reference laboratory may also be considered to analyze cross-reactive samples.
[7] Consider performing neutralization in a reference laboratory to confirm the case.
[8] If the sample was collected less than 5 days after the onset of symptoms, request the collection of a second sample and repeat the IgM ELISA.
[9] Investigate the cases and perform clinical differential diagnosis.

Figure 2. Algorithm for laboratory confirmation of Usutu virus (USUV) infection. *CSF: cerebrospinal fluid; SLEV: St. Louis encephalitis virus; WNV: West Nile virus.*

Sample storage

- Keep refrigerated (2 - 8°C) if processed (or sent to a reference laboratory) within 48 hours.
- Keep frozen (-10 to -20°C) if processed after 48 hours but within 7 days.
- Keep frozen (-70°C or lower) if processed more than one week after collection. The sample is adequately preserved at -70°C for prolonged periods of time.
- It is recommended to aliquot samples into at least two vials.
- Avoid multiple freeze-thaw cycles.

Shipping of samples to the reference laboratory by air

- Ensure the cold chain preferably with dry ice, or with refrigerant gels. Always use triple packaging.
- Ship the samples preferably within the first 48 hours.
- The original samples should be packaged, marked, appropriately labeled, and documented as **category B.**
- The complete clinical and epidemiological record must accompany the shipment.

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