INFRAVEC2 PROTOCOL

Standardisation protocol for Toscana virus-sand fly infections

Protocol Information

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Authors: CUNI, UG
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Scientific Coordinator: Kenneth Vernick

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OBJECTIVES

Toscana virus (TOSV) is one of the most important viruses transmitted by sand flies. A clinical outcome in human infected by TOSV is going from non-symptomatic forms through febrile illness to CNS disease. It is the major causative agent of meningitis during warm seasons in Mediterranean area. Among viruses transmitted by sand flies it is the only one which exhibits neurotropic activity (Charrel et al., 2012). It circulates on large geographic area, from Turkey to Morocco (Charrel et al., 2012; Es-Sette et al., 2015) and is usually connected with the presence of suspected vectors *Phlebotomus perniciosus* and *Phlebotomus perfiliewi*. TOSV was detected also from *Phlebotomus tobbi* in Cyprus (Ergunay et al., 2014), *Phlebotomus longicuspis* and *Phlebotomus sergenti* in Morocco (Es-Sette et al., 2014; Es-Sette et al., 2015), *Sergentomyia minuta* in Marseille (Charrel et al., 2006), but role of these sand fly species in virus circulation remains still unknown. Experimental studies demonstrated oral (Ciufolini et al., 1985; Maroli et al., 1993), transstadial, transovarial (Tesh and Modi, 1987; Tesh et al., 1992; Maroli et al., 1993) and venereal (Tesh et al., 1992) transmission of TOSV between *P. perniciosus* sand flies. However, there have been no report of experimental studies since 90´s and a standardization approach is necessary to study phlebovirus transmission and sand fly borne-pathogens coinfections (e.g. *Leishmania* sp.–TOSV).

Here we established an experimental protocol for oral TOSV infection of sand flies, **initially with TOSV lineage B.** All experiments with sand flies were performed at CUNI. All necessary information and procedures how to infect sand flies with TOSV within Biosafety Level 2 (BSL2) are given below.

1. EQUIPMENT AND CONSUMABLES REQUIRED

- Facility and large equipment requirements:
  - incubator/room with controlled temperature and humidity for keeping sand flies
  - biological safety cabinet class II
  - glove box for blood feeding and handling infected sand flies
  - water bath with external circulation for blood feeding
  - binocular microscope for dissections
  - CO_2 incubator for incubation of cells for TCID50
  - inverted microscope for checking cells and TCID50 evaluation
- Small equipment and consumables requirements:
  - battery powered aspirator for sand flies sorting
  - nylon mesh cages
  - glass feeders
  - chicken skin
  - chemical stand
  - parafilm
  - entomological pins
  - tweezers
  - tissue grinding pestles
  - set of multichannel pipettes, set of normal pipettes, pipetboy
  - sterile plastic (filtered tips, tubes, 96-well plates, cultivation flasks, safety lock eppendorf tubes etc.)
  - counting chamber
  - protective mask
  - longer and normal size gloves
  - laboratory coat

- Chemicals requirements:
  - DMEM 4,5 g/L D-Glc, L-Glutamine, pyruvate
  - fetal bovine serum (FBS)
  - amphotericin B
  - nystatin
  - gentamycin
  - penicillin-streptomycin
  - PBS
  - trypsin
  - Virkon™ desinfectant

2. DESCRIPTION OF WORK

1. Safety rules
2. Sand fly colony maintenance
3. Cultivation of BSR cells
4. Production of TOSV
5. Blood feeding process
6. Post feeding manipulations and dissection of sand flies
7. Evaluation of sand fly infection by TCID50

1. Safety rules

TOSV is handled in BSL2 conditions at CUNI and this may change depending on country- please verify! Briefly, experimenter should wear laboratory coat, two pairs of gloves (long and short), mask and protection glasses. All manipulations with virus (preparing of infectious blood, homogenization, TCID50) should be done in biological safety cabinet class II and all manipulations with infected sand flies should be performed in glove box (blood feeding, sorting, dissection). Virus inactivation is achieved by using 1% Virkon™ solution.

2. Sand fly colony maintenance

If the colony is not available in the laboratory/institution, it could be sent from another workplace. Detailed information about establishment and maintenance of sand fly colonies were published in Volf and Volfova (2011) or Lawyer et al. (2017). For experimental infection in CUNI, we used *P. perniciosus* and *P. tobbi* females (originated from Spain and Turkey respectively) which were maintained under standard conditions (26°C±1°C with 60-70% relative humidity; photoperiod light 14h:10h dark; 50% sucrose solution) as described in publications mentioned above.

3. Cultivation of BSR cells

For the production of TOSV and for TCID50 the BSR cells (BHK21 clone) were needed. Cultivation of the cells was performed in CO_2 incubator (5% CO_2, 37°C), in the plastic tissue culture flasks (150 ml) with liter screw cap, which were placed horizontally in the incubator. The grow DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin was used for standard cultivation.

4. Production of TOSV

Virus stocks were prepared by infecting BSR cells in DMEM media supplemented with 4% FBS. Supernatant were collected at D3 or D4 post-infection, when obvious CPE was observed. The supernatant was clarified by centrifugation (200g for 5 min) and subsequently ultracentrifugated through a sucrose cushion (20% in PBS) at 27,000 g for 3 hours (4°C). The viral pellet was resuspended in PBS in order to concentrate 100 times the virus stocks (i.e. 300 µl for 30 ml of viral supernatant), aliquoted and stored at -70°C.

5. Blood feeding process

One day before infection approximately 150-200 females (3-7 days old) were separated per cage and starved (without sugar meal). Feeding system with glass feeder, proven for leishmania infection (Volf and Volfova, 2011), was used as sand flies refused to feed on hemotek membrane system. Open part of glass feeder was covered by chicken skin which was attached by parafilm. TOSV virus (infectious dose: approx. 10^6 PFU/ml of blood, more details listed in result section) were mixed with defibrinated sheep blood and 3 ml of this mixture were filled to glass feeder. Glass feeder was clamped onto a stand and water bath with external circulation was connected by tubes and heated to 38°C±1°C. Part of glass feeder with chicken skin was placed directly to cage through sleeve and elastic bands to prevent sand fly escaping. Sand flies were allowed to feed through membrane for 90-120 minutes once the blood feeding was done, glass feeder was disinfected in Virkon™ solution.

6. Post feeding manipulation and dissection of sand flies

After blood feeding, non-fed females were removed by battery powered aspirator and killed in Virkon™ solution. Five blood fed females from each group were transferred into a plastic pot and cooled on ice to anesthetize them. They were homogenized individually by crushing pestle in 1 ml of crushing medium (DMEM; 4% FBS; amphotericin B 2.5 g/ml; nystatin 100 U/ml; gentamycin 50 g/ml; penicillin-streptomycin 50g/ml) and stored in -80°C until used. Blood fed females were kept in incubator and subsequently sampled at D4, D8 and D14 post-infection. In order to determine TOSV dissemination, head with attached salivary glands (H) was separated from the body (B). Dissections were done under binocular microscope in PBS solution using fine tweezers and entomological pins. Dissected parts were further processed as

described for whole females above. To evaluate infection rate, intensity of infection and dissemination we used TCID50 method which is described in the next paragraph.

7. Evaluation of sand fly infection by TCID50

96-well plates were filled by 100 µl DMEM media (supplemented by 4% FBS; 1% penicillin-streptomycin) except first line, where 111 µl of each sample were added. Each sample was tested in quadruplicates and for each series of experiments were used also blood fed non-infected females as negative control. Serial dilutions 1:10 were performed by transferring 11 µl from one line to the other (15 flushes is recommended to mix well each dilution before transfer) and 11 µl from the last line was removed. Subsequently 100 µl of BSR cells (concentration $4x10^4$/ml) was added to each well. Plates were incubated for 6 days in CO_2 incubator. The detection of virus induced cytopathic effects was assessed under inverted microscope. The numbers of positive and negative wells were recorded and TOSV infectious titer in each sample was calculated by Reed and Muench method.