Detection of Toscana virus from an adult traveler returning to Australia with encephalitis

Research Article

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Detection of Toscana virus from an adult traveller returning to Australia with encephalitis[1].

Running Title: TOSV in a traveller returning to Australia

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Abstract

Toscana virus (TOSV) is identified in sandflies, animals and humans around the Mediterranean Sea. TOSV has not been reported in Australia. During investigations of cerebrospinal fluid samples from patients with encephalitis, TOSV genetic sequences were identified in a traveller returning to Australia from Europe. TOSV should be considered, especially during May to October, in travellers to Australia who embarked in countries in and around the Mediterranean Sea and who subsequently present for medical care because of neurological symptoms.

KEYWORDS.

Toscana virus, Phlebovirus, travel, high throughput sequencing, encephalitis

Introduction.

Members of the family *Bunyaviridae*, genus *Phlebovirus*, are enveloped viruses containing a tripartite negative-stranded RNA genome comprised of large (L), medium (M) and small (S; encoding non-structural [NS] and nucleocapsid [N] proteins) segments.[1] Phleboviruses are transmitted to humans through the bite of an infected insect vector and may result in an initial flu-like illness that can progress to neurotropic disease resulting in aseptic meningitis or mild meningoencephalitis.[2, 3] Humans are considered dead-end hosts.[1]

Toscana virus (TOSV) is a phlebovirus transmitted by sandflies of the *Phlebotomus* genus (including *P. perniciosus* and *P. perfiliewi*) which has been identified in the cerebrospinal fluid (CSF), plasma and urine of human cases of fever and suspected viral encephalitis. Cases occur in areas around the Mediterranean Sea and peak during summer months in parallel with the life-cycle of its arthropod host.[2-5] Serological studies indicate that human infections may also occur without neurological involvement.[6] Reports indicate that dogs, cats, goats, sheep and bats may also be infected by TOSV.[7-11]

Neurological symptomatology resulting from TOSV infection is clinically indistinguishable from that due to other viral causes so laboratory testing is essential to confirm a TOSV infection.[3, 12] Molecular characterization has defined three lineages among TOSV strains; A, B and C although there are no complete gene segment lineage C sequences and it is unclear whether these represent one or several serotypes.[4] The global distribution of TOSV lineages has yet to be investigated.

Identifying a putative pathogen in the CSF can be difficult and a cause of infection is frequently not found.[13] Culture-based virus isolation techniques are insensitive and brain-biopsy approaches are highly invasive, so PCR-based diagnostics using CSF fluids are considered a vast improvement and a preferred approach.[14, 15] A negative PCR result is considered reliable for central nervous system (CNS) specimens. The usefulness of target-specific molecular diagnostics depends on what assays are available in a laboratory,

which assays are included during analysis, or whether analysis of a target is clinically requested during patient investigations.[15]

Molecular detection methods based on “deep” or high throughput sequencing (HTS) are unbiased and although cost, turnaround time and data analysis demands still limit their uptake, they have the capacity to identify unexpected or unsought pathogens from cases of disease. This was the case for TOSV in a traveller returning to Australia.

Methods.

Specimen selection, preparation and sequencing

Retrospective selection of specimens from patients who had tested negative for a known cause of acute encephalitis but were clinically defined as suffering from an acute infection were chosen as part of a study into the utility of unbiased, HTS for the detection of unknown infectious agents directly from clinical samples.

100 to 500µL of frozen CSF was spun through a size exclusion centrifuge column (Centricon) at 14,000rpm for 15 minutes. A 15µl concentrated eluate was retrieved and RNA was purified (Roche nucleic acid kit) into a 50µl volume. The 50µl solution was submitted to the Australian Genome Research Facility for RNA-seq library construction, without mRNA purification or RNA fragmentation according to the manufacturer's protocol (Illumina). Briefly, RNA underwent random primed cDNA synthesis and second strand cDNA

synthesis followed by DNA end-repair, A-tailing and adapter ligation. RNA-seq libraries were sequenced on an Illumina HiSeq 2500 (San Diego, CA, USA) with 100bp paired end chemistry.

Ethical approvals

Studies were approved by the Children's Health Queensland Hospital and Health Service Human Research Ethics Committee (HREC/10/QRCH/100 and HREC/14/QRCH/231) and The University of Queensland Medical Research Ethics Committee (201001474). Access to patient samples was approved by Pathology Queensland (PQ14172) and restricted access to confidential information was permitted in accordance with Public Health Act 2005, approval number RD005886.

Bioinformatics analysis

The sequences were trimmed with a minimum quality score of 30 and a minimum length of 90bp after removal of adapter sequence.[16] Contaminating human, bacterial, plant and fungal sequences were removed using Deconseq [17] by screening using settings of 90% identity and coverage. The remaining reads were assembled using IDBA-tran [18] with default parameters. The assembled contigs were compared by BLAST to the NCBI nucleotide database using BLAST+ (version 2.2.30).[19]

Available complete or near-complete (>99% coverage) TOSV gene sequences were downloaded from GenBank, trimmed to the coding region

(encompassing the start of NS and the end of N for the S segment) and aligned together with the study sequences.

Sequence alignments were carried out using the MAAFT algorithm in Geneious v8.1.8.[20] Neighbor-joining p-distance phylogenetic trees with pairwise deletion were constructed using 500 bootstraps in Mega v7.0.1.[21]

Results.

Medical chart review

On July 2014, a 51-year old man presented to the emergency department with headache and fever. Two days prior, he had returned from a holiday in Italy where he visited Sicily, Positano and Naples. After two and a half weeks, he flew to Paris for four days then returned to Brisbane, Australia via Dubai, United Arab Emirates where he stopped over for three days. He began to feel ill with fatigue and a mild headache while in Italy, becoming progressively worse, and two days after his return home, he presented to hospital. He had experienced a short period of right-sided weakness and a possible petechial rash one week prior to his return. He experienced no neck stiffness or photophobia. He denied any insect bites. A diagnosis of viral encephalitis was made.

Blood, urine and CSF cultures yielded no microbial growth. CSF white blood cells were 82 x 10^6/L, red blood cells were 14 x 10^6/L, of which 90% were

mononuclear cells and 10% polymorphonuclear cells, glucose 3.2 mmol/L, protein 710 mg/L. Full blood count and electrolytes were normal on admission but the patient developed a mild transaminitis during hospitalisation. Radiological investigations including chest X-ray and CT scan of the head did not detect evidence of disease.

PCR tests on CSF did not detect enteroviruses, herpes simplex type I and II, varicella zoster virus, *N. meningitidis* or *S. pneumoniae*. A throat swab tested negative for enterovirus by RT-PCR. No PCR or serology testing was requested for other arboviruses.

Treatment was commenced in the emergency department with intravenous ceftriaxone, benzylpenicillin and dexamethasone. Acyclovir and ceftriaxone were continued in the ward then stopped when relevant cultures and molecular tests yielded negative results. The patient improved symptomatically and was discharged home after four days.

Genetic analyses

Illumina HiSeq sequencing identified the near complete L genome segment (Fig 1a; 6,285nt; missing start codon), the 4,181nt M segment (Fig 1b; missing start codon) and the 1,826nt S genome segment (Fig 1c; complete NS and N coding regions) of TOSV from the CSF sample. The variant was named Toscana virus H.sapiens-wt/Italy/2014/Genotype-A_BB8, most closely grouping with other TOSV A lineage variants. Genbank accession numbers for these three sequences are KY626660-62. Each nucleotide was sequenced

between two to 194 times. The segments shared 90.3% to 99.5% amino acid identity with other TOSV strains.

Figure 1. Phylogenetic representation of complete and near complete Toscana virus (TOSV) lineage L (1a), M (1b) and S (1c) gene nucleotide segments compared to a Sandfly fever Naples virus (SFNV). Lineage A and B strains are indicated by blue (solid outline) and green boxes (dashed outline), respectively. A circle indicates the TOSV strain H.sapiens-wt/Italy/2014/Genotype-A_BB8, described here. MEGA7 was used to construct Neighbor-Joining trees with distances calculated using the p-distance method (500 bootstraps; percentage replicate tree branching patterns are shown next to initial branches). Figshare https://doi.org/10.6084/m9.figshare.4696888.v1

Conclusion.

To the authors' knowledge this is the first use of HTS to deduce a near complete TOSV genome and the first report of TOSV infection of an Australian with a clinical diagnosis of encephalitis who returned from a summer visit to the Mediterranean region. It remains unknown how many such cases may contribute to currently undiagnosed but clinically suspected viral encephalitis in Australia. Due to the retrospective nature of this investigation, it was impossible to determine precisely where or when the traveller acquired his infection. He paused in the Arabian peninsula for three days during his return to Australia but the majority of his time was spent in Italy, where TOSV is

endemic.[3] Because his symptoms began during his time in Italy, it is likely that is where the infection was acquired.

This laboratory process was rapid, using a centrifugal enrichment step and kit-based total nucleic acids extraction process. The study was limited by a paucity of CSF which meant virus isolation was impossible. Exhaustive systematic, microbe-specific analyses were not conducted and no serology assays were requested or conducted. However, no other viral or bacterial sequences were identified using unbiased HTS or any specific laboratory assay. This result does not exclude the possibility of one or more other pathogens being the cause of the acute encephalitis in this patient. Arbovirus serology would be a useful addition to future investigations of this sort.

HTS made it possible to detect a viral pathogen that was not included in any routine diagnostic screening panels and can help to better characterize the global transport of viruses via infected humans. These findings support further prospective and retrospective investigations of greater numbers of CSF samples from clinically suspect but otherwise laboratory negative acute CNS infections, using HTS and sensitive TOSV-specific PCR methods. It may also be prudent to examine the fitness of Australian arthropods to host, replicate and transmit TOSV and to examine whether local animal species may have antibodies to TOSV, given that infected travellers do visit Australia.

The authors suggest that TOSV be considered, especially during May to October, in the laboratory screening of samples collected from travellers to Australia who embarked in countries around the Mediterranean Sea and

subsequently presented for medical care with signs and symptoms of acute neurological disease.

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References

1. Murray PR, Rosenthal KS, Pfaller MA. Bunyaviridae and Arenaviridae. In: Murray PR, Rosenthal KS, Pfaller MA, editors. Medical Microbiology. 7 ed. Philadelphia: Elsevier; 2013. p. 561-6.e1.

2. Baldelli F, Ciufolini MG, Francisci D, Marchi A, Venturi G, Fiorentini C, et al. Unusual presentation of life-threatening Toscana virus meningoencephalitis. Clin Infect Dis. 2004;38(4):515-20. doi: 10.1086/381201. PubMed PMID: 14765344.

3. Valassina M, Cusi MG, Valensin PE. A Mediterranean arbovirus: the Toscana virus. Journal of neurovirology. 2003;9(6):577-83. doi: 10.1080/13550280390247678. PubMed PMID: 14602570.

4. Ergunay K, Kaplan B, Okar S, Akkutay-Yoldar Z, Kurne A, Arsava EM, et al. Urinary detection of toscana virus nucleic acids in neuroinvasive infections. J Clin Virol. 2015;70:89-92. doi: 10.1016/j.jcv.2015.07.297. PubMed PMID: 26305827.

5. De Lamballerie X, Tolou H, Durand JP, Charrel RN. Prevalence of Toscana virus antibodies in volunteer blood donors and patients with central nervous system infections in southeastern France. Vector Borne Zoonotic Dis. 2007;7(2):275-7. doi: 10.1089/vbz.2006.0637. PubMed PMID: 17627448.

6. Fezaa O, M'Ghirbi Y, Savellini GG, Ammari L, Hogga N, Triki H, et al. Serological and molecular detection of Toscana and other Phleboviruses in patients and sandflies in Tunisia. BMC Infect Dis. 2014;14:598. doi: 10.1186/s12879-014-0598-9. PubMed PMID: 25398483; PubMed Central PMCID: PMCPMC4236749.

7. Alwassouf S, Maia C, Ayhan N, Coimbra M, Cristovao JM, Richet H, et al. Neutralization-based seroprevalence of Toscana virus and sandfly fever Sicilian virus in dogs and cats from Portugal. J Gen Virol. 2016;97(11):2816-23. doi: 10.1099/jgv.0.000592. PubMed PMID: 27589865.

8. Ergunay K, Ayhan N, Charrel RN. Novel and emergent sandfly-borne phleboviruses in Asia Minor: a systematic review. Rev Med Virol. 2016. doi: 10.1002/rmv.1898. PubMed PMID: 27531681.

9. Verani P, Ciufolini MG, Caciolli S, Renzi A, Nicoletti L, Sabatinelli G, et al. Ecology of viruses isolated from sand flies in Italy and characterized of a new Phlebovirus (Arabia virus). Am J Trop Med Hyg. 1988;38(2):433-9. PubMed PMID: 3128131.

10. Dincer E, Gargari S, Ozkul A, Ergunay K. Potential animal reservoirs of Toscana virus and coinfections with Leishmania infantum in Turkey. Am J Trop Med Hyg. 2015;92(4):690-7. doi: 10.4269/ajtmh.14-0322. PubMed PMID: 25711610; PubMed Central PMCID: PMCPMC4385760.

11. Navarro-Mari JM, Palop-Borras B, Perez-Ruiz M, Sanbonmatsu-Gamez S. Serosurvey study of Toscana virus in domestic animals, Granada, Spain. Vector

Borne Zoonotic Dis. 2011;11(5):583-7. doi: 10.1089/vbz.2010.0065. PubMed PMID: 20925529.

12. Jaijakul S, Arias CA, Hossain M, Arduino RC, Wootton SH, Hasbun R. Toscana meningoencephalitis: a comparison to other viral central nervous system infections. J Clin Virol. 2012;55(3):204-8. doi: 10.1016/j.jcv.2012.07.007. PubMed PMID: 22867730; PubMed Central PMCID: PMCPMC3445752.

13. Glaser C, Long SS. Encephalitis. In: Long SS, Pickering LK, Prober CG, editors. Principles and Practice of Pediatric Infectious Diseases. 4 ed. China: Elsevier; 2012.

14. Valassina M, Meacci F, Valensin PE, Cusi MG. Detection of neurotropic viruses circulating in Tuscany: the incisive role of Toscana virus. Journal of medical virology. 2000;60(1):86-90. PubMed PMID: 10568768.

15. Jeffery KJ, Read SJ, Peto TE, Mayon-White RT, Bangham CR. Diagnosis of viral infections of the central nervous system: clinical interpretation of PCR results. Lancet. 1997;349(9048):313-7. doi: 10.1016/S0140-6736(96)08107-X. PubMed PMID: 9024374.

16. Jiang H, Lei R, Ding SW, Zhu S. Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. BMC Bioinformatics. 2014;15:182. doi: 10.1186/1471-2105-15-182. PubMed PMID: 24925680; PubMed Central PMCID: PMCPMC4074385.

17. Schmieder R, Edwards R. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. 2011;6(3):e17288. doi: 10.1371/journal.pone.0017288. PubMed PMID: 21408061; PubMed Central PMCID: PMCPMC3052304.

18. Peng Y, Leung HC, Yiu SM, Chin FY. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics. 2012;28(11):1420-8. doi: 10.1093/bioinformatics/bts174. PubMed PMID: 22495754.

19. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinformatics. 2009;10:421. doi: 10.1186/1471-2105-10-421. PubMed PMID: 20003500; PubMed Central PMCID: PMCPMC2803857.

20. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28(12):1647-9. doi: 10.1093/bioinformatics/bts199. PubMed PMID: 22543367; PubMed Central PMCID: PMCPMC3371832.

21. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33(7):1870-4. doi: 10.1093/molbev/msw054. PubMed PMID: 27004904.

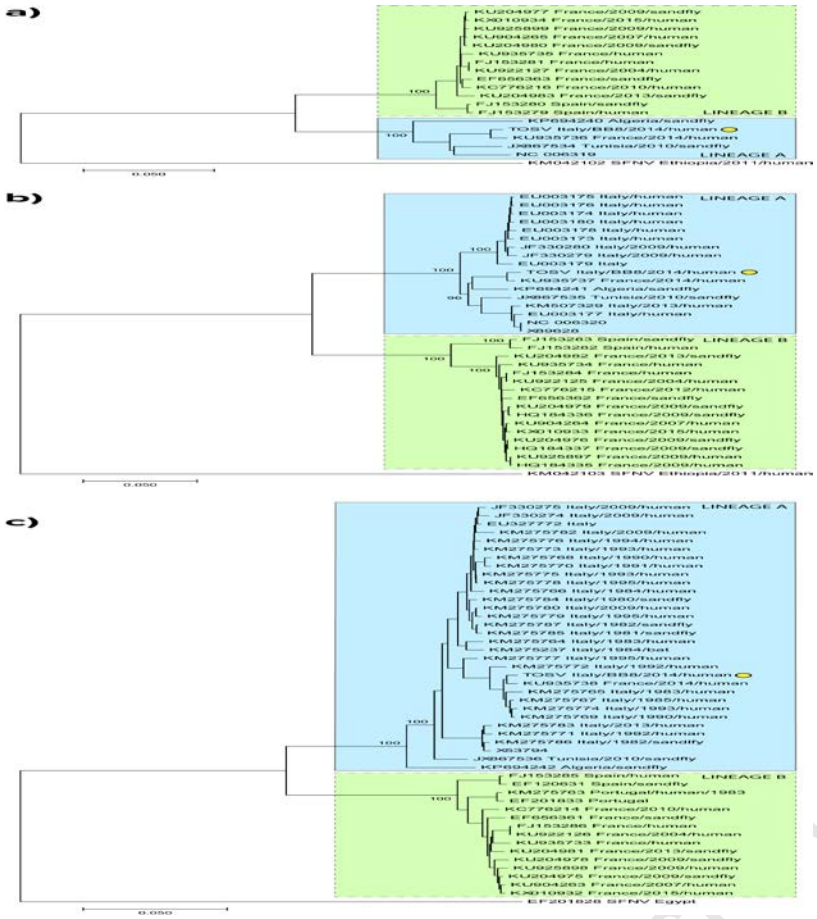
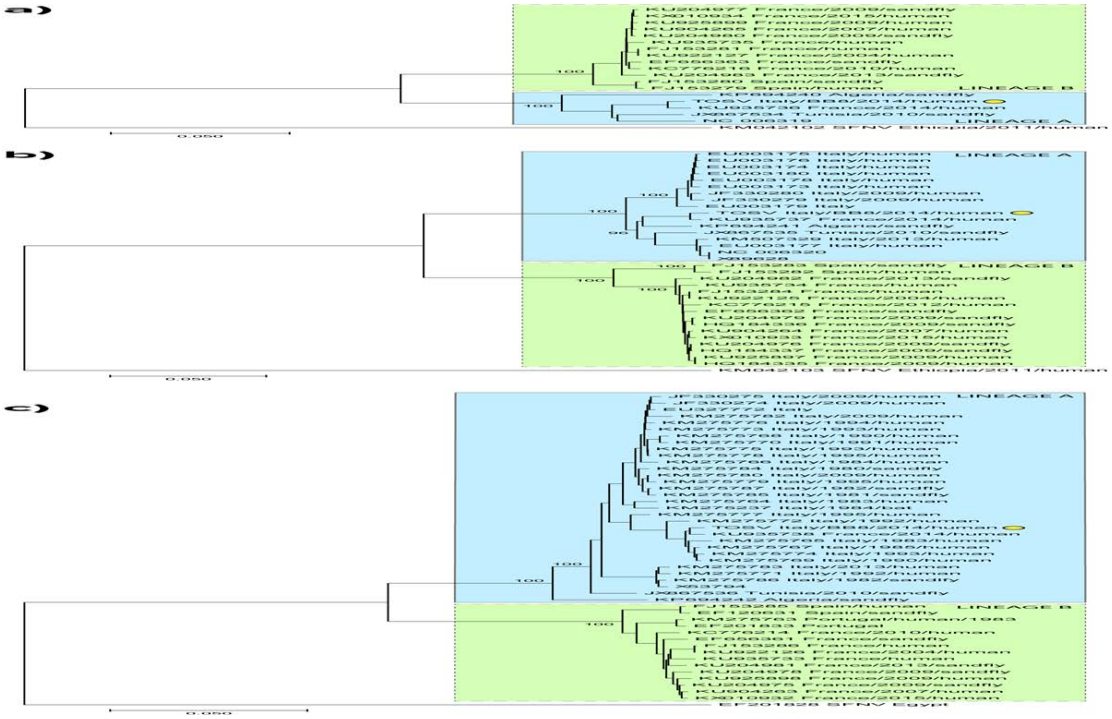


Figure 1



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