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Epidemiology, Isolation, and Genetic Characterization of Toscana Virus in Algerian Patients Displaying Neurological Infection, 2016–2018

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ABSTRACT

Purpose: The current study reports the results of the diagnosis of neuro-invasive Toscana virus (TOSV) infection in Algeria between 2016 and 2018 and describes the first isolation of TOSV strain from human samples in North Africa.

Material and methods: Cerebrospinal fluid (CSF) and sera samples were obtained from 720 hospitalized patients displaying neurological infection symptoms of unknown etiology, of which 604 were screened for TOSV. The diagnosis was performed by serological and/or RT-PCR tests. In addition, TOSV was isolated *in vivo* and *in vitro* from CSF and genetically characterized.

Results: 23 cases of TOSV neurological infections were detected. Cases were located in 11 Wilayas (administrative provinces), mainly in northern Algeria. In addition, we report the isolation of TOSV strain belonging to lineage A from human samples with its complete coding sequence.

Conclusion: Even though the number of infections is probably underestimated, TOSV is endemic in Algeria, with several cases of neuro-invasive diseases in humans recorded each year. Therefore, the diagnosis of TOSV should be included in the differential diagnosis of neurological diseases, especially aseptic meningitis, during the period of activity of the phlebotomine vector. Further studies are required to measure precisely the nationwide prevalence of TOSV in Algeria.

Introduction

Several viral pathogens can be responsible for neurological infections. The most common agents causing aseptic meningitis and encephalitis are enteroviruses (EV), herpes simplex virus (HSV), mumps virus (MuV), and neurotropic arboviruses, including West Nile virus (WNV) [1]. However, the etiology remains unknown in approximately 50% of cases [2]. In the Mediterranean basin, Toscana virus (TOSV) is one of the most common emerging viruses causing neurological infection during warm season [3]. TOSV (genus *Phlebovirus*, family *Phenuiviridae*) is an arthropod-borne enveloped RNA virus transmitted by sand flies belonging to the genus *Phlebotomus* [4]. In humans, TOSV infection is commonly asymptomatic or causes mild disease [5]. However, it can

sometimes cause neuro-invasive forms [6,7]. Diagnosis is performed by detecting (i) viral RNA in CSF, (ii) IgM antibody in serum at the acute stage of infection, or by (iii) demonstrating a seroconversion [6]. Viral isolation is not used as a standard diagnostic tool, but it is helpful for the genetic characterization of recovered strains.

Despite the fact that many studies have revealed the circulation of TOSV in sand flies in North African countries [3,8], its implication in viral neuro-invasive diseases remains largely unknown. Fezaa et al. have shown in a Tunisian cohort that 12.6% of patients with meningeal syndrome tested positive for anti-TOSV IgM [9]. Seroprevalence of TOSV in Tunisia stands around 8.5% [10]. Although there have been no reports of neuro-invasive infection caused by TOSV in other North African countries, some seroprevalence studies provided evidence of TOSV infection

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in humans and animals in Algeria [11,12]. One study revealed that over than 45% of the healthy population of the Kabylia region (northern Algeria) was positive for IgG anti-TOSV [11]. In another study, serum analysis showed that the seroprevalence of TOSV in Algerian canine population was 4.5% [12].

Molecular epidemiology studies have revealed the widespread circulation of three viral lineages in the Mediterranean basin [8,13]. Lineage A is common in Italy, France, and Tunisia [9,14], lineage B is present in France, Portugal, Spain, and Morocco [3,15], whereas lineage C is present in Croatia and Greece [16]. In Algeria, a strain belonging to lineage A was isolated in 2013 from sand flies [11].

This work aimed to describe the distribution of neuro-invasive TOSV cases in Algeria and attempt virus isolation along with a genetic characterization.

Materials and methods

Sample collection and case diagnosis strategy

Samples have been collected in the framework of the National Surveillance Program for WNV (Supplementary Table S1) from patients with neurological symptoms admitted to healthcare facilities and submitted for diagnosis to the National Reference Center for Arbovirus and Emerging Viruses (Institut Pasteur of Algeria).

CSF and serum were obtained from 720 patients between 2016 and 2018. Samples had been previously tested for EV, HSV, MuV, Measles virus (MeV), Rubella virus (RuV), and WNV. TOSV diagnosis was carried out on negative samples collected during the vector's activity period (01st April to 30th November) [8,14].

CSF and serum collected from each patient during the first week of the illness were tested for the presence of TOSV RNA and IgM anti-TOSV, respectively. A second serum was collected a week later for the detection of specific IgG.

Quantitative RT-PCR

Total viral RNA was extracted using Pure-link[®] viral RNA Kit (Invitrogen) from 200 μ L of CSF according to the manufacturer's instructions and tested by specific TOSV Real-Time Reverse Transcription PCR (RT-PCR) as previously described [17]. 5 μ L of total RNA was added to 20 μ L of a mix containing 12.5 μ L of 2X reaction buffer (Superscript[®] III one-step with Platinum[®]Taq polymerase) (ThermoFisher Scientific), 0.4 μ M of each primer, 0.2 μ M of probe, 0.5 μ L of SuperScript[®] III/Platinum[®]Taq Mix, and 4 μ L of deionized distilled water. The cycling program was 50°C for 30 min, 95°C for 2 min, and 40 cycles consisting of 15 seconds at 95°C and 1 min at 60°C, in Applied Biosystems[™] 7500 system (ThermoFisher Scientific).

Serology tests

Serological analyses to detect TOSV IgM and IgG were conducted using an Indirect ImmunoFluorescence Test (IIFT) (Sandfly-fever virus Mosaic, Euroimmun[®]) according to the manufacturer's instructions.

Geographic distribution

The geographical distribution of TOSV cases data was gathered and visualized using QGIS[®] software version 3.20.3-Odense (Open Source Geospatial Foundation Project. <http://qgis.osgeo.org>).

Virus isolation

Virus isolation in cell culture

Virus isolation was attempted on TOSV RNA-positive CSF using Porcine kidney Stable (PS) or African green monkey kidney (Vero) cells as previously described [11]. Cells were grown in 5.5 cm² Nunc[™] tubes.

At 70% confluence, cells were inoculated and incubated for 1 hour with 50 μ L of CSF and then washed with PBS. 3 mL of Leibovitz's medium (L-15) and Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% Fetal Bovine Serum (FBS) and 1% Peni-Streptomycine were added to PS, and Vero cells respectively. Daily observation was done to detect a cytopathogenic effect (CPE). On day 6, 300 μ L of supernatant was used for a blind passage in a 25 cm² flask. The same steps were repeated twice unless CPE was observed earlier. After centrifugation, the supernatant was tested by RT-PCR.

Virus isolation from neonatal murine brain

TOSV RNA-positive CSF showing cycle threshold values (Ct) lower than 30 were inoculated in newborn mice brains except for sample 3/16 due to its limited volume. One-day-old BALB/c mice were assigned to two experimental groups: 6 mice were used as control and inoculated intracerebrally (i.c) with 20 μ L of inoculation medium (L-15, 2% FBS, 1% Peni-Streptomycin), and 12 mice were inoculated i.c with 20 μ L of sample 3/18 diluted at 1:10².

Mice were monitored for neurological signs. A daily clinical score was assigned to each animal: 0: healthy, 1: partial limb paralysis, 2: limb paralysis with weight loss, 3: limb paralysis with weight loss and mild deterioration of health status, and 4: displaying severe deterioration of health status leading to euthanasia or death. Dead and sacrificed mice were conserved at -20°C. At day 10, animals were sacrificed, and brains were collected. The brains of each group were homogenized using MM200[®] mixer (Retsch, Germany). After centrifugation, supernatants were conserved at -80°C, and 500 μ L was used to detect isolated strain by RT-PCR.

Sequencing

TOSV strain passage 2 on Vero cells was selected for complete genome sequencing using Next Generation Sequencing (NGS). 200 μ L of culture supernatant was incubated at 37°C for 7h with 25 U of Benzonase (Novagen) and MgCl₂. RNA extraction was performed using Viral RNA Mini kit on the BioRobot EZ1-XL Advanced (Qiagen). RT-PCR was realized using tagged random primers, ProtoScript[®] II Reverse Transcriptase kit (New England Biolabs), specific primers, and Platinum[®] Taq High Fidelity polymerase enzyme (ThermoFisher Scientific). The purified PCR products were used for sequencing using the Ion PGM sequencer (Life Technologies). CLC genomics software v.21 (Qiagen) was used to process reads. Reads were mapped to the reference sequence (GenBank accession no. KP694240) for at least 50% of its length with a minimum of 80% identity.

Additionally, a partial genome sequencing of the NSs gene was performed directly on available positive CSF samples as described earlier [18].

Phylogenetic analysis

Segment S, M, L, and NSs gene sequences were blasted against the GenBank database and aligned with homologous sequences using Muscle software. A maximum likelihood tree was constructed using MEGA X [19,20]. For each tree, 500 bootstrap replicates were performed.

Statistical Analysis

Disease scores data are presented as mean \pm standard error of the mean (SEM) and were statistically analyzed using two-way analysis of variance (ANOVA) with Sidak post-hoc tests.

Results

TOSV infection diagnosis

From 2016 to 2018, 720 CSF samples were obtained from hospitalized patients displaying neurological symptoms. Among them,

Table 1

Classification of TOSV infection based on the detection of TOSV genomic RNA (RT-PCR) and anti-TOSV antibodies in the CSF and sera samples of the patients.

Neuro-invasive form		2016	2017	2018	Total	Age years, median (min-max)	Sex ratio (M/F)
Acute infection	RT-PCR+, IgM-	1	0	3	13	28 (7-60)	1.17
	RT-PCR+, IgM+ IgG+/-	3	3	3			
Recent infection	IgM+ IgG+/-	0	5	5	10	19.5 (5-72)	2.33
Total		23				25 (5-72)	1.55

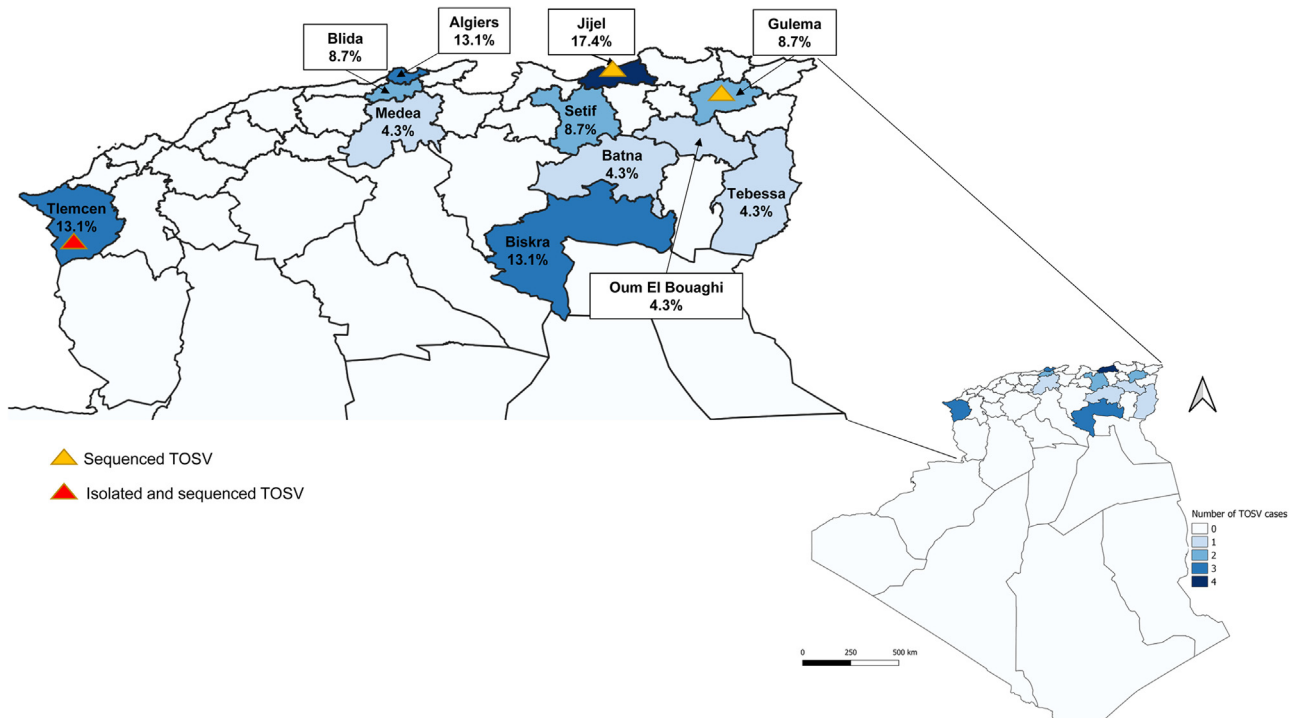


Figure 1. Map of Algeria showing the localization of TOSV human cases described in the current study.

604 samples were screened for TOSV while 116 CSF were not included in the study: 43 were diagnosed out of arboviral activity period, 66 tested positive for EV/HSV/MeV/MuV/RuV, and seven were positive for WNV. TOSV infection was confirmed in 23 out of 604 patients (3.8 %) (Supplementary table S2) with a sex ratio of 1.55 (M/F). The ages ranged from 5 to 72 years, with a median age of 25 (Table 1).

Out of the 23 confirmed cases, 13 (56.52%) were in the acute phase of the disease (four were TOSV RNA-positive and negative for IgM, while nine tested positive for both TOSV RNA and anti-TOSV antibodies). 10 patients (43.47%) presented recent TOSV infection with only positive IgM (Table 1). Furthermore, 13 CSF samples collected 2 to 4 days after the onset of symptoms showed a Ct value that ranged from 26.85 to 36.92 (cut off ≤ 37) (Supplementary table S3). Samples collected seven days after the illness onset were negative for TOSV RNA. The IIFT showed a seroreactivity for both SFNV and TOSV in all sera, except for two samples, which were only positive for TOSV. However, the fluorescence intensity was higher for TOSV (Supplementary figure S1).

Geographical distribution of TOSV cases

The 23 TOSV cases were detected in 11 Wilayas (administrative provinces), mainly restricted to northern Algeria, occurring in the arid and humid bioclimate stages. The region with the highest percentage of cases was Jijel (17.4%), followed by Tlemcen, Algiers, and Biskra

(13.1% each), Guelma, Blida, and Setif (8.7% each), Medea, Batna, Tebessa and Oum El Bouaghi (4.3% each) (Figure 1).

TOSV strain isolation

Two samples (3/16 and 3/18) yielded positive viral cell cultures among the 13 CSF that tested positive for TOSV RNA. Both CSF samples had initial Ct values <30 (Supplementary table S3). The inoculated Vero and PS cells, respectively, showed a CPE at 4 and 5 days post-inoculation of the second passage (Figure 2A-D). RT-PCR results showed more efficient replication of TOSV in Vero with a Ct value of 10 and low viral load in PS with a Ct value of 33.98, which became undetectable after the 3rd passage suggesting the lack of active replication (Figure 2E).

In addition to cell culture, sample 3/18 was i.c. inoculated to newborn mice. The control group (n=6) maintained optimal growth and remained asymptomatic (Figure 3A, B). The lethality was 33% (2/6), with two deaths occurring on day 2 and 3 post-inoculation (Figure 3C). On the other hand, mice inoculated with sample 3/18 (n=12) displayed a progressive increase in the clinical sickness score from 1 to 4. Few mice (3/12) began to show weakness and partial limb paralysis on day 4. At the end of the experiment, mice were scored between 2 and 4. All mice showed limb paralysis with weight loss, among which 25% (3/12) displayed a mild deterioration of health status (Figure 3A,B). The limb paralysis with weight loss and severe deterioration of health status led to death in 50% of the mice (6/12) (Figure 3C). RT-PCR showed an efficient replication of TOSV in mice brains with a Ct value of 16.19 (Figure 3D).

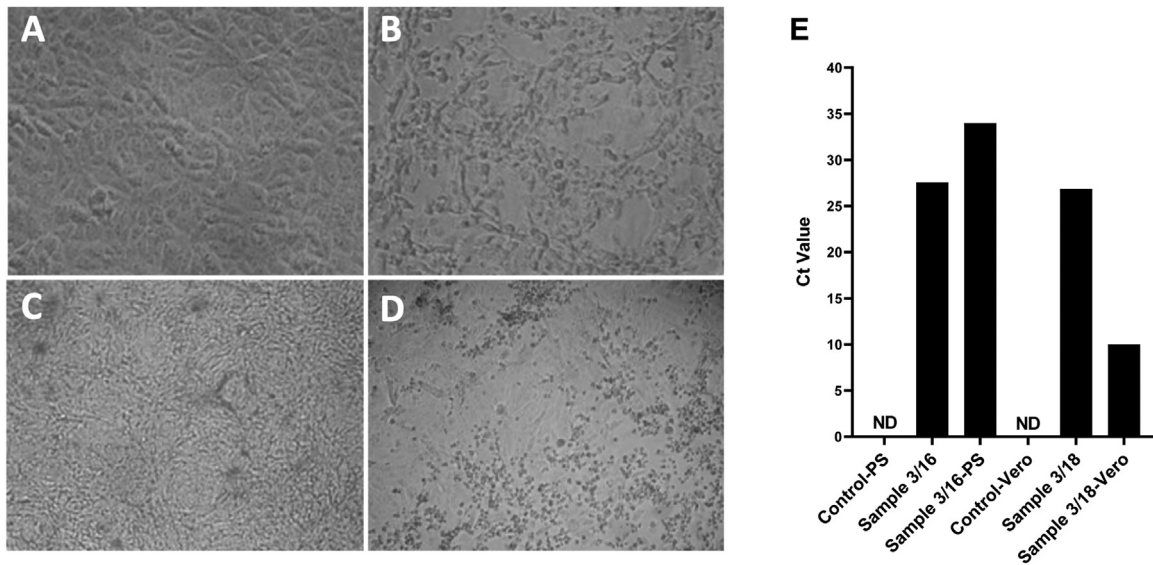


Figure 2. Cytopathic effects (CPE) in PS and Vero cells inoculated with positive TOSV CSF (Ct value<30). [A] PS control cells. [B] CPE in PS cells, five days post-inoculation with sample 3/16. [C] Vero control cells. [D] CPE in Vero cells, four days post-inoculation with sample 3/18. Cytopathic effects are displayed as clusters of small, rounded detached cells. [E] Ct value in CSF (Sample 3/16 and Sample 3/18) and inoculated PS and Vero cells.

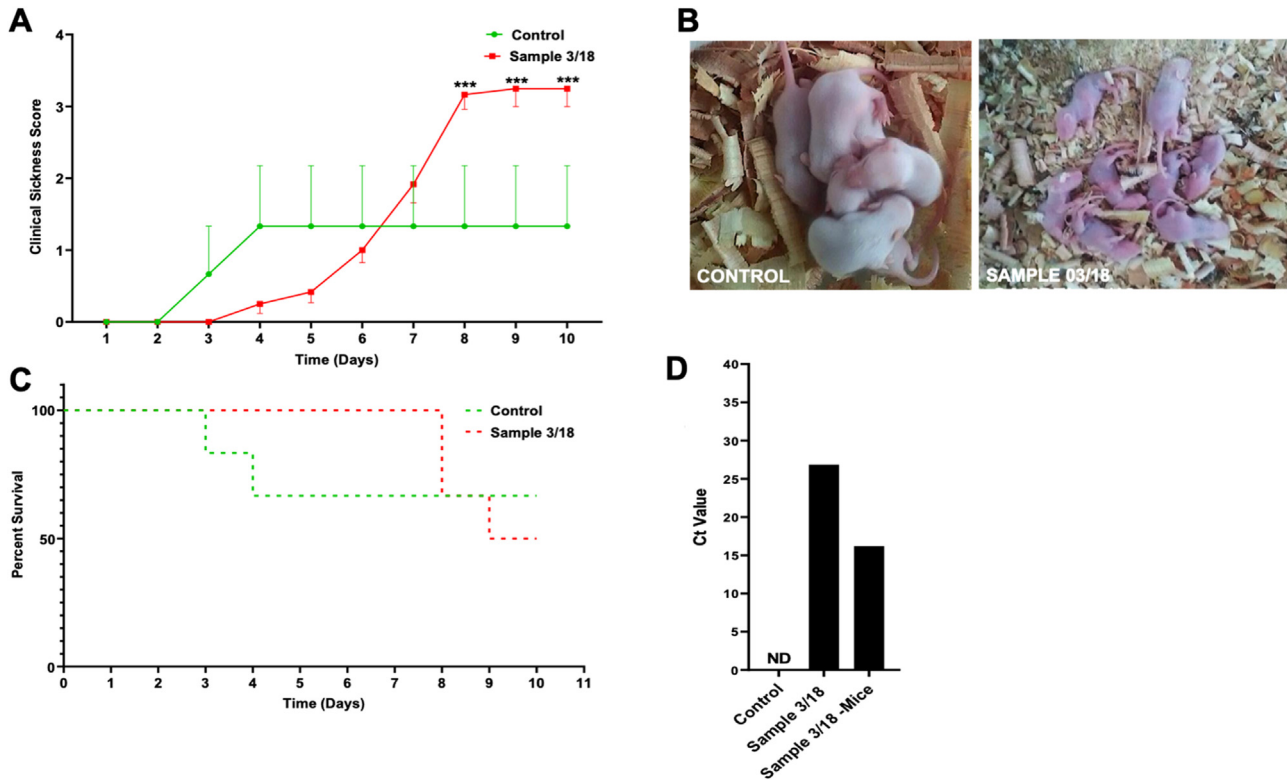


Figure 3. (A) Clinical sickness score of mice inoculated with sample 3/18 (red) compared to control mice (green). The clinical sickness scores ranged from 0 to 4: 0, healthy; 1, partial limb paralysis; 2, limb paralysis with weight loss; 3, limb paralysis with weight loss and mild deterioration of health status; 4, severe deterioration of health status leading to euthanasia or death. (B) Relative changes in body weight of neonatal mice inoculated with sample 3/18 and control groups at day 7 post-infection (C). Survival curve of mice inoculated with sample 3/18 (red), compared to control mice (green). (D) Efficiency of TOSV replication in neonatal mice brains assessed by RT-PCR. Ct values were obtained from mice inoculated with samples 3/16 and 3/18.

TOSV strain sequencing

Whole genome sequencing of the isolated TOSV strain confirmed the identity with 1786 nts for segment S (GenBank accession no. ON165939), 4215 nts for segment M (GenBank accession no. ON165938), and 6371 nts for segment L (GenBank accession no. ON165937).

The phylogenetic analysis of the 3 sequences revealed that the isolated strain belonged to lineage A (Figure 4) and was grouped with previous sequences of Algeria, Tunisia, and Italy. Interestingly, the analysis of each segment revealed a high degree of genetic similarity with a previously identified Algerian strain [11], with 100% identity for segment L and 97.2% for segment M. In contrast, the S segment displayed a lower similarity (90.8%).

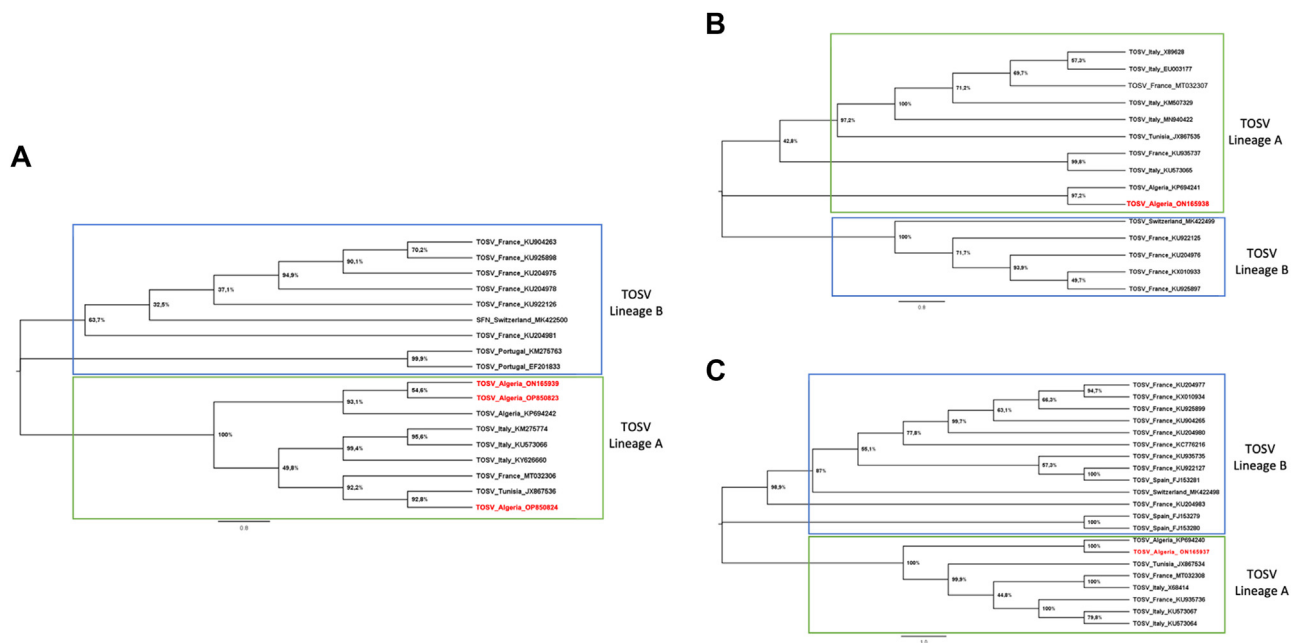


Figure 4. Phylogenetic tree analysis of the three segments of Toscana virus (TOSV) isolated in Algeria from Vero cell culture. NSs genes from CSF samples and homologous sequences of other TOSV described in the literature. (A) Small segment (S) and NSs genes; (B) Medium segment (M); (C) Large segment (L). Green: TOSV Lineage A; Bleu: TOSV Lineage B.

The partial NSs sequences obtained from samples 2/16 and 3/16, with respectively, 492 nts (GenBank accession no. OP850823) and 894 nts (GenBank accession no. OP850824), were blasted and compared to the previous GenBank sequences of the S segment.

The phylogenetic analysis showed that the two strains belong to lineage A (Figure 4). Interestingly, the strain (GenBank accession no. OP850824) was most closely related to the Tunisian strain, while the strain (GenBank accession no. OP850823) was highly similar to the previously identified strains in Algeria [11] and the strain isolated in this study.

Discussion

TOSV is one of the main endemic viral agents involved in neuro-invasive human infections during the summer season in the Mediterranean area [3,13,21]. In recent years, studies have demonstrated an increasing number of reported cases [13,22,23].

In our study, TOSV infection was confirmed in 3.8% of patients with neurological disorders, particularly those presenting meningitis (Supplementary table S4). In comparison, a previous study showed a higher seroprevalence (>45%) in a northern region in Algeria [11]. In reality, the diagnosis of TOSV infection is undoubtedly underestimated and limited to a few laboratories [24]. One of the primary reasons is that the infection is mostly asymptomatic [5], leading to unreported cases. While diagnosing TOSV mild infection is not critical as it is often self-resolutive, it is crucial for the neuro-invasive forms.

We observed a higher proportion of TOSV infections among adults than among children (Supplementary table S4), with a median age of 25 years, which contrasts with a previous study describing a higher rate of asymptomatic TOSV infection in young people (91%) [21]. Furthermore, infection was important among males compared to females (Sex Ratio M/F 1.55) because they are likely to be more involved in evening outdoor activities during sand fly activity [25].

In line with previous reports, our data revealed that TOSV RNA and IgM antibodies were present in patients within 2 to 4 days after the onset of neurological symptoms [26]. On the other hand, 10 out of 23 patients tested positive for TOSV IgM and/or IgG, while the virus RNA

was not detected in their CSF. It is important to highlight that some phleboviruses present a significant homology with TOSV, resulting in a cross-reactivity with TOSV antigens in the IIFI test [3]. Indeed, several sand fly fever viruses, such as Naples, Sicilian [3,8,9], and Punique [3], have been detected in North Africa. It is worth noting that Sand fly Naples Fever and Sand fly Sicilian Fever viral infections are associated with fever, headache, malaise, photophobia, myalgia, and retro-orbital pain [8], whereas there is no available data on the pathogenicity of Punique virus.

Previous studies investigating TOSV circulation in Algeria focused exclusively on seroprevalence and virus detection in sand flies [11,12]. As far as we know, this is the first study addressing TOSV neuro-invasive disease in Algeria.

The geographical distribution of TOSV neuro-invasive cases showed localization in northern Algeria. As in other studies conducted in the Maghreb, most patients were living in coastal provinces [13,10]. Interestingly, TOSV cases were also identified in inland Wilayas known for the high activity of sand flies and where TOSV-specific antibodies were detected in dogs [27,12]. Among these Wilayas, two are bordering Tunisia and Morocco, where TOSV circulation was described [15,10]. Interestingly, here, no TOSV cases were detected in the region of Kabylia despite a previously reported high seroprevalence [11]. This highlights the underestimated number of reported cases, likely due to the limited access to hospitals in some areas and the lack of public awareness about neurotropic arboviruses. We can also hypothesize that the high seroprevalence rate in this region may confer natural immunity and prevent severe cases.

Given the low viral load in CSF and the lack of a standardized protocol for phlebovirus isolation, we decided to use two cell lines and i.c inoculation in neonatal mice brains for TOSV isolation. We successfully isolated TOSV from Vero and mice, conversely to the PS cells, which did not result in a significant CPE after the 3rd passage.

Our *In vivo* experiment showed that the TOSV strain isolated from CSF could efficiently replicate in newborn mice brains leading to fatal neurological manifestations. These data are in line with a previous study that established a mouse model of TOSV infection [28] and demonstrated that neurons are the target cells in TOSV i.c infected mice. However, it is worth noting that in contrast with the strain described by

Grazia Cusi et al. [28], our strain did not require prior passaging to induce a neurological pathology in newborn mice.

To our knowledge, this study is the first to isolate and characterize the TOSV strain from human samples in North Africa. The strain recovered from CSF of a patient living in Wilaya of Tlemcen was genetically close to the strain isolated from sand flies collected in the region of Kabylia [11], which is 595 km away. This strain belonged to lineage A together with those originating from Italy and Tunisia [9,14]. Interestingly, this Wilaya borders Morocco, where the circulation of lineage B has been reported [15]. This suggests that the co-circulation of different genetic lineages previously described in southern France and in Turkey might be more common than previously believed [29,30].

In conclusion, our data clearly show that TOSV is an important etiologic agent of neuro-invasive disease in Algeria and should be included in the differential diagnosis of neurological diseases, especially aseptic meningitis during vector activity season.

Conflict of interest

None of the authors have any conflict of interest to disclose.

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Ethical Approval statement

Patient samples were submitted for diagnosis of arbovirus infection to the National Reference Center for Arbovirus and Emerging Viruses, Virology department (Institut Pasteur of Algeria) in frame of surveillance program of Algerian Health Ministry. Hence, informed consent was not necessary as patients with suspected viral neuro-invasive disorders were included in a national diagnostic protocol.

The protocol of virus isolation from neonatal murine brains was approved by the biosafety and biosecurity committee of Institut Pasteur of Algeria. All experimental procedures were conducted in a level 2 biosafety facility by respecting the World Organization for Animal Health regulation (https://www.oie.int/fileadmin/Home/fr/Health_standards/tahc/current/chapitre_aw_research_education.pdf) (accessed on 10 January 2021).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2023.03.012.

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