

## Rapid Identification of Toscana Virus by Nested PCR during an Outbreak in the Siena Area of Italy

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**The sand fly-transmitted Toscana virus is recognized as an etiologic agent of an aseptic meningitis with a long convalescence. This infection has been reported overall in many tourists or in a seronegative population circulating in endemic Mediterranean areas (Italy, Portugal, Egypt, and Cyprus). We report a cluster of acute Toscana virus infections in the local population during the summer of 1995. Twenty-one clinical cases of meningitis were investigated for the presence of Toscana virus by nested PCR performed on the S segment of the virus RNA extracted from cerebrospinal fluid samples.**

Sandfly fever viruses are transmitted in endemic areas by the sandfly (*Phlebotomus* spp.) and can cause headache, myalgia, ocular symptoms, and fever. These viruses belong to the *Bunyaviridae* family, genus *Phlebovirus*. The enveloped virions have a segmented RNA genome consisting of three noncovalently closed, circular RNA species, designed small (S), medium (M), and large (L) (1, 2, 13). The genus *Phlebovirus* is composed of 38 different viruses isolated throughout the world (19, 22, 23). Four viruses in this genus are known to cause serious human diseases: the Rift Valley fever virus, the sandfly fever Naples (SFN) virus, the Sandfly fever Sicilian (SFS) virus, and the Toscana (TOS) virus. SFN, SFS, and TOS viruses are spread in the Mediterranean area. While SFN and SFS viruses are responsible for mild illness, the TOS virus is associated with cases of human aseptic meningitis or meningoencephalitis, which is more severe in adults (15, 21). This neuropathic infection is more frequent during summer, with a peak in August, because of the life cycle of the insect vectors (*Phlebotomus perniciosus* and *Phlebotomus perfliewi*) (6, 20) and because of increased tourism in endemic areas (3, 8, 9, 17). In this article, we report data from an outbreak of TOS virus during the summer of 1995 in the Siena area and evaluate a new nested-PCR assay for detection of TOS virus. PCR results are compared with tissue culture and serological data by immunofluorescence assaying (IFA) of sera drawn at the time of the acute phase of the illness.

### MATERIALS AND METHODS

**Patients.** From June to August 1995, 21 patients were hospitalized with neurological disease (severe frontal headache, vomiting, ocular pain, myalgia, and neck rigidity) which was described as aseptic meningitis or meningoencephalitis. All of the patients observed were adults or younger adults (15 to 58 years old; mean age, 28.1; 8 females and 13 males) residing in the Siena area of Tuscany, Italy.

**Clinical samples.** Blood and cerebrospinal fluid (CSF) were drawn from the patients at the time of hospitalization.

**Cell culture.** Vero cells (ATCC CCL 81) were used for virus propagation and were maintained in Eagle's minimal essential medium (Life Technologies, Milan, Italy) supplemented with 5% fetal calf serum (Life Technologies) and penicillin (200 U/ml)-streptomycin (200 µg/ml) (Sigma Co.). The reference strain of TOS virus was kindly provided by C. Giorgi (Istituto Superiore di Sanità, Rome, Italy). All of the CSF specimens were inoculated once in Vero cells at 37°C for 60 min, and these were then incubated at 37°C in Eagle's minimal essential medium. The

virus was harvested at the appearance of a lytic cytopathic effect on cell culture, which was confirmed by hemadsorption with goose erythrocytes. The negative cell cultures were maintained for 14 days after the supernatant was harvested and blind passaged on cells.

**Serological test.** An IFA was performed to detect anti-TOS virus immunoglobulin M (IgM) and IgG according to a procedure described elsewhere (24). Briefly, cells infected with the isolated TOS virus were harvested and collected on spot slides (10<sup>4</sup> cells per spot); uninfected cells were collected in the same manner as that used for the negative control. The slides were air dried and fixed in cold acetone for 10 min. When the slides were not immediately used, they were stored at -70°C. For detection of anti-TOS IgM, all of the sera were previously incubated with rheumatoid factor absorbents at a dilution of 1:5 (affinity-purified IgG-rheumatoid factor; The Binding Site, Birmingham, United Kingdom). The solution was mixed for 30 min at room temperature and was centrifuged to remove the immune complexes. The sera were then diluted 1/40 and screened for IgM; a negative control serum (drawn from a healthy baby) and a positive control serum (kindly provided by C. Giorgi) were included in each assay. The specimens were incubated for 90 min at 37°C and, after being washed with phosphate-buffered saline for 10 min at room temperature, fluorescein isothiocyanate-conjugated anti-human IgM (Argene Biosoft, Varillhes, France) was added, and the mixtures were incubated for 30 min at 37°C. Positive sera, which were determined by the presence of typical intracytoplasmatic fluorescence, were further diluted from 1:40 to 1:1,280 and were tested to evaluate the endpoint titers. The same procedure was followed for the detection of anti-TOS virus IgG, except for the use of the rheumatoid factor absorbents. A fluorescein isothiocyanate-conjugated anti-human IgG (Argene Biosoft) was used.

**RNA extraction.** A 200-µl aliquot of each CSF sample was vortexed with 600 µl of extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 2% Sarkosyl, 0.1 M mercaptoethanol, 2 µg of tRNA). Then, 50 µl of 2 M sodium acetate (pH 4.0), 400 µl of water-saturated phenol, and 100 µl of a chloroform-isoamyl alcohol mixture (49:1) were added (5). The mixture was vortexed and placed on ice. After 10 min, the mixture was centrifuged at 10,000 × g for 15 min and the aqueous phase was collected. RNA was precipitated by the addition of 2.5 volumes of cold ethanol, and subsequently the pellet was washed with 70% cold ethanol. The RNA was resuspended in 5 µl of sterile diethylpyrocarbonate-treated water and was analyzed by reverse transcriptase-PCR (RT-PCR). This procedure was followed for RNA extraction from cell cultures infected by the virus, starting from 2 × 10<sup>6</sup> cells and without the addition of tRNA.

**cDNA synthesis and PCR.** The following primers (Genset) were used for the detection of TOS virus: sense primer TV1 (5'-CCAGAGGCCATGATGAAGA AGAT-3'; N gene; nucleotides [nt] 256 to 278) and antisense primer TV2 (5'-CCACTCTATGAGCAGCTTCT-3'; N gene; nt 721 to 701) (12). The cDNA was synthesized in a 25-µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM MgCl<sub>2</sub>, 1 mM deoxyribonucleoside triphosphate (dNTP) mixture, 20 U of RNase inhibitor (Boehringer, Mannheim, Germany), 40 U of Moloney murine leukemia virus reverse transcriptase (Boehringer), and the anti-sense primer (0.25 µM). The mixture was incubated at 37°C for 30 min and then denatured for 3 min at 94°C and cooled quickly on ice. The PCR amplification was carried out in a 100-µl volume containing 25 µl of the cDNA product, 10 µl of the PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 25 mM MgCl<sub>2</sub>), 20 pmol of each primer, a 0.125 mM dNTP mixture, and 2 U of *Taq* DNA polymerase (Boehringer). The samples were subjected to 35 cycles of thermal cycling for 1 min at 94°C, 30 s at 56°C, and 30 s at 72°C. Then, a nested PCR was performed on 5 µl of the amplified product with inner primers TV3 (5'-AACCTGATTTCAGTCTACCAGTT-3'; N gene [nt 308 to 330]) and TV4

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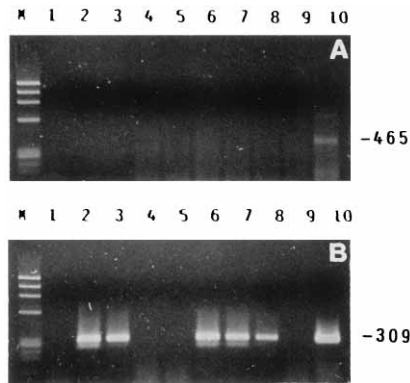


FIG. 1. Visualization by gel electrophoresis of representative data from RT-PCR (A) and nested PCR (B) products. (A) Lanes: 1, negative control; 2 to 9, RT-PCR performed on some CSF samples for TOS virus detection; 10, positive-control TOS virus; (B) lanes: 1 to 10, nested PCR performed on the same samples. M, molecular weight standard ( $\Phi$ x174 digested with *Hae*III).

(5'-TTGTTCTCAGAGATGGATTATG-3'; N gene [nt 617 to 595]) (12). The samples were subjected to further 30 cycles for 45 s at 94°C, for 25 s at 58°C, and for 30 s at 72°C. PCR was carried out according to the recommended guidelines, including three negative controls and one positive control in each assay. PCR products were analyzed by electrophoresis on a 3% agarose gel and were visualized by staining of the gel in a 1- $\mu$ g/ml solution of ethidium bromide.

## RESULTS

The patients with neurological symptoms were hospitalized, and CSF and blood samples were drawn in the early phase of the acute illness in order to detect neurotropic viruses. The laboratory data relating to the samples from all of these patients showed CSF cell counts of  $>10^3/\text{mm}^3$  and protein amounts of  $>50$  mg/dl, the blood samples showed glucose concentrations of  $>60\%$ . The clinical picture associated the epidemiological data with the summer season and gave a diagnosis of TOS virus. All of the CSF specimens were stored in duplicate; 500  $\mu$ l was used for cell culturing and 200  $\mu$ l was used for the PCR assay. In two cases, it was possible to isolate TOS virus in tissue culture 3 days postinoculation. Another sample gave a positive result in tissue culture only after a blind passage on Vero cells. A 200- $\mu$ l volume of the remaining aliquot of CSF samples was used for RNA extraction in order to detect the TOS virus genome by RT-PCR. The results for 13 of 21 cases were positive by nested PCR with the TV3 and TV4 primers, as shown in Fig. 1. A 309-bp fragment was amplified on the S RNA segment of the TOS virus (12). No positive results were detected by performing only a single RT-PCR on the CSF samples. RNA extracted from cell cultures infected by the three isolates, in contrast, gave positive results by RT-PCR. We tested the sera of the patients collected at the time of hospitalization by an IFA procedure for the presence of anti-TOS IgM. The presence of IgM antibodies was assumed to

indicate a recent infection and confirmed the diagnosis (15). Serum drawn 2 weeks after the onset of illness was not available. A total of 9 of 21 tested serum samples gave positive IgM results, with a titer range from 1:40 to 1:160; among these, all but one confirmed the positive results obtained by nested PCR (Table 1). The only sample that was IgM positive, with an IgG titer corresponding to 1:1,280, was drawn from a patient who did not reveal the presence of the TOS virus genome. Four IgM-seronegative subjects were positive for the presence of the viral genome (Table 1). We included in the study a control group of 20 healthy individuals for anti-TOS IgM screening; none of these was positive. The patients' sera were also tested for the presence of anti-TOS IgG antibodies. A total of 17 of 21 serum samples were positive, with IgG titers ranging from 1:40 to 1:640. The samples (13 of 21) with IgG titers higher than 1:80 were those that also gave positive results by nested PCR. The anti-TOS IgG titers in the control group ranged from 1:40 to 1:160 in 8 cases; the remaining 12 serum samples gave negative results (titers of  $\leq 1:40$ ).

## DISCUSSION

Most major outbreaks of TOS virus, as well as of SFN or SFS virus, have occurred when a large nonimmune population moves into an endemic area. Under these circumstances, TOS virus can cause severe morbidity; less clear is the importance of TOS virus in the native population of the endemic region (10). Studies have indicated that when infection is acquired during infancy, the clinical manifestations are mild or subclinical, except in a few cases that have been described elsewhere (7). In the present study, we report 21 cases of meningitis discovered during the summer of 1995 in the local population. Clinical infections are often associated with travel-related virus infection of a tourist or military population while staying in endemic areas (middle Italy, Portugal, Egypt, Spain, and Cyprus) (3, 8, 9, 11, 17). Clinical findings include neurological symptoms in adults, even if TOS virus infection could occur without signs of disease, as reported in a few serological studies in which anti-TOS-specific IgM antibodies were detected in the control group (15, 18). The detection of TOS virus by nested PCR has been very useful, and this technique (14, 16, 25) shows a level of sensitivity and specificity higher than those of tissue culture and the IFA IgM test. The only discrepant result, i.e., the patient who was negative by nested PCR of CSF and positive for IgM and IgG by IFA, was explained by the fact that the CSF and the serum were collected 6 days after the onset of illness. This result is in accordance with the observation that other samples drawn from patients who had been positive at the onset of illness became negative for the presence of the TOS genome 1 week after the appearance of symptoms. The RT-PCR assay not associated with the nested PCR did not appear to be a valid method to detect the TOS virus, since no sample was positive by the first reaction. This fact could be due to the very low amount of virus in CSF samples; therefore, it

TABLE 1. Correlation of results obtained by direct and indirect assays

No. of samples	RT-PCR	Nested PCR	Tissue culture	IgM	IgG (titer)
3	Negative	Positive	Positive	Positive	Positive (1:80–1:640)
5	Negative	Positive	Negative	Positive	Positive (1:80–1:640)
1	Negative	Negative	Negative	Positive	Positive (1:1,280)
3	Negative	Negative	Negative	Negative	Positive (1:40–1:160)
4	Negative	Negative	Negative	Negative	Negative ( $<1:40$ )
5	Negative	Positive	Negative	Negative	Positive (1:80–1:320)

appears evident that only a second step of amplification allows detection of the virus genome. The PCR product was identified as a fragment of the TOS virus genome by sequencing (data not shown) and by comparing it with the genome of a reference strain of TOS virus (12). Even if the serological data are not complete and a second serum sample is not available for any patient, IgM serology still represents a valid backup to nested PCR; in fact, it was possible to diagnose infection by TOS virus in one patient who was negative for the presence of the virus but who had specific anti-TOS IgM at a high titer. It is important to test samples for the presence of the virus at the onset of symptoms, since the viremic phase is very short after infection and specimens drawn later could give negative results. This fact could explain why we failed to detect TOS virus in the blood and serum specimens. Since the actual rate of successful isolation of TOS virus from CSF is rather low, the nested PCR provides an efficient tool to detect this virus. Studies are now in progress to characterize the TOS virus strains circulating in the same area. The diagnostic approach described in this report could also be very useful for clinical and epidemiological studies.

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