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Laboratory Diagnosis of Toscana Virus Infection by Enzyme Immunoassay with Recombinant Viral Nucleoprotein

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A recombinant enzyme immunoassay (rEIA) to detect serum immunoglobulin M (IgM) and IgG to Toscana virus (TOSV) was developed with the aim of establishing a simple and easily available assay for diagnosing acute and/or previous infections. The rEIA, based on the recombinant nucleoprotein of TOSV expressed in *Escherichia coli*, was evaluated with 97 serum samples collected in an area where TOSV is endemic and compared to an analogous assay based on cell-derived TOSV. Discordant results were resolved by immunoblotting (IB). Twenty-two of these samples, obtained from subjects hospitalized during the summer season with meningitis of suspected TOSV etiology, were further characterized by indirect immunofluorescence and IB, and detection of specific TOSV RNA sequences in the cerebrospinal fluid of these patients was attempted by nested PCR. The results indicated that rEIA was able to diagnose acute TOSV infection by detection of specific serum IgM in all of the subjects with TOSV meningitis confirmed by nested PCR or serology. The overall sensitivity and specificity of rEIA were both 100% for IgM detection and 100 and 96.6%, respectively, for IgG detection. Thus, rEIA appears to be a simple and reliable laboratory test for the diagnosis of acute TOSV infection and for the assessment of immune status.

Sandfly fever viruses (SFV) (genus *Phlebovirus* in the *Bunyaviridae* family) comprise three serotypes: Sicilian (SFVS), Naples (SFVN), and Toscana (TOSV) (9); these viruses cause human illness and are transmitted by the sandfly (*Phlebotomus* spp.) in the Mediterranean region (17). While SFVS and SFVN infection are usually self-limited diseases characterized by fever, myalgia, and headache (1, 8), TOSV infection is known to cause meningitis and meningoencephalitis (11, 15). TOSV is endemic in the Mediterranean area, as indicated by studies of residents (2, 3, 7, 11, 19) and by case reports of infection among tourists (4, 5, 6, 12, 14, 15). In endemic regions TOSV infection may account for a high proportion of human aseptic meningitis during the summer months (3). Various diagnostic methods have been used for the laboratory diagnosis of TOSV infection, including virus isolation from cerebrospinal fluid (CSF) (11), amplification of viral RNA sequences from CSF (13, 19), indirect immunofluorescence assay (IIFA) (14), plaque reduction neutralization test (6), enzyme immunoassay (EIA) (14), and immunoblotting (IB) (16).

On the whole, these methods are technically demanding or time-consuming and are restricted to specialized laboratories because of the need to propagate infectious virus in cell culture. Here we describe an EIA that is based on the recombinant nucleoprotein (rN) of TOSV that can be used for the laboratory diagnosis of TOSV infection. The advantages of this assay appear to be its simplicity, its safety of use, and the possibility of standardization, in addition to good sensitivity and specificity.

MATERIALS AND METHODS

Virus and cell culture. Vero cells (ATCC CCL81) were cultivated in Eagles' minimal essential medium (MEM) (Life Technologies, Milan, Italy) supplemented with 5% fetal bovine serum (Life Technologies) and penicillin-streptomycin (100 U/ml and 100 μg/ml) (Life Technologies). TOSV strain 1812 was isolated from the CSF of a patient with aseptic meningitis (19) and was propagated in Vero cells in MEM supplemented with 2% fetal bovine serum and penicillin-streptomycin. To prepare TOSV antigen for EIA, confluent monolayers of Vero cells in 850-cm roller bottles (Corning-Costar, Concorezzo, Italy) were infected with TOSV at a multiplicity of infection of 1 50% tissue culture infective dose per cell and incubated until a complete cytopathic effect was evident. Cells and culture medium were frozen and thawed three times and centrifuged at low speed to remove debris. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C; the virus pellet was then resuspended in TNE buffer (10 mM Tris, pH 7.4; 150 mM NaCl, 1 mM EDTA), loaded on a sucrose gradient made of two layers that contained 3 ml each of 20% (wt/wt) and 60% (wt/wt) sucrose in TNE buffer, and centrifuged at 100,000 ×*g* for 3 h at 4°C. The virus band was collected from the interface between the two sucrose layers, diluted with TNE, and centrifuged at 100,000 × *g* for 90 min at 4°C. The sedimented virus was resuspended in TNE and stored at −80°C until use.

Recombinant nucleoprotein. *Escherichia coli* BL21 (λDE3), which contained the pDSTV-1 plasmid derived from pET15b (Novagen, Madison, Wis.) by insertion of the cDNA coding for the nucleoprotein of TOSV in frame with a 25-amino-acid sequence containing a polyhistidine tag (20), were cultivated in Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) supplemented with 100 μg of ampicillin (Sigma-Aldrich, Milan, Italy) per ml. Cultures were induced to produce rN by the addition of 1 mM isopropyl-β-thiogalactopyranoside. After 2 h at 37°C, the culture was centrifuged and the bacterial cells were resuspended in phosphate buffer (50 mM phosphate, pH 8.0; 300 mM NaCl) and lysed by ultrasonication. After clarification at 10,000 × *g*, the supernatant containing the soluble rN was applied to an Ni-NTA column (Qiagen, Hilden, Germany), and the adsorbed protein was eluted by a 0 to 0.5% imidazole gradient. The purified rN was biotinylated for use in the μ-capture EIA for IgM by using *N*-hydroxysuccinimidobiotin in 0.1 M carbonate buffer (pH 8.7) with a 1:10 weight ratio with respect to the protein content.

Serum panels. Three groups of sera were used during this study. The first was a group of 22 sera from patients resident in the Siena area of Tuscany, Italy, and hospitalized with neurological symptoms of meningitis during an outbreak of TOSV infection in the summer season (group I). These sera were evaluated for the presence of TOSV-specific IgM and IgG by EIA with cell-derived virus and rN, IIFA, and IB. CSF from these patients was tested for the presence of TOSV RNA sequences by nested PCR as described previously (19). In group II were 22 sera from children aged 1 to 5 years without any neurologic symptoms; these sera were obtained during the winter season. Finally, 53 unselected sera (group III) from routine viral diagnosis were obtained from people living in the Tuscany region of Italy. Group II and III sera were used to estimate the frequency of IgG and IgM anti-TOSV. Sera of groups II and III were evaluated by EIA with cell-derived virus and rN, and discordant results were resolved by IB.

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TABLE 1. Comparison of nested PCR, rEIA, and other serological methods for detecting TOSV infection in group I sera[a]

Serum sample	PCR	TOSV detection results							
		IgM				IgG			
		IIFA	TOSV-EIA (OD_{450})	IB	rEIA (OD_{450})	IIFA	TOSV-EIA (OD_{450})	IB	rEIA (OD_{450})
1	POS	POS	2.694	POS	3.061	POS	3.023	POS	3.041
2	NEG	NEG	0.086	NEG	0.072	NEG	0.092	NEG	0.210
3	NEG	NEG	0.352	NEG	0.387	POS	1.201	POS	1.160
4	POS	POS	3.625	POS	2.940	POS	3.387	POS	3.271
5	NEG	NEG	0.121	NEG	0.126	NEG	0.056	NEG	0.293
6	POS	POS	0.607	POS	0.454	NEG	0.076	POS	0.138
7	NT	POS	3.761	POS	3.456	POS	1.348	POS	1.095
8	POS	NEG	0.997	POS	0.767	POS	0.616	POS	0.737
9	NT	NEG	0.945	POS	0.889	NEG	3.275	POS	3.238
10	POS	NEG	2.872	POS	2.682	NEG	3.234	POS	3.291
11	POS	POS	2.292	POS	3.307	POS	0.883	POS	0.444
12	POS	POS	3.273	POS	2.799	POS	3.388	POS	3.029
13	POS	POS	3.275	POS	3.751	POS	3.370	POS	3.495
14	POS	NEG	1.464	POS	1.895	POS	1.884	POS	1.442
15	POS	NEG	1.871	POS	1.731	POS	1.723	POS	1.330
16	POS	POS	3.739	POS	3.117	POS	2.950	POS	2.667
17	NT	NEG	3.363	POS	3.504	NEG	2.409	POS	1.448
18	NEG	NEG	2.721	POS	3.343	POS	3.111	POS	3.518
19	POS	POS	3.505	POS	3.126	POS	3.235	POS	2.444
20	POS	NEG	2.625	POS	2.292	POS	1.779	POS	2.837
21	NEG	NEG	2.494	POS	2.218	POS	0.849	POS	3.479
22	POS	NT	2.025	POS	2.590	POS	2.975	POS	1.008

[a] POS, positive; NEG, negative; NT, not tested.

IgG EIA. Microtiter plates (Labsystem, Helsinki, Finland) were coated overnight at room temperature with either the purified rN or TOSV in 0.1 M carbonate buffer (pH 9.6). After the plates were washed with washing buffer (10 mM phosphate, pH 7.4; 150 mM NaCl; 0.05% Brij-35 [Sigma-Aldrich]), 100 μl of test serum diluted 1:100 in incubation buffer (washing buffer plus 2% newborn calf serum [BioSpa, Milan, Italy]) was added to each well. After 45 min of incubation at 37°C, the plates were washed and 100 μl of a peroxidase-conjugated mouse monoclonal antibody (MAb) to human IgG (Diesse, Monteriggioni, Italy), diluted 1:20,000 in incubation buffer, was added to each well. The plates were incubated for another 45 min at 37°C and then washed; the substrate (tetramethylbenzidine) was then added. After 15 min of incubation at room temperature (RT), the enzymatic reaction was stopped with 1 N H_2SO_4, and the optical density at 450 nm (OD_{450}) was measured in a microplate reader (Labsystem). Those samples giving an OD_{450} of >0.360 were considered to be positive. The cutoff value was the mean OD_{450} of 24 negative samples, which were confirmed by IB, plus three standard deviations.

IgM EIA. A direct method was used with cell-culture-derived virus and was performed as described for IgG EIA, except that peroxidase-labeled MAb to human IgM (Diesse), diluted 1:6,000 in incubation buffer, was used instead of MAb to human IgG. The μ-capture method was used with the rN as follows: 100 μl of test serum, diluted 1:100 in incubation buffer, was added to each well of a microtiter plate coated with MAb anti-human IgM (Diesse). After 45 min at 37°C, the plates were washed with washing buffer and 100 μl of incubation buffer, containing the biotinylated rN and peroxidase-conjugated streptavidin (Boehringer Mannheim, Milan, Italy), was added to each well. The plates were incubated 45 min at 37°C and washed, and then the substrate (tetramethylbenzidine) was added. After 15 min at RT the enzymatic reaction was stopped with 1 N H_2SO_4, and the OD_{450} was read in a microplate reader. Those samples giving an OD_{450} of >0.400 were considered to be positive. The cutoff value was the mean OD_{450} of 22 negative samples, which were confirmed by IB, plus three standard deviations.

IB. Purified TOSV was subjected to electrophoresis in a 12% polyacrylamide gel according to Laemmli (10). Separated proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, Calif.) with a semidry apparatus (Bio-Rad) at 0.05 mA/cm^2 for 30 min with transfer buffer (25 mM Tris-HCl; 192 mM glycine, pH 8.3; 20% [vol/vol] methanol). The membrane was blocked with 5% milk powder in TBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl) for 1 h at RT and cut into strips. Each strip was incubated overnight at RT with test serum diluted 1:100 in TTBS (TBS, 0.05% Tween 20 [Sigma-Aldrich]) containing 5% milk powder. After four washings with TTBS, alkaline phosphatase-conjugated goat anti-human IgG or anti-human IgM (Sigma-Aldrich) was added. After 1 h of incubation at RT, recognized bands were detected by the addition of the substrate (NBT-BCIP). Samples reactive with the 28-kDa band corresponding to N were scored as positive. For IgM detection, sera were preliminarily screened for the presence of rheumatoid factor, and positive sera were not included in the present study.

PCR. Nested PCR for detection of TOSV RNA sequences in CSF was performed as already described (19).

IIFA. IIFA was performed according to a procedure already described (19).

RESULTS

Of the 22 subjects with meningitis of suspected TOSV etiology (group I), 14 were positive by PCR (Table 1). When tested for the presence of TOSV-specific IgM in serum, eight were positive by all of the serological methods used (samples 1, 4, 6, 11, 12, 13, 16, and 19), and the other six were positive by EIA with rN (rEIA), by EIA with TOSV (TOSV-EIA), and IB (samples 8, 10, 14, 15, 20, and 22). Of five serum samples from subjects negative by PCR, three were also negative by all of the serological methods (samples 2, 3, and 5), and two were positive by rEIA, TOSV-EIA, and IB (samples 18 and 21). When the same sera were tested for TOSV-specific IgG, 12 of 14 samples from subjects positive by PCR were positive by all of the serological methods used (samples 1, 4, 8, 11, 12, 13, 14, 15, 16, 19, 20, and 22), 1 was positive by rEIA, TOSV-EIA, and IB (sample 10), and 1 was positive by IB only (sample 6). Three of the five samples negative by PCR were positive (samples 3, 18, and 21) and two were negative (samples 2 and 5) by all of the serological methods. One of the three samples not tested by PCR was positive by all the serological methods (sample 7), while the other two were positive by rEIA, TOSV-EIA, and IB (samples 9 and 17) for both IgM and IgG. All of the 22 sera obtained during the winter season from children living in the Siena area of Tuscany (group II) were negative by both rEIA and TOSV-EIA for TOSV-specific IgM (Table 2) and IgG (Table 3). All of the sera from the routine diagnosis (group III) were negative for TOSV-specific IgM by both rEIA and TOSV-EIA (Table 2). When tested for TOSV-specific IgG, 13 were positive and 30 were negative by both EIAs, while 8 were

TABLE 2. Correlation between rEIA and TOSV-EIA for the detection of TOSV-specific IgM[a]

Group	rEIA (*n*)	TOSV-EIA	
		Positive	Negative
I	Positive (19)	19	0
	Negative (3)	0	3
II	Positive (0)	0	0
	Negative (22)	0	22
III	Positive (0)	0	0
	Negative (53)	0	53

[a] Correlation between the two EIAs was 100% in all of the serum groups.

positive by rEIA only and 2 were positive by TOSV-EIA only (Table 3). When discordant samples were tested by IB, six of the eight sera positive by rEIA and negative by TOSV-EIA were confirmed to be positive, and the two samples negative by rEIA and positive by TOSV-EIA were confirmed to be negative (data not shown).

DISCUSSION

Viral nucleoprotein has been shown to be the major immunogen during acute TOSV infection (16). We expressed this antigen in *E. coli* and used it to develop an EIA to detect TOSV-specific IgM and IgG. This assay was compared to other serological methods based on cell-culture-derived TOSV and to PCR for the diagnosis of infection. Recombinant EIA was able to detect TOSV-specific IgM in all of the sera from subjects with TOSV meningitis confirmed by PCR or by at least two different serological methods (Table 1). Diagnosis of TOSV infection by detection of anti-TOSV IgM by rEIA had the same sensitivity and specificity (both 100%) as TOSV-EIA and IB and the same specificity but a better sensitivity than IIFA and PCR (50 and 87.5%, respectively). rEIA was also able to detect TOSV-specific IgG in all of the subjects in group I which were positive by both TOSV-EIA and IB. In only one case (serum 6), did IB seem to be more sensitive than EIA for the detection of IgG. The correlation between rEIA and TOSV-EIA was 100% for both IgM and IgG detection (Tables 2 and 3). To further investigate the specificity of rEIA, we compared it to TOSV-EIA with sera from groups II and III. As expected, sera from both groups were all negative for the presence of TOSV-specific IgM by rEIA and TOSV-EIA, confirming a very good specificity for the IgM assay. Pediatric sera (group II) were also negative by both EIAs for anti-TOSV IgG.

TABLE 3. Correlation between rEIA and TOSV-EIA for the detection of TOSV-specific IgG[a]

Group	rEIA (*n*)	TOSV-EIA	
		Positive	Negative
I	Positive (19)	19	0
	Negative (3)	0	3
II	Positive (0)	0	0
	Negative (22)	0	22
III	Positive (21)	13	8
	Negative (32)	2	30

[a] Correlation between the two EIAs was 100% in groups I and II and 84% in group III.

However, there were differences between the two assays in the detection of IgG in group III. Successive IB analysis of discordant samples confirmed results obtained with rEIA in 8 of 10 serum samples. The frequency of IgG positivity obtained with rEIA after resolution of discordant samples with IB was 35.8%, which compares well with the frequencies of positivity found by Braito et al. (2) in two different groups of residents in an endemic area (16.2 and 53.3%). The remaining two serum samples discordant with IB appear to be rEIA false positives. Schwartz et al. (16) demonstrated that TOSV-specific IgG cross-reacted with SFVS and Tesh et al. (18) showed that TOSV was recognized by SFVN-specific antibodies in the complement fixation test. Thus, the possibility exists that false-positive reactions could be caused by cross-reacting antibodies directed towards other SFVs. The reasons why we did not observe the same cross-reactivity by IB and TOSV-EIA are not evident, but it can be hypothesized that cross-reactivity is caused by some conformational epitope present in the viral N protein that is lost during sodium dodecyl sulfate-polyacrylamide gel electrophoresis before blotting, while it can be assumed that TOSV-EIA detects mainly antibodies directed towards pericapsidic glycoproteins. The fact that the two false-positive samples were found in subjects who experienced past infection and might well have been infected with other SFVs seems consistent with the hypothesis of cross-reacting antibodies. Comparison of the deduced amino acid sequence of the N protein of TOSV with those of Punta Toro, Rift Valley fever, and Sandfly fever (Sicilian) revealed 52.2, 49.8, and 39.2% identities, respectively. It remains to be ascertained whether and to what extent this similarity contributes to the cross-reactivity between members of the *Phlebovirus* genus, since conserved sequences could not represent major immunogens or be readily accessible to antibodies. However, the issue of cross-reactivity among SFVs needs to be further investigated, especially in view of more-accurate epidemiological investigations to define the distribution of TOSV. Cases of infection have been reported in central Italy (2, 3, 11, 19), in Cyprus (7), and among tourists returning from Tuscany, Italy (4, 12, 14, 15), Portugal (5), and Spain (6), but the effective diffusion of TOSV and TOSV-related disease in the Mediterranean area is unknown. The reasons which underlie this uncertainty may be varied, but the lack of a simple method accessible to the standard laboratory of serology is probably one of the main obstacles to the study of the epidemiology of TOSV infection. In conclusion, rEIA showed an excellent sensitivity and specificity when used to detect TOSV-specific IgM and an equal sensitivity (100%) and acceptable specificity (96.6%) when used to detect IgG. Thus, rEIA appears to be a simple and readily accessible assay for diagnosing TOSV infection. These characteristics make rEIA a useful assay for a wide range of diagnostic and epidemiological purposes.

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