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# Serosurvey and laboratory diagnosis of imported sandfly fever virus, serotype Toscana, infection in Germany

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## SUMMARY

Of eight acute infections in German tourists caused by sandfly fever virus, serotype Toscana (TOS), and diagnosed clinically and serologically, seven were acquired during visits to Tuscany, Italy, and one to Coimbra, Portugal. An indirect immunofluorescence assay (IFA) using infected cells, and a newly developed enzyme-immunoassay (EIA) using crude virus antigen prepared from infected Vero-E6 cells was used to detect anti-TOS IgM and IgG. In a seroepidemiological survey of 859 health care workers and medical students, anti-TOS IgG was detected in 1·0% by IFA, and in 0·7% by EIA. In 2034 German patients hospitalized for various diseases, 1·6% were positive for anti-TOS IgG by IFA, and 0·8% by EIA. Anti-TOS IgG was detected in 43 samples of commercial immunoglobulins at titres of 10–1000 by EIA. Although the seroprevalence of antibodies to TOS is low in Germany, TOS infection should be considered in patients returning from endemic areas who complain of fever, and headaches, and have symptoms of meningitis.

## INTRODUCTION

Clinical infections of sandfly fever (pappataci fever), now known to be caused by sandfly fever (SF) viruses, were described first in Herzegovina in 1886 [1]. Three serotypes, Sicilian (SFS), Naples (SFN), and Toscana (TOS), were associated with clinical infections in humans. SFS and SFN infections are endemic in several Mediterranean countries, the Middle East, and Central Asia [2–9], and these viruses are transmitted by bites of the insect vector *Phlebotomus papatasi* (sandfly) [10, 11]. In the early 1980s, TOS was isolated in Italy from *Phlebotomus perniciosus* [5]. Phlebotomus sandflies are active in some Mediterranean countries, as indicated by the presence of leishmaniasis which is also transmitted by these vectors [12, 13]. Recent studies in endogenous populations and case reports of infection in tourists indicate that TOS is endemic in the Italian regions of Tuscany and Marche, and also in Algarve, Catalonia and Cyprus [14–19].

SFS and SFN generally cause a febrile illness with headaches lasting for several days [7, 14, 20, 21], whereas TOS infection is characterized by high fever, severe headaches, and aseptic meningitis with long convalescence [16–18, 22]. At present,

only a few cases of TOS infection have been reported in individuals from non-endemic areas [14–19]. Because millions of visitors travel to the Mediterranean countries each year, there is a potential risk of travel-related TOS infections, but many clinicians in most non-endemic countries are unaware of this possibility.

In the early phase of acute illness, sandfly fever virus can be propagated in Vero-E6 cells from serum or cerebral spinal fluid, and virus antigens can be detected and typed by specific antisera. Specific antibodies to the three SF virus serotypes can be detected by indirect immunofluorescence assay (IFA), enzyme-immunoassay (EIA), complement fixation, haemagglutination-inhibition assay, and plaque reduction neutralization test (PRNT) [22, 23].

This paper describes the clinical and epidemiological significance of imported TOS infections in Germany, and the evaluation of a new EIA for the detection of anti-TOS IgM and IgG.

## MATERIALS AND METHODS

### *Study groups*

*Patients with suspected pappataci fever*

From July 1993–July 1994, 12 samples of serum from 8 patients with suspected sandfly fever were sent for serological confirmation to the Max von Pettenkofer Institute. All eight had visited Mediterranean countries before or at onset of illness. From five patients (1–5), only one serum was available for testing TOS-specific antibodies. From patient 6, two consecutive sera obtained 6 (6B) and 18 (6C) weeks after onset of illness were available. Second specimens taken 7 (7B) and 3 (8B) weeks after onset of illness were obtained from patients 7 and 8 (Table 1).

*Healthy individuals*

To determine the antibody prevalence in selected groups of healthy German individuals, sera from 451 health care workers from Freiburg, and 408 medical students from Munich, were tested for anti-TOS IgG.

*Patients with various clinical diseases*

To assess the significance of TOS infection in various clinical diseases, 2034 sera from hospitalized patients were tested for anti-TOS IgG. These sera, obtained from different diagnostic departments of the Max von Pettenkofer Institute, Munich, included pregnant women ($n = 249$), premature babies ($n = 41$), patients with hepatitis ($n = 212$), meningitis and encephalitis ($n = 200$), unexplained fever ($n = 168$), human immunodeficiency virus (HIV) infection ($n = 133$), lymph-adenopathy ($n = 124$), sera from patients with suspected virus infections ($n = 132$), sera from patients with atypical exanthems ($n = 97$), atypical pneumonia and bronchitis ($n = 96$), arthralgia and arthritis ($n = 95$), neuropathy ($n = 86$), leukaemia ($n = 75$), carditis ($n = 61$), acute inner ear disorders ($n = 61$), Lyme disease ($n = 50$), uveitis ($n = 45$), nephropathy ($n = 30$), inflammatory gastro-intestinal diseases ($n = 29$), neoplastic diseases ($n = 37$) and chronic fatigue ($n = 13$).

Table 1. *Serological responses in eight patients with sandfly fever*

| | | | IFA | | EIA | |
|---|---|---|---|---|---|---|
| Patient | Serum | Weeks* | IgM | IgG | IgM | IgG |
| 1 | A | 3 | 64 | 256 | 10000 | 10000 |
| 2 | A | 3 | 128 | 256 | 10000 | 1000 |
| 3 | A | 12 | Neg | 128 | 1000 | 1000 |
| 4 | A | 14 | Neg | 512 | 1000 | 100 |
| 5 | A | 12 | 16 | 64 | Neg | 100 |
| 6 | A | 1 | 64 | 2048 | 10000 | Neg |
| 6 | B | 6 | 128 | 512 | 100000 | 10000 |
| 6 | C | 18 | 16 | 2048 | 1000 | 10000 |
| 7 | A | 3 | 64 | 2048 | 100000 | 10000 |
| 7 | B | 7 | 64 | 4096 | 100000 | 100 |
| 8 | A | 1 | 32 | 256 | 100000 | 1000 |
| 8 | B | 3 | 64 | 512 | 100000 | 1000 |

* Weeks after onset of illness.

*Immunoglobulins*

Forty-three batches of commercial immunoglobulin (Immuno, Vienna, Austria, Armour Pharma, Eschwege, Germany) were tested for anti-TOS IgG by EIA. Titres were determined by end-point titration of 10-fold dilutions.

## *Serological tests*

*Preparation of virus antigen*

Virus antigen was prepared from TOS-infected Vero-E6 cells. Confluent monolayers of cells in 25 $mm^3$ tissue culture flasks were infected with 1 ml of TOS suspension, and cultivated for 7 days in Eagle's MEM supplemented with 5% foetal calf serum. Supernatants were collected and centrifuged for 10 min at 4000 g to remove cell debris. A total volume of 70 ml culture supernatant was then centrifuged for 4 h at 75000 g. Supernatant was discarded, and the pellet resuspended in 1 ml buffer (0·15 M NaCl, Tris-HCl (pH 7·4), EDTA (pH 8·0), sonicated for 10 min, aliquoted and stored frozen at −70 °C.

*Indirect immunofluorescence assay*

The IFA used is based on the method described for detection of antibodies to Lassa fever virus [24]. Vero-E6 cells were infected with TOS and incubated for 3 days. 'Spot slides' were prepared by allowing approximately $\frac{1}{2}$ infected and $\frac{1}{2}$ uninfected Vero-E6 cells to attach in the same spot. The spots were air-dried and fixed in acetone for 10 min at −20 °C. Slides were stored at −70 °C until use. For detection of anti-TOS IgG, all sera were diluted 1/32 in phosphate-buffered saline (PBS), and the IFA performed as described for detection of arboviral antibodies [25]. Spots showing the typical intracytoplasmic fluorescence were regarded as positive. In sera of patients with sandfly fever, end point titres were determined by titration. To study the antibody prevalence in selected groups, all sera were screened at a dilution of 1/32.

To detect specific IgM, sera were incubated with RF absorbens (Behringwerke,

Marburg, Germany) before being added to the spots, and incubated for 30 min at 37 °C. Then, FITC-conjugated anti-human IgM was added, and incubated for 30 min at 37 °C. End-point titres were determined by titration.

*Enzyme-immunoassay (EIA)*

As additional test for detection of TOS specific antibodies of the IgM and IgG class, an EIA was established using native virus antigen. Specificity of the assays was determined by testing sera of 50 patients with other known acute or past virus infections.

*IgG–EIA*

Wells of flat-bottom microtitre plates were coated with TOS antigen diluted 1/400 in 0·2 M carbonate buffer, pH 9·5. After washing three times with buffer containing 1% Tween-20, 50 $\mu$l of 10-fold dilutions of test sera from $10^{-2}$–$10^{-4}$ in PBS containing 2% Tween-20, 3% foetal calf serum (dilution buffer), were added to the wells in duplicate, and incubated for 2 h at 37 °C. After washing, 50 $\mu$l of peroxidase-conjugated anti-human IgG (Dako, Hamburg, Germany; dilution 1/1000 in dilution buffer) were added and incubated for 1 h at 37 °C. Finally, plates were washed three times and the enzymatic reaction performed as described previously for parvovirus B19 IgM and IgG [26]. Sera with a positive/negative (P/N)-ratio of $\geqslant 2{\cdot}1$ were regarded as positive. For screening sera, a dilution of 1/100 was chosen.

*IgM–EIA*

For IgM–EIA, the same buffers were used as described for IgG–EIA. Wells were coated with 50 $\mu$l of anti-$\mu$ (Dako) diluted 1/2000. After washing, 50 $\mu$l of 10-fold dilutions of test sera from $10^{-2}$–$10^{-4}$, were added, and incubated for 2 h. Wells were washed, virus antigen diluted 1/400 was added, and incubated overnight at 4 °C. After washing, wells were incubated with a polyclonal mouse antibody to TOS (kindly supplied by C. Giorgi, Rome) diluted 1/500, for 2 h followed by addition of peroxidase-conjugated anti-mouse IgG (Dako; dilution 1/1500) for 1 h. The enzymatic reaction was carried out as described [26]. Sera were regarded as positive if the P/N-ratio was $\geqslant 2{\cdot}1$. For screening, sera were tested in a dilution of 1/100.

## RESULTS

*Epidemiological findings*

All eight patients with sandfly fever had visited TOS endemic areas during June–September 1993, or June or July 1994 before or at onset of illness. Seven patients had visited Tuscany, Italy, and one was infected near Coimbra, Portugal.

*Clinical findings*

Six of the eight patients with sandfly fever were male. The median age was 33·9 (range 20–53) for males, and 43·0 (range 42–44) years for females. All patients complained of fever, severe headaches, and photophobia. Headaches usually lasted up to 4 weeks and in two patients, prolonged courses were noted. Aseptic meningitis occurred in one patient.

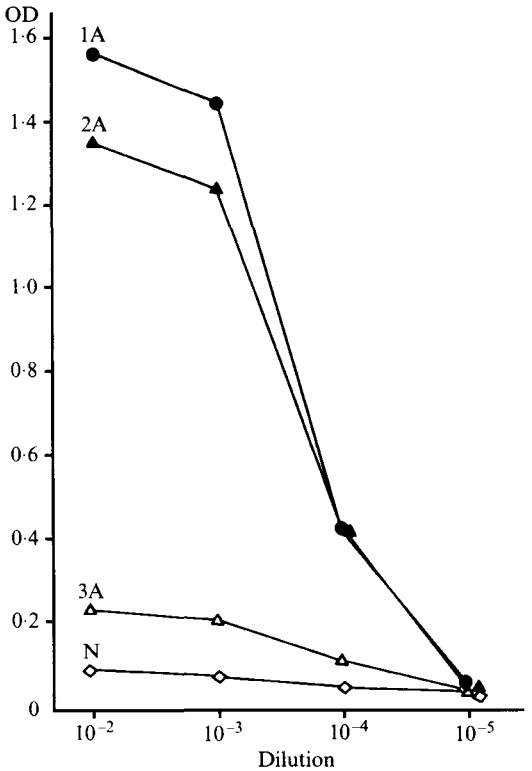


Fig. 1. Detection of anti-TOS IgM by IgM–EIA. Serum 1A and 2A were taken 3 weeks and 3A 12 weeks after onset of sandfly fever. Serum N is a negative control. Sera were tested by end-point titration after 10-fold dilution.

*Detection of anti-TOS IgM in patients with sandfly fever by EIA*

To determine anti-TOS IgM in clinical specimens, an EIA was established using crude virus suspensions. The IgM–EIA discriminated well between anti-TOS-positive and -negative sera as shown for sera of patients 1 and 2 taken 3 weeks after onset of sandfly fever (Fig. 1). End-point titres of 10000 were determined, and anti-TOS was still demonstrated at a titre of 1000 in a specimen taken 12 weeks after clinical illness (Fig. 1).

For further testing, all sera were diluted 1/100. All 50 sera of patients with other known acute or past virus infections were negative by IgM–EIA.

*Comparison of IFA and EIA for anti-TOS IgM*

To compare both methods for detecting anti-TOS IgM, end-point titres were determined in sera of patients with sandfly fever by IgM–IFA and IgM–EIA. As shown in Table 1, both assays detected anti-TOS IgM in the early phase of infection. However, IFA was not as sensitive as EIA for detecting anti-TOS IgM in sera taken 12 or more weeks after onset of illness. Two of 12 sera (3A, 4A) still positive by IgM–EIA were negative by IgM–IFA, but one serum (5A) positive by IgM–IFA was negative by IgM–EIA. The latter serum (5A) had been frozen and thawed several times before being tested by IgM–EIA. Since there is no 'gold

Table 2. *Sensitivity and specificity of IFA and EIA in determining anti-TOS IgM and anti-TOS IgG in 103 (anti-TOS IgM) and 2955 (anti-TOS IgG) sera*

| | EIA versus IFA | | IFA versus EIA | |
|---|---|---|---|---|
| | Sensitivity | Specificity | Sensitivity | Specificity |
| Anti-TOS IgG | 63·0% | 99·9% | 100·0% | 99·3% |
| Anti-TOS IgM | 90·0% | 97·8% | 81·8% | 98·9% |

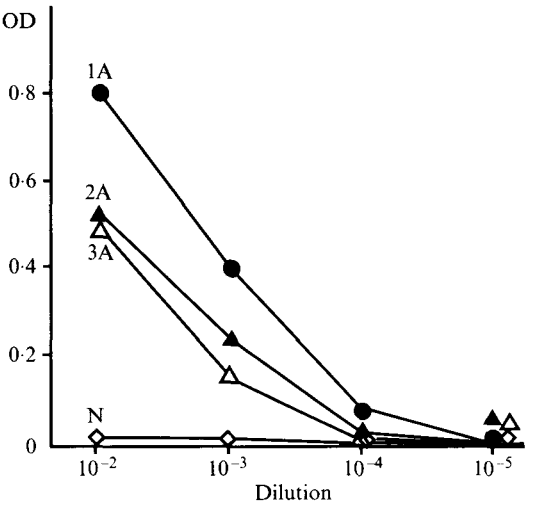


Fig. 2. Detection of anti-TOS IgG by IgG–EIA. Serum 1A and 2A were obtained from patients 3 weeks, and serum 3A 12 weeks after onset of TOS infection. Serum N is a negative control. Titres were determined by end-point titration after 10-fold dilution.

standard' for anti-TOS, sensitivity and specificity were determined by comparing the results of all 103 sera tested for anti-TOS IgM by IFA and EIA as shown in Table 2. Concordant results were obtained in 100/103 (97·1%) sera.

*Detection of anti-TOS IgG by EIA in patients with sandfly fever*

The EIA assay allowed excellent discrimination between positive and negative sera as shown for sera of patients 1–3 (Fig. 2). The titres were 1/10000 for serum 1, and 1/1000 for patients 2 and 3. None of the 50 sera of patients with known other acute or past virus infections reacted positively for anti-TOS by EIA.

*Comparison of IFA and EIA for detecting anti-TOS IgG*

Sera of patients 1–8 were tested for anti-TOS IgG by IgG–IFA and IgG–EIA. As shown in Table 1, all 12 sera were positive by IgG–IFA, whereas only 11 reacted positively by EIA. The discrepant serum (6A), negative by IgG–EIA but positive by IgG–IFA, was taken in the first week after onset of illness. No 'gold standard' is available for determining anti-TOS IgG. Therefore, sensitivity and specificity of the IFA and EIA were calculated for all 2955 sera tested for anti-TOS IgG by both tests. Results are shown in Table 2. Concordant results were found in 2935/2955 (99·3%) sera.

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Table 3. *Seroprevalence of anti-TOS IgG determined by IgG–IFA, and confirmed by IgG–EIA in selected groups of 2034 patients with various diseases*

| Diagnosis (no. of sera) | Anti-TOS IgG positives (% in brackets) IFA | EIA |
|---|---|---|
| Chronic fatigue (13) | 1 (7·7) | 1 (7·7) |
| Atypical exanthema (97) | 4 (4·1) | 2 (2·1) |
| Atypical pneumonia and bronchitis (96) | 4 (4·2) | 1 (1·0) |
| Leukaemias (75) | 3 (4·0) | 1 (1·3) |
| Inflammatory gastrointestinal diseases (29) | 1 (3·4) | 0 (0·0) |
| Nephropathy (30) | 1 (3·3) | 1 (3·3) |
| Unexplained fever (168) | 4 (2·4) | 2 (1·2) |
| Lymphadenopathy (124) | 3 (2·4) | 3 (2·4) |
| Hepatitis (212) | 4 (1·9) | 3 (1·4) |
| Carditis (61) | 1 (1·6) | 1 (1·6) |
| Meningitis and encephalitis (200) | 3 (1·5) | 0 (0·0) |
| Neuropathy (86) | 1 (1·2) | 1 (1·2) |
| Arthralgia and arthritis (95) | 1 (1·1) | 0 (0·0) |
| Screening for virus diseases (132) | 1 (0·8) | 1 (0·8) |
| Neoplastic diseases (37) | 0 (0·0) | 0 (0·0) |
| Lyme disease (50) | 0 (0·0) | 0 (0·0) |
| HIV infection (133) | 0 (0·0) | 0 (0·0) |
| Uveitis (45) | 0 (0·0) | 0 (0·0) |
| Acute inner ear disorders (61) | 0 (0·0) | 0 (0·0) |
| Pregnant women (249) | 0 (0·0) | 0 (0·0) |
| Premature babies (41) | 0 (0·0) | 0 (0·0) |
| Total (2034) | 32 (1·6) | 17 (0·8) |

*Seroprevalence of TOS in healthy individuals*

Sera from 859 health care workers and medical students were screened for anti-TOS IgG by IgG–IFA. Anti-TOS IgG was detected in nine sera. The median age of the sero-positives was 26·5 years. The anti-TOS IgG-positive individuals were all negative for anti-TOS IgM by IgM–IFA. All sera positive for anti-TOS IgG by IgG–IFA were retested by IgG–EIA, and 6 of the 8 were also positive for anti-TOS IgG by IgG–EIA. Thus, the overall prevalence was 1·0% by IgG–IFA, and 0·7% by IgG–EIA.

*Seroprevalence of TOS in patients with various diseases*

Sera of 2034 patients with various clinical diseases were tested for anti-TOS IgG by IgG–IFA (Table 3). In total, 32 sera were positive for anti-TOS IgG by IgG–IFA. The median age of the seropositives was 47·7 years. Anti-TOS IgM was not detected in these 32 patients by IgM–IFA. Seventeen of the 32 (53·1%) sera reactive by IgG–IFA were also positive by IgG–EIA. The antibody prevalence was 1·6% determined by IgG–IFA, and 0·8% by IgG–EIA.

*Immunoglobulins*

To determine whether commercial immunoglobulin preparations contain antibodies to TOS, 43 batches were tested for anti-TOS IgG by IgG–EIA. Specific antibodies were detected in all 43 batches. Four (9·3%) batches were positive for anti-TOS IgG at a titre of 10, 26 (60·5%) at 100, and 13 (30·2%) at 1000.

## DISCUSSION

This study reports acute infections with sandfly fever virus, serotype Toscana, in German tourists who had visited endemic areas in Italy and Portugal, and is the first report of specific antibodies to TOS in non-endogenous human populations. The prevalence of anti-TOS IgG in selected groups of healthy individuals and hospitalized patients was between 1·0 and 1·6% by IgG–IFA, and 0·8–1·0% by IgG–EIA. Commercial immunoglobulins were all positive for anti-TOS IgG by IgG–EIA.

TOS infection is endemic in some Mediterranean areas. A recent study showed that TOS is an important agent in virus-associated meningitis and meningoencephalitis in some parts of central Italy [22], and another seroepidemiological study found an antibody prevalence of 20% in the endogenous population of Cyprus [3]. At present, no data on the seroprevalence of antibodies to TOS are available from Spain and Portugal but as clinical infections have been reported from these areas, it is likely that the virus is present. To date, only a few cases of TOS infection have been reported in visitors from non-endemic areas travelling to Italy, Cyprus, Spain, and Portugal [15–18], and in view of the large number of visitors travelling to these areas during the months of vector activity, the infrequent reports of sandfly fever are surprising. In this study, most tourists had been infected in Tuscany, Italy but one patient had acquired the infection in Coimbra, Portugal, an area from where TOS infection has never been reported.

To date, seroepidemiological studies on the antibody prevalence to TOS have not been carried out in non-endogenous populations. By screening sera of 859 healthy individuals by IgG–EIA, a prevalence of anti-TOS IgG of 0·7% was found. In sera of 2034 hospitalized patients with various diseases, an antibody prevalence of 0·8% was determined by IgG–EIA. Anti-TOS IgG was not associated with any specific clinical disease in these patients. Since infections with TOS mainly occur between June–October [22], patients returning from endemic areas presenting with fever, headaches, photophobia, and meningitis, should be tested for sandfly fever. Unnecessary diagnostic procedures may thus be avoided in patients with TOS-related neurological symptoms or prolonged convalescence.

All 43 batches of immunoglobulins tested in this study contained TOS-specific IgG. It is unknown whether commercial immunoglobulins protect against TOS, or what titres are protective. A vaccine against hepatitis A virus (HAV) is already available in some countries, and immunoglobulins are now less-commonly used for travellers to Mediterranean countries. If such immunoglobulins protected against TOS infection in the past, it may be that more cases of sandfly fever will occur in travellers who receive HAV vaccine rather than immunoglobulin.

Serological diagnosis of TOS infection is based on the detection of specific antibodies. At present, there is no method which has been defined as a 'gold standard' for detection of antibodies to TOS. In this study, the results of the two assays for demonstrating anti-TOS IgM and IgG in acute infections agreed well. Anti-TOS IgM was still detectable up to 18 weeks after onset of illness by IgM–EIA and IgM–IFA with variable end-point titres. IgM–EIA appears to be more sensitive in detecting anti-TOS IgM in sera taken more than 12 weeks after infection. Following acute infection with SFS, detection by EIA of specific IgM

was reported up to 9 months [23]. One study demonstrated that IFA and PRNT produced identical results in acute SFS infection [14]. At present, it is not known how long IgG to sandfly fever viruses can be detected after acute infection. Recently, the absence of detectable antibodies in a serum obtained 8 months after sandfly fever virus infection tested by IFA was reported [7]. In testing sera from Cypriots, PRNT was significantly less sensitive in detecting anti-SFS IgG than EIA and IFA [3], and for detecting acute TOS infection, EIA was more sensitive than the haemagglutination-inhibition assay [22]. In the present study, 1·6% of sera from 2034 patients with various diseases were anti-TOS IgG-positive by IgG–IFA, whereas only 0·8% were reactive by IgG–EIA. The discrepancy may be explained by the different dilutions in which sera were tested in the two assays but it may be that anti-TOS IgG titres fall to a low level in individuals from non-endemic areas, if not boosted by repeated exposure to the virus.

TOS infection is a travel-related virus disease of visitors to Mediterranean countries. With the increase of vector activity in the Mediterranean countries [12, 13, 27], more clinical cases of sandfly fever may be seen in future.

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