Diagnostic approaches for oculoglandular tularemia: advantages of PCR

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Aim: The authors describe a diagnostic approach that proved to be particularly valuable in rare cases of ocular tularemia registered during the tularemia outbreak in 1997–2005 in Bulgaria. The authors describe the laboratory findings and diagnosis of four cases with an oculoglandular form of infection.

Methods: Several different specimens from each patient were analysed. Oculoglandular tularemia was diagnosed in four patients either by culture, immunofluorescent antibody analysis (IFA), serology or by a polymerase chain reaction (PCR) assay.

Results and Discussion: Three *F tularensis* strains were isolated and characterised. One of these was isolated from a conjunctival swab specimen obtained from a seronegative patient. The authors report for the first time a successful application of diagnostic PCR performed directly on conjunctival swab specimen. From all analysed specimens IFA was diagnostically effective only in the case of lymph node aspirates and was not sensitive enough for conjunctival swabs or blood samples. The authors also describe the histological picture of a conjunctival granuloma in course of infection. All patients were successfully treated with ciprofloxacin.

Conclusions: Some of the proposed laboratory diagnostic strategies (swab PCR) are not invasive and could represent a new approach for resolving rare and hard-to-diagnose cases of oculoglandular tularemia.

Tularemia is a zoonotic disease caused by the gram-negative bacterium *Francisella tularensis*. It affects a variety of mammalian species, mostly humans, hares and rodents and survives in protozoa.^{1–2} The *Francisellaceae* family is in the γ subclass of *Proteobacteria* and range over closely-related microorganisms incorporated within a single genus *Francisella* with two species *F tularensis* and *F philomiragia*.³ *F tularensis* with its four subspecies (*ssp tularensis*, *ssp holarctica*, *ssp mediasiatica* and *ssp novicida*) is the causative agent of tularemia disease in North America, Europe, Asia and recently Australia.⁴ *F tularensis ssp tularensis* is highly virulent (infectious dose less than 10 CFU) for various mammals including humans and is considered by the Centers for Disease Control and Prevention (CDC) a category A potential bio-weapon agent. The genetic basis of its high virulence and pathogenicity is currently unknown. The second species *F philomiragia* is an opportunistic pathogen, rarely causing disease in mainly immunocompromised patients and often associated with water.

Depending on the route of entry, tularemia occurs in several clinical forms: glandular, pneumoglandular, oculoglandular, oropharyngeal, intestinal, pneumonic and typhoidal. Some authors accept only two forms—ulceroglandular (local) and typhoid (septic).⁵ The oculoglandular form of tularemia is one of the rarest ones comprising up to only 4% of all cases.^{6–7} Humans may acquire this disease form by direct contact with infected materials, often via contaminated hands or by

aerosolised contaminated particles. The intracellular bacterium appears to enter and replicate in macrophages via a cytoplasm-B-insensitive pathway and without triggering a respiratory burst.⁸ The subsequent clinical presentation, known as Parinaud oculoglandular syndrome, is a rare medical condition characterised by granulomatous conjunctivitis, associated with homolateral cervical and/or pre- or retroauricular lymph adenopathy.⁹ It has been associated with several infectious diseases including tularemia which is rarely considered in the differential diagnosis.¹⁰

Herein we discuss our findings in four patients with Parinaud syndrome diagnosed during a tularemia outbreak.

METHODS

All tularemia cases were diagnosed according to the case definitions of the CDC.¹¹ Clinically relevant information was gathered by interview, referral to hospitals, and questionnaires and sent to general practitioners in the region for submission to the reference centres for epidemiological analysis. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study, and the overall study was in accordance with the Declaration of Helsinki. Serum samples for serological assays for specific anti-*Francisella* antibodies were collected from all patients 1–4 weeks after the onset of the disease (S1). Second and third samples were obtained after 14 days (S2) and three months later respectively (S3). All samples were tested with a serum tube agglutination and haemagglutination kit (BulBio-NCIPD, Bulgaria). Serial dilutions of sera, starting from 1:20, and antigen prepared from *F tularensis* strain Srebarna 19 were mixed and after overnight incubation at 37°C the agglutination was evaluated by unaided eye. Serum specimens with $\geq 1:80$ or at least fourfold titre rise in S2 or S3 samples were interpreted as positive results.^{12–13}

Biopsy specimens from cervical lymph nodes (2 patients), conjunctival swab (1 patient), surgically extracted conjunctival granuloma (1 patient), and 3 ml blood samples (4 patients) were collected for culture and PCR detection.

Half of the volume from each specimen suspension was cultured on modified Thayer–Martin agar plates (containing Gc medium base, haemoglobin and IsoVitaLex at 37°C with 5% CO₂ in air) and the other half was processed for PCR.⁴ All manipulations were carried out in a biosafety level 3 cabinet. DNA from biopsy specimens/aspirates (500 μ l), blood samples (1.5 ml) or swabs was isolated following standard phenol-chloroform protocol with slight modifications.¹⁴ DNA (100 ng) was used in PCR with *tul4* and *RD1* *F tularensis* specific primers according to the protocols by Johansson and Broekhuijsen.^{15–16}

Direct immunofluorescence assay (IFA) with FITC-conjugated anti-*Francisella* sera (BulBio-NCIPD) was used to detect *F tularensis* antigens in all specimens except blood. Preparations (smears) from conjunctival lymph nodule (patient 3) and smears were finally counterstained with 0.25% Evans blue.

Abbreviation: CDC, Centers for Disease Control and Prevention

Figure 1 Picture of a tularemia infected eye (patient 1). Informed consent was obtained for publication of this figure.

The histological techniques were as follows: briefly, 4% p-formaldehyde was used for fixation and 4 µm paraffin embedded tissue sections were microscopically examined.

RESULTS

A typical clinical picture of oculoglandular tularemia was observed in all patients (fig 1).

In addition to the Parinaud syndrome, two patients showed signs of cervical lymphadenopathy. IFA was positive only in the lymph node specimens but no visible fluorescence was detected in the swab smears. Except for one patient significant and/or rising agglutination titres were detected in all the other patients.

Surgically extracted conjunctival nodule with dimensions 5×2 mm was white-yellow in colour. We observed an area surrounded by stratified non-keratinised, squamous epithelium with regions of parakeratosis and submembrane manifested mononuclear infiltrations, pronounced vascularisation and single Langhans cells. A granuloma with central necrosis was detected in the centre of the nodule.

The systematic observations of all other diagnostic findings are summarised in table 1.

Direct bacteriological isolation was achieved only from cervical lymph node aspirates from two patients. We did not succeed with attempts for primary direct isolation of the causative agent from the conjunctival swab and blood specimens. However, *F tularensis* was isolated from conjunctival swab suspension (500 µl) in one patient by intraperitoneal inoculation in a guinea pig and subsequent culturing of spleen material. All animals were treated in compliance with the

Guide for the Care and Use of Laboratory Animals.¹⁷ *F tularensis* was detected by PCR in both types of ocular specimens—conjunctival swab (patient 4) and surgically extracted conjunctival nodule (patient 3). The blood samples from these patients were also PCR positive (fig 2). With the RD1 subspecies specific primers all isolates produced characteristic electrophoretic bands (900 bp) for ssp *holarctica* (fig 2). In three cases, the consecutive serological samples exhibited marked titre dynamics, which further supported diagnosis. However, in patient 4 seroconversion did not take place and she remained seronegative for six months (when the last sample was tested) after successful therapy. This case had a clinical history of an extensive abdominal intervention shortly before the infection took place, and was resolved by positive PCR from conjunctival swab and blood but also with consecutive isolation of the causative agent (see table 1).

All patients were successfully treated with ciprofloxacin 500 mg twice daily for 21 days. The rationale for this type of treatment was the specific drug pharmacodynamics characterised by good tear mucosal excretion.

DISCUSSION

A large number of studies discussing tularemia outbreaks in various regions have been published in the last 15 years.⁸ There are several publications describing outbreaks in the Balkan Peninsula with significant healthcare importance. The first ocular case was reported in Bulgaria in 2003.¹⁶

As the ocular forms are extremely rare and the overall index of suspicion is low, they often remain unresolved and under-reported.¹⁸ The clinical picture of Parinaud syndrome is non-indicative for a particular causative agent and more often is associated with *Bartonella henselae*, *Mycobacterium tuberculosis* or *Herpes* infections.

To our knowledge the direct detection and/or isolation of *F tularensis* from conjunctival swab material have not been reported in the literature. In the last decade PCR assays have proved to be of great clinical and diagnostic value, providing rapid and definitive results. Several such assays have been published for the detection of tularemia in ulcers, blood, ticks and so on but none for diagnosing ocular tularemia. Because of the requirements for special biosafety conditions when handling *F tularensis* and the very low isolation rate (~ 5%), cultures are not routinely performed in most of the laboratories. The fastidious nature of the microorganism prevents its detection in the common microbiological laboratories where specific media are not routinely applied. In these cases the Parinaud syndrome remains “idiopathic” with no aetiological diagnosis. With respect of tularemia the specific antibodies are not detectable in the early stages of the disease (up to the 14th day). In such cases the PCR assays performed either with ulcerous or conjunctival swabs are very useful alternatives for early confirmation of clinical diagnosis and proper treatment. Our diagnostic approach concerning swab analysis is not invasive or traumatic and appears to be highly effective and straightforward. In addition, it is supportive in cases where no specific immune response appears or is delayed. This was

Table 1 Diagnostic findings observed in four Parinaud syndrome patients

	Isolation from eye	Isolation Ly node	PCR eye	PCR Ly node	PCR blood	S1 titre	S2 titre	S3 titre	Histology
1	–	+	NA	+	NA	1:320	1:640	1:1280	NA
2	–	+	NA	+	NA	1:160	1:320	1:640	NA
3	–	NA	+ (Nodule)	NA	+	–	1:160	1:640	+
4	+	NA	+ (Swab)	NA	+	<1:80	<1:80	<1:80	NA

Figure 2 PCR on clinical specimens with tul4 and RD1 primers. (A) (1) *F. tularensis* ssp. *holarctica* strain LVS; (2) negative control, ddH₂O; (3) lymph node, patient 1; (4) lymph node, patient 2; (5) blood, patient 3; (6) blood, patient 4; (7) conjunctival nodule, patient 3; (8) conjunctival swab, patient 4; M, molecular size marker 100–3000 bp. (B) (1) *F. tularensis* ssp. *tularensis* strain Schu; (2) *F. tularensis* ssp. *holarctica* strain LVS; (3) lymph node, patient 1; (4) lymph node, patient 2; (5) blood, patient 3; (6) blood, patient 4; (7) conjunctival nodule, patient 3; (8) conjunctival swab, patient 4; (9) negative control, ddH₂O.

clearly demonstrated in one of our patients (patient 4), having no positive serology at the onset of disease or afterwards. The patient had a history of recently performed serious abdominal surgical intervention that had resulted in a significant level of immunosuppression.

These data suggest that we should strongly recommend a broader application of this methodology when oculoglandular tularemia is suspected.

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Informed consent was obtained for publication of figure 1.

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