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CASE REPORT

Fluorescent in situ hybridization can be used as a complementary assay for the diagnosis of *Tropheryma whipplei* infection

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Abstract

Background Immunohistochemistry and Periodic acid–Schiff (PAS) staining have been routinely used for the diagnosis of Whipple's disease (WD). However, these methods present limitations. As a result, the last years, Fluorescence in situ hybridization (FISH) has been increasingly used as a complementary tool for the diagnosis of WD from various tissue samples.

Case report In this study, we visualized, by FISH, *Tropheryma whipplei* within macrophages of a lymph node from a patient with WD. Moreover, we report in this study a patient with a pulmonary biopsy compatible with WD by PAS, immunostaining and FISH, although the specific molecular assays for *T. whipplei* were negative. Sequencing analysis of the 16S rDNA revealed a *T. whipplei*-related species with unknown classification.

Conclusion FISH can be a valuable method for the detection of *Tropheryma* species in formalin-fixed paraffin-embedded tissues. FISH cannot replace the other already approved diagnostic techniques for WD, it can be used as a complementary tool and can provide supplementary information in a relatively short time.

Keywords *Tropheryma whipplei* · Fluorescent in situ hybridization · Lymphadenopathy · Respiratory infection · Whipple's disease

Introduction

Tropheryma whipplei is the causative agent of Whipple's disease (WD), a multisystem disorder with prominent intestinal manifestations such as diarrhea, weight loss, lymphadenopathy, and polyarthritis [1, 2]. Lymphadenopathy, mainly mediastinal and mesenteric, have been observed in 50–55% of cases of classic WD [3]. *T. whipplei* DNA was also previously amplified in bronchoalveolar lavage (BAL) samples of children and adults showing an involvement in respiratory disease [4]. WD diagnosis is based on histopathologic examination with Periodic acid–Schiff (PAS) staining, immunochemistry (IHC) and real time PCR (qPCR) [5]. Immunohistochemical analysis using specific mouse or rabbit polyclonal antibodies allows the direct visualization of bacteria in samples and show specificity superior to that one of PAS staining. *T. whipplei* specific quantitative real-time PCR (qPCR) has been previously validated as an important tool for the detection and diagnosis of *T. whipplei* [4, 6, 7]. Indeed, this method was described as sensitive, specific and rapid for the screening and diagnosis of WD [5, 8]. However, they exist discrepancies among the different assays for

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the diagnosis of WD and it was previously described that duodenal samples become rapidly negative by qPCR but PAS staining and immunohistochemistry positive after the beginning of treatment [9, 10]. Moreover, PAS staining can be positive in other circumstances, such as *Mycobacterium* infection [11].

Fluorescence in situ hybridization (FISH) has been increasingly used as a complementary tool for the diagnosis of WD from various tissues [12, 13]. Very few cases reported the detection by FISH of *T. whipplei* from lymph node specimens [10, 12, 14] and no publication was found in case of respiratory infection. We report in this study two cases of WD diagnosed by FISH on a lymph node and pulmonary samples.

Methods

DNA was extracted from the tissue biopsy specimens using a DNA Tissues Kit on an EZ1 Advanced XL device (Qiagen, Courtaboeuf, France). A specific quantitative real-time PCR (qPCR) assay, using specific oligonucleotide TaqMan probes to target *T. whipplei* repeated sequences, was performed to detect DNA from this bacterium in the specimens, as described previously [15]. A case was defined as positive if two independent qPCR assays targeting different repeated sequences were positive, with cycle threshold (C_t) values of < 35. The biopsy specimens were formalin-fixed and paraffin-embedded. For each tissue specimen, serial cuts 3-µm thick were obtained to perform hematoxylin-eosin, diastase-digested PAS staining, and immunohistochemical investigations. Immunohistochemical analysis was performed on paraffin-embedded tissue sections using a rabbit anti-*T. whipplei* polyclonal antibody at a dilution of 1:2000, as described elsewhere [16]. A negative control was performed using an irrelevant monoclonal antibody. FISH was performed with universal EUB338 probe (labelled with Alexa Fluor 555) and specific Tw16S-652 probe (labelled with Alexa Fluor 488) for *T. whipplei*. NonEUB probe (labelled with Alexa Fluor 647) was used as negative control for non-specific binding. The specificity of TW-652 probe was tested against non-target bacterial species (Supplementary Table 1). The FISH procedure was performed as previously described [17]. Slices were de-paraffinized by incubation for 10 min at 65 °C, then for 10 min in substitute xylene solution. The tissue was rehydrated in a descending ethanol series (100, 70 and 50%, 5 min each), rinsed in water and air dried. Slides were incubated at 37 °C for 20 h with hybridization buffer containing 0.9M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% (w/v) SDS, 30% Formamide and 1 µM of probes. The hybridization step was performed using a hybridizer device (Dako, Agilent.com). For the washing step, slides were incubated at 37 °C for 15 min in washing buffer containing 0.112 M NaCl, 5 mM 0.5M EDTA, 20 mM Tris-HCL pH 8.0 and 0.01% (w/v) SDS. Slides were rinsed once again with water, then dried and mounted with mounting media containing the antifading agent 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) used to visualize microorganisms and host cell nuclei. Slides were visualized under Confocal laser scanning microscopes (CLSM) using the Leica SP5 resonant Scanner with appropriate filter sets fluorescence and an 60× oil immersion objective as previously described [10]. *T. whipplei* spotted cells and human duodenal biopsies infected with *T. whipplei* were used as positive control. Non-infected human tissues were used as negative controls. Samples were also, analyzed in the absence of any probe to check for auto-fluorescence.

Case 1: Patient 1 was a 68-year-old French woman with a polyarthritis affecting her ankles and knees and was receiving a treatment with corticoids. The last 6 months, she was suffering from fever and had lost 10 kg. Abdominal CT scan revealed many retro peritoneal lymphadenopathy with a hypodense centre and a lymph node biopsy was performed. Stool samples, duodenal and lymph node biopsies were all positive by qPCR targeting *T. whipplei*. On serial cuts 3-µm thick of the lymph node we performed hematoxylin–eosin, diastase-digested Periodic acid–Schiff (PAS) staining and immunohistochemical investigations as previously described [16]. Microscopic examination revealed abundant foci of foamy macrophages in the enlarged lymphatic sinuses and among the lymphoid cells stuffed with dense and granular material that was positive on the PAS staining and resistant to diastase (Fig. 1a). Bacilli were identified in the lymph node by immunostaining as coarse granular immunopositive material in macrophage cytoplasm (Fig. 1b). FISH revealed large positive regions with bacteria mainly localized within the intra-cytoplasmic area of macrophages of the inflammatory cellular infiltrates and appeared in clusters as multiple rounded structures (Fig. 1c, d and Supplementary Figure 1). Treatment with doxycycline and hydroxychloroquine was introduced and symptoms retreated.

Case 2: Patient 2 was a 40-year-old Belgian woman with lupus nephritis who experienced fever and breathing-related thoracic pain [18]. The patient underwent a thoracoscopic lung biopsy of the left lower lobe (Supplementary Figure 2) and a formalin-fixed paraffin-embedded (FFPE) sample was sent to our laboratory in Marseille. This pulmonary biopsy was negative by the *T. whipplei* specific qPCR. PAS and immunostaining revealed foamy macrophages in interalveolar walls and in bronchioloalveolar air spaces with dense and granular material and resistant to diastase (Fig. 2a, b). FISH was also positive for *T. whipplei* and bacteria were visualized directly in infected lung cells, mainly in the alveolar air spaces (Fig. 2c, Supplementary Figure 3) and they were also

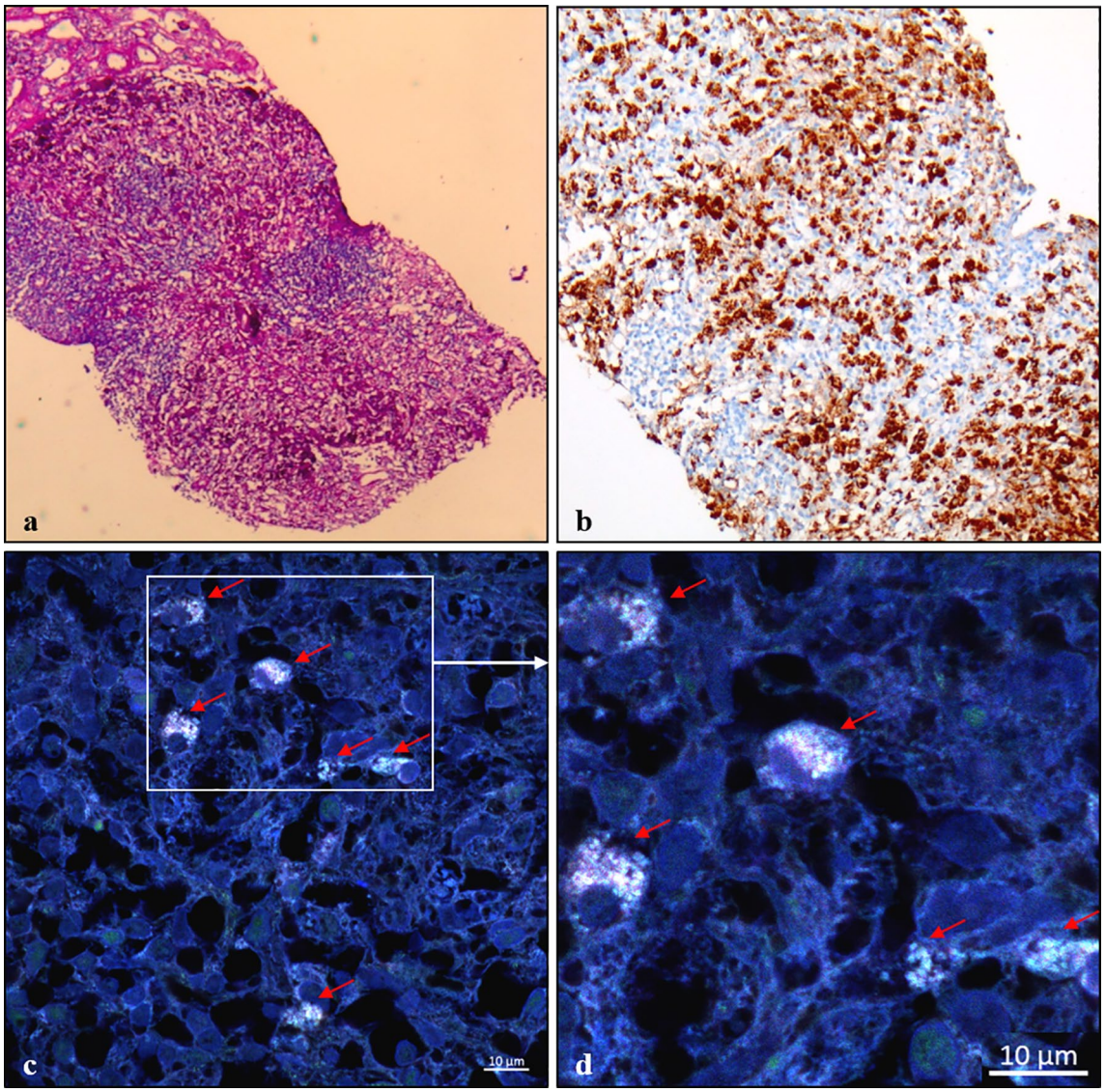

Fig. 1 PAS, Immunohistochemistry and FISH staining on lymph node biopsy specimen from patients with Whipple disease. **a** Observation of the large infiltration of foamy macrophages in the lymph node pulp, these macrophages contain many PAS-positive particles in their cytoplasm (original magnifications ×50); **b** demonstration of *T. whipplei* by immunohistology: bacilli are packed as granular immunopositive material in macrophage cytoplasm (original magnifications ×100); **c, d** bacteria are visualized by FISH into macrophage with merged signals (white) of the green channel (TW-652) and the red channel (EUB338), the nuclei are counterstained with 4′6-diamidino-2-phénylindole (DAPI) in blue. Original magnifications ×63 and ×63, respectively, and appear in clusters as multiple rounded structures

detected in the interalveolar area (Fig. 2d, Supplementary Figure 4). As a result, we diagnosed the patient as having WD.

Discussion

We detected *T. whipplei* in a lymph node and a pulmonary biopsy by FISH with specific 16S rRNA targeted probes. Previous studies revealed that FISH-based diagnosis can be considered as an ancillary diagnostic tool for the feasibility of *T. whipplei* [12, 19]. Moreover, in a series of duodenal biopsies from patients with WD, FISH showed the localization in CD68+ compartment of macrophages and in the cytoplasmic area of the CK20+ epithelial cells [10]. Among this series, FISH was positive for three negative immunochemistry samples and 20 samples for which the qPCR result was superior to 34C_t and was considered negative [10]. However, FISH has recently been found to be much more sensitive than immunostaining to the detection of intracellular agents [17]. A limitation of our study was that, for the first case, although a duodenal biopsy was also received in

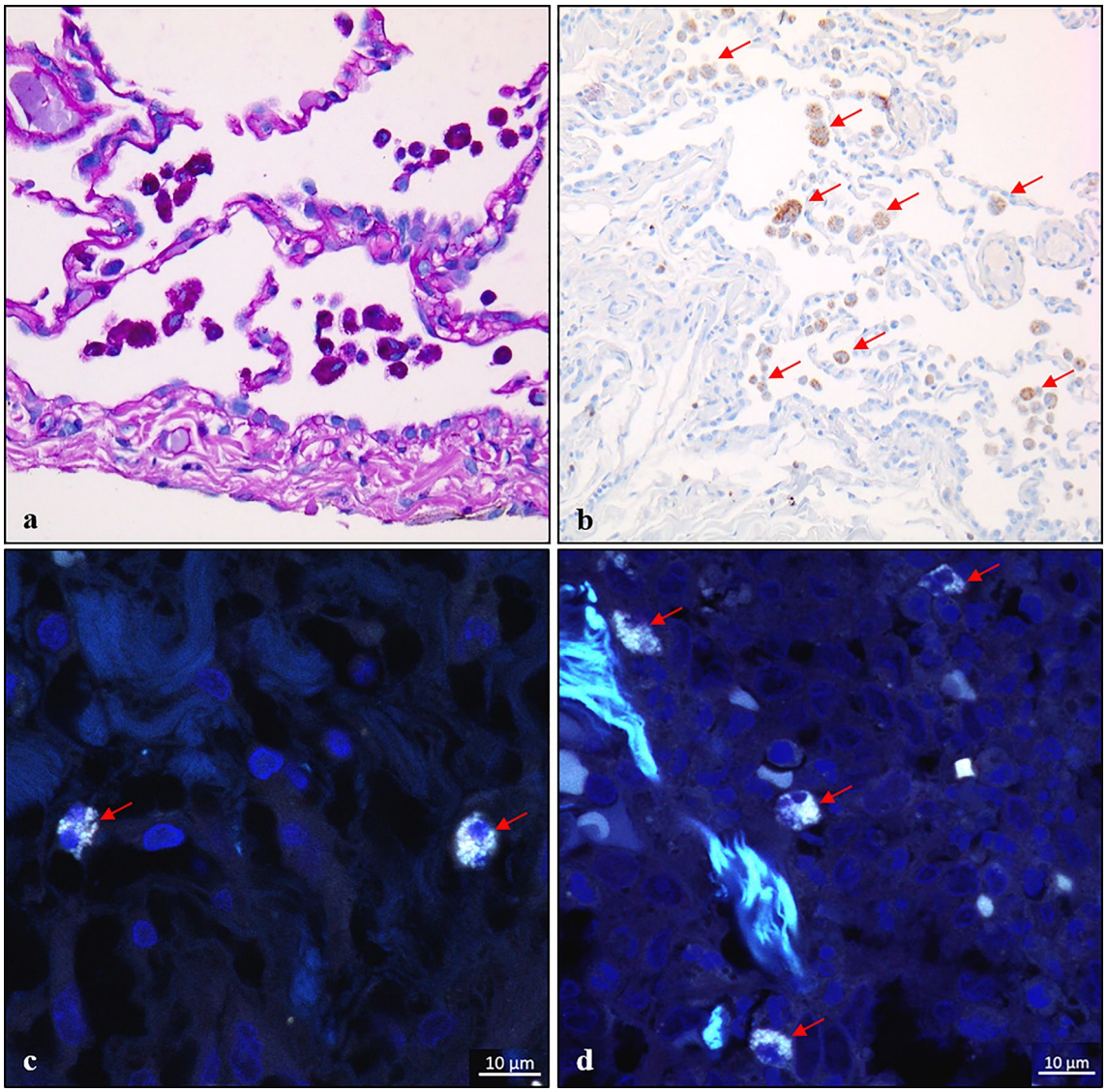

Fig. 2 PAS, Immunohistochemistry and FISH staining on pulmonary biopsy specimen from patients with Whipple disease. **a** *T. whipplei* was observed into macrophages seen in alveolar air spaces containing many PAS-positive particles in their cytoplasm (original magnifications ×200); **b** cytoplasmic vacuoles of macrophages in inflammatory infiltrates are packed with coarse granular immunopositive material (original magnifications ×100). The overlay (white) of the green and red filter sets shows rounded clusters of *T. whipplei* FISH positive localized in **c** alveolar air spaces **d** interalveolar areas (original magnifications ×63 and ×63, respectively)

our laboratory and was tested positive by molecular assays, its few quantity did not permitted to realize FISH.

Pulmonary involvement was estimated in 30–40% of patients with classical WD [11]. However, this is the first case of WD diagnosed by FISH in a pulmonary biopsy. *T. whipplei* has also been isolated from the bronchoalveolar fluid, reinforcing its role as a respiratory pathogen [4]. Moreover, there are few studies that reported a *T. whipplei* detection by FISH in lymph node [10, 12, 14] and recent studies on large series of lymphadenitis revealed a low identification of *T. whipplei* from lymph nodes [8, 15]. Although histology and FISH were compatible with WD, the pulmonary biopsy of the patient in case 2 was negative by the *T. whipplei* specific qPCRs [15, 18]. However, 16S and 23S rDNA sequencing performed in OLV Hospital Aalst (Belgium) revealed that this patient was infected by a *T. whipplei*-related species with unknown classification presenting only 98% similarity with *T. whipplei* for 16S rDNA and a unique 23S rDNA sequence [18]. This information was not available at the time the FISH analysis was performed in Marseille. BLAST analysis revealed that the *T. whipplei* FISH probe presented 100% similarity with both *T. whipplei* and this *Tropheryma* sp. (Supplementary Figure 5) explaining our FISH positive results but *T. whipplei* specific qPCR negative results.

In conclusion, we provide additional evidence that FISH on FFPE tissue can be a valuable method for the diagnosis of WD. Although FISH cannot replace the other already approved diagnostic techniques for WD, this method can be used as a complementary tool.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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