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Original Article

Pathological features of *Tropheryma whipple*-induced infective endocarditis in Northern China

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Abstract: Whipple's disease (WD) is a rare, chronic, and recurrent disease with diverse and non-characteristic clinical symptoms. WD is caused by the pathogen *Tropheryma whipplei* (TW). TW-induced infective endocarditis (TWIE) is rarely reported. This study aimed to understand the pathology of TW infection in patients with blood culture-negative infective endocarditis (BCNE) in northern China. 21 formalin-fixed and paraffin-embedded (FFPE) tissue specimens of heart valves from 18 BCNE patients were subjected to hematoxylin-eosin (HE) staining and Periodic acid-Schiff (PAS) staining, as well as PCR analysis and gene sequencing. HE staining showed macrophage-derived foam cell infiltration in 8 specimens from 5 patients, and PAS staining was positive in 15 specimens from 13 patients. Detection of TW 16S rRNA by PCR showed that 2 patients were positive for TW, which was confirmed by gene sequencing. In conclusion, this study is the first to detect nucleic acids of TW pathogens in specimens from WD patients in China.

Keywords: Whipple's disease, *Tropheryma whipplei*, infective endocarditis, 16S rRNA

Introduction

Whipple's disease (WD) was first reported by George Hoyt Whipple, an American pathologist, in 1907. The clinical symptoms of WD include multiple migratory arthritis, cough, diarrhea, steatorrhea, small intestinal malabsorption, progressive emaciation, and mesenteric lymphadenitis [1]. In 1991, Wilson et al. used PCR to confirm that WD was caused by a type of newly discovered bacteria *Tropheryma whipplei* (TW) [2]. TW is a widely present microorganism. However, WD patients are very rare, and contact with TW does not necessarily lead to the development of WD, which indicates that WD patients have genetic susceptibility [3].

TW can induce both chronic and acute infections and have asymptomatic carriers. There are several types of chronic infections induced by TW including classic WD, which primarily presents with histological damage to the gastrointestinal tract combined with various clinical symptoms, and local infection type of WD, which does not cause histological damage to the gastrointestinal tract but may lead to endocarditis, infection of the nervous system, spine and joint infections, and uveitis [4-6]. The prevalence of endocarditis ranks second in chronic WD patients. The most common patients are men with an average age of approximately 60 years, and they usually experience joint pain or arthritis before the diagnosis of endocarditis [5]. There are very few clinical symptoms of infection with suggestive signs, and the Duke Criteria for the diagnosis of endocarditis cannot be used for cases caused by TW infection [5]. Currently, diagnosing endocarditis caused by TW infection is still a challenge and requires molecular biology detection techniques on heart valves obtained from surgery [7].

In this study we performed pathological analysis of TW infection in patients with blood culture-negative infective endocarditis (BCNE) in northern China. We examined 21 formalin-fixed, paraffin-embedded (FFPE) tissue specimens of heart valves from 18 BCNE patients and detect-

Table 1. Primers used in this study

Primer	Nucleotide sequence 5'-3'	Molecular target	Fragment length	Reference
TW27F	TGTTTTGTACTGCTTGTAACAGGATCT	TW Chromosome repeats	156 bp	[8]
TW182R	TCCTGCTCTATCCCTCCTATCATC			
13F	TGAGTGATGGTATGTCTGAGAGATATGT	TW Chromosome repeats	151 bp	[8]
163R	TCCATAACAAAGACAACAACCAATC			
pW3FE	AGAGATACGCCCCCCGCAA	TW 16S rDNA	266 bp	[9]
pW2RB	ATTCGCTCCACCTTGCGA			

ed TW pathogens using molecular biology techniques.

Materials and methods

Specimen

A total of 21 FFPE heart valve tissue specimens were collected from 18 BCNE patients hospitalized at the Department of Cardiothoracic Surgery of Daqing Oilfield General Hospital between 2012 and 2014.

Histopathological analysis

21 FFPE heart valve tissue specimens from 18 patients were sectioned at 10-μm thick and subjected to hematoxylin and eosin (HE) and Periodic acid-Schiff (PAS) staining according to standard protocols. The stained sections were observed under a microscope.

DNA extraction

DNA was extracted from 5-8 FFPE heart valve tissue specimen sections using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

Primer design

Primers were designed based on the literatures as follows: TW27F-TW182R primer pair: amplification of one non-coding specific repetitive sequence in the TW chromosome with a 105 bp length [8]; TW13F-TW163R primer pair: amplification of one non-coding specific repetitive sequence in the TW chromosome with a 98 bp length [8]; and pW3FE-pW2RB primer pair: amplification of one 266-bp specific DNA sequence fragment from the TW 16S rRNA [9]. The primer sequences were shown in **Table 1**.

Real-time PCR

The two specific DNA repetitive sequences in the TW chromosome were detected using the SYBR Green I reagent (TaKaRa, Dalian, China) in the Roche LightCycler 2.0 fluorescence quantitative PCR machine. TW27F-TW182R primer pair was used to detect 105-bp specific DNA repetitive sequence in the TW chromosome. PCR conditions were as follows: 95°C denaturation for 30 s and 40 cycles of 95°C denaturation for 5 s and 60°C annealing and extension for 30 s. The single-channel signal collection mode was used for the melting curve analysis. If the result of the first quantitative PCR assay was positive, a second quantitative PCR assay was performed for validation. TW13F-TW163R primer pair was used to detect 98-bp specific repetitive DNA sequence in the TW chromosome, using the same system and conditions as described above.

Sequencing

pW3FE-pW2RB primer pair was used to amplify a specific DNA sequence in TW 16S rRNA. PCR products were sent to Shanghai Sangon Biotech for sequencing analysis.

Results

Histopathological analysis

HE staining showed that among the 18 patients, 8 heart valve tissue specimens from five patients (No. 2, 5, 9, 11, and 15) contained foam-like macrophages. The features included vacuoles and granular substances in the cytoplasm (**Figure 1**, **Table 2**). Among the 18 patients, 15 specimens from 13 patients showed positive PAS staining. The features included many red amylase-resistant, Ziehl-Neelsen staining-negative inclusion bodies in the foam-like macrophages (**Figure 1**, **Table 2**).

Genetic analysis

Quantitative PCR analysis showed that TW was detected in two patients (No. 2 and 5). TW 16S

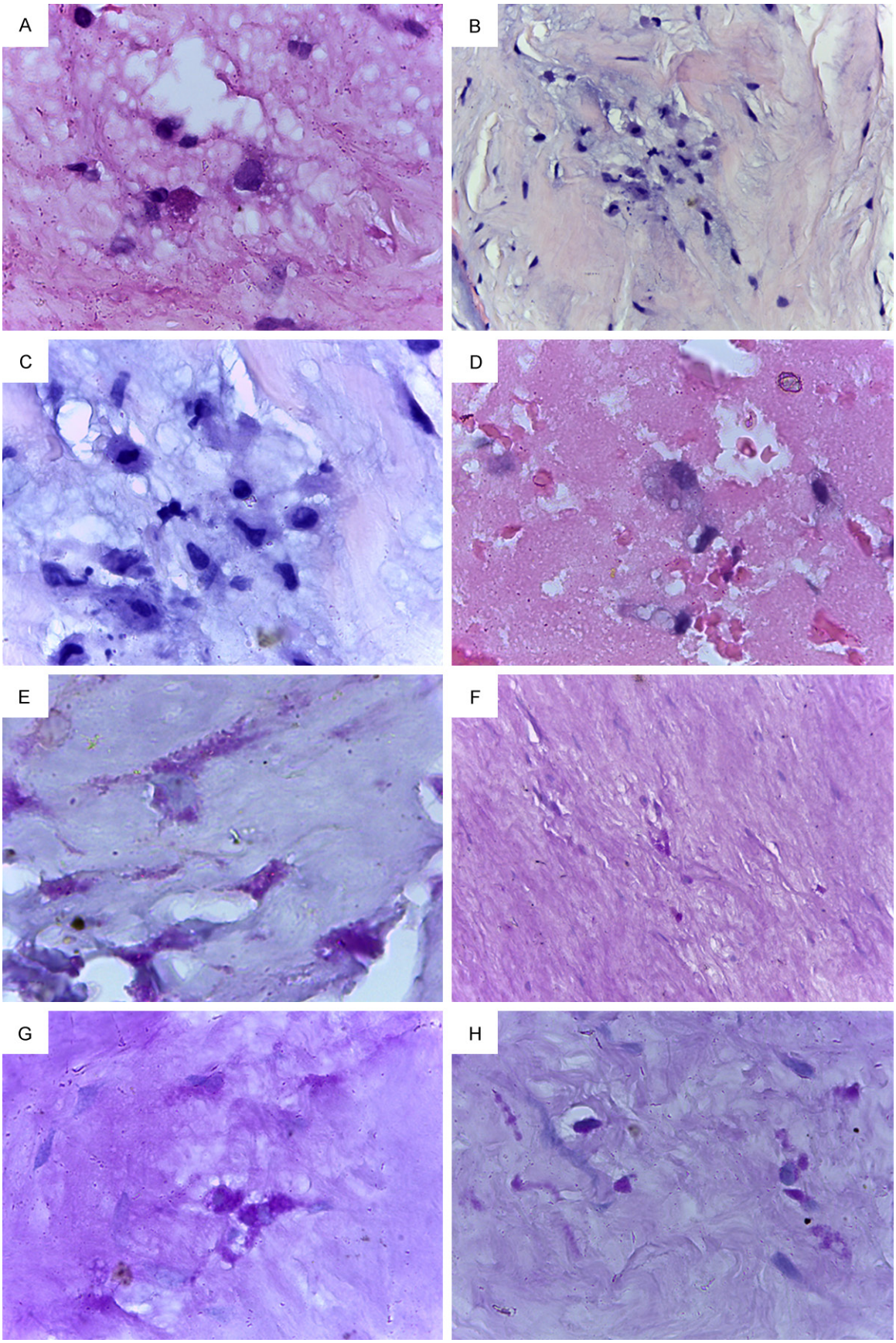
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Figure 1. HE staining and PAS staining of FFPE heart valve specimens. A-D: HE staining (specimen No. 2, 5, 5, and 9, respectively): specimens with macrophage infiltration, foam-like macrophages could be observed; E-H: PAS staining (specimen No. 2, 5, 5, and 17, respectively): specimens with positive PAS staining foam-like macrophages containing vacuoles, red granules, and rod-like substances could be observed.

Table 2. HE staining, PAS staining, and PCR results in 18 patients with BCNE

Patient	Specimen number	Gender	Age at diagnosis (year)	Specimen	Foam-like macrophages	PAS staining	PCR
1	M1	F	54	Mitral valve			
2	M2	F	54	Mitral valve	+	+	+
3	M3	F	50	Mitral valve		+	
4	M4	F	55	Mitral valve		+	
5	M5	F	64	Left atrium, Mitral	+	+	+
6	M6	M	64	Mitral valve			
7	M7	F	59	Mitral valve		+	
8	M8	F	68	Mitral valve		+	
9	M9	F	45	Mitral valve	+		
	M10			Mitral valve	+		
10	M11	M	58	Mitral valve		+	
11	M12	F	69	Mitral valve	+	+	
	M13			Mitral valve	+	+	
12	M14	F	71	Mitral valve		+	
13	M15	F	45	Mitral valve			
14	M16	F	49	Mitral valve		+	
15	M17	F	54	Left atrium, Mitral	+	+	
	M18			Left atrium, Mitral	+	+	
16	M19	M	43	Mitral valve		+	
17	M20	F	48	Mitral valve		+	
18	M21	F	64	Mitral valve			

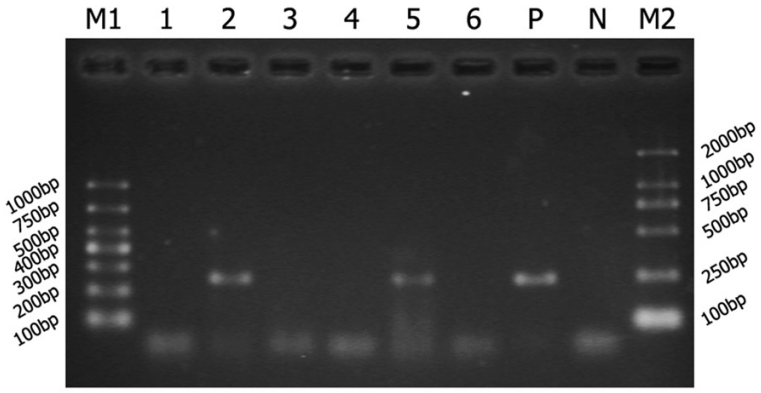

Figure 2. Agarose electrophoresis of PCR products of TW 16S rRNA-specific fragment. M1: DNA marker (DL1000); M2: DNA marker (DL2000); P: positive; N: negative control; Lanes 1-6: PCR products of the specimen No. 1-6, respectively.

rRNA specific primers pW3FE-pW2RB gave to amplified products of 266 bp (**Figure 2**, **Table 2**). The amplified products were used for sequencing analysis (**Figure 3**). The sequences were completely consistent with TW 16S rDNA sequence in GenBank (accession number AE014184).

Discussion

WD is a very rare disease with a prevalence of approximately 1 in 1 million, and the patients are mainly middle-aged Caucasian men. The male to female ratio is approximately 8:1 and the prevalence in farmers is higher [10]. With the advancement of detection techniques and the successful *in vitro* isolation and culture of TW in recent years, the number of confirmed WD patients has increased rapidly

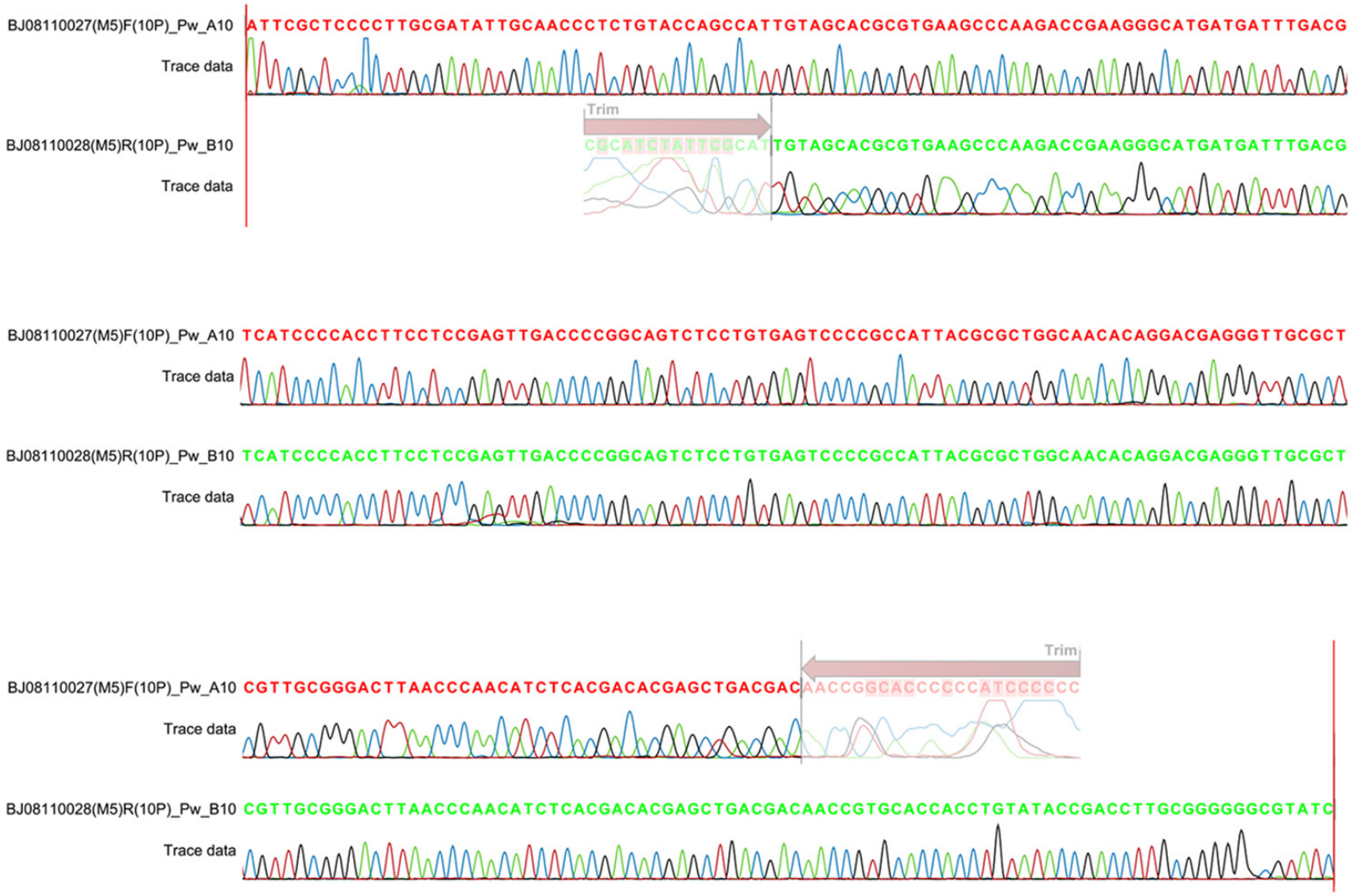

Figure 3. The sequencing of PCR product of TW 16S rRNA-specific sequence from specimen No. 5.

and approximately 1,500 cases have been confirmed in Europe [11]. In contrast, reported WD cases are rare in Asia. Until 2013, there were only 14 confirmed WD cases in Japan [12]. WD case was first reported in China in 1982, and only 16 cases have been reported since then [13]. In addition to genetic differences between Caucasians and Asians and regional differences in the distribution, differences in the awareness and diagnosis of WD may contribute to the difference in the number of cases. In China, all reported 16 cases of WD were diagnosed based on clinical symptoms and histopathological examinations of the patients, and molecular biology techniques were not used to confirm the presence of TW pathogen.

In this study, we used PCR to examine 18 specimens from BCNE patients and confirmed the results by gene sequencing. Therefore, we definably detected the pathogens in 2 cases of TW-induced infective endocarditis. Our results confirmed for the first time endocarditis caused by TW infection in northern China. Therefore, physicians in China should promote the clinical application of molecular biology techniques in the diagnosis of WD to increase the rate of diagnosis.

Currently, IE pathogens are mainly diagnosed clinically through blood culture. If patients use antibacterial drugs before blood culture or if the pathogens are fastidious microbes, blood culture results will be negative. The prevalence of BCNE has been reported to be only 2.5-31% [14]. Therefore, the early diagnosis of BCNE is very important. The amplification of 16S rRNA by PCR and sequencing techniques has been utilized for the diagnosis of TWIE [15]. Currently, more than 100 cases of TWIE have been reported worldwide [16]. The diagnosis of TWIE requires specimens obtained through heart valve replacement surgery. Therefore, clinical diagnosis is more difficult. A study revealed that the most common IE causing bacteria are *Streptococcus*, *Staphylococcus*, *Enterococcus*, and TW in the order. TW has the fourth highest isolation rate (6.3%) and is the most common bacterium that causes BCNE [16]. In this study, TW detection rate among the 18 cases of BCNE was 11.1%. Further studies are needed to identify other pathogens of BCNE.

TW grows slowly, which makes the culture very difficult [17, 18]. PCR is currently the preferred detection method for WD diagnosis. Although 16S rRNA was first used, the repetitive gene sequences of TW increase the detection sensitivity without changing the specificity, and have been used to detect TW in various specimens including saliva, synovial fluid, cerebrospinal fluid, stool, heart valve tissue, and all types of biopsy tissues [19]. However, PCR results need to be confirmed by gene sequencing to exclude false positive results [20]. TWIE is currently diagnosed by the detection of TW nucleic acids in heart valve specimens using PCR [16]. In this study, 2 specific non-coding repetitive sequences in TW chromosome were targeted, and PCR results were positive in 2 cases. After validation by sequencing of 16S rRNA specific sequences, we confirmed that the pathogen in these 2 cases of BCNE was TW.

In the past, the diagnosis of WD relied mainly on histological characteristics such as the presence of macrophage-derived foam cells and positive PAS staining. PAS staining of duodenal biopsy specimens was once the preferred diagnostic test for WD [21, 22]. However, PAS staining is not specific, and infections induced by certain pathogens such as *Mycobacterium avium* complex, *Rhodococcus equi*, *Bacillus cereus*, *Corynebacterium, Histoplasma*, and fungi can also exhibit positive PAS staining [23-25]. In this study, PAS staining of the FFPE samples from 13 patients were positive, but only 2 cases of TWIE were confirmed by PCR. Therefore, our results further confirm the non-specificity of PAS staining in the diagnosis of TW and the specificity of molecular biology methods.

In conclusion, this is the first study to combine pathological analysis and molecular genetic techniques to analyze TWIE samples in northern China. With an in-depth understanding of TW and wide application of molecular biology techniques for TW detection, the rate of misdiagnosed TWIE will be reduced.

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Disclosure of conflict of interest

None.

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