Evaluation of the Biotoxis qPCR Detection Kit for Francisella tularensis Detection in Clinical and Environmental Samples

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ABSTRACT Rapid and reliable detection and identification of *Francisella tularensis* (a tier 1 select agent) are of primary interest for both medical and biological threat surveillance purposes. The Biotoxis qPCR detection kit is a real-time quantitative PCR (qPCR) assay designed for the detection of *Bacillus anthracis*, *Yersinia pestis*, and *F. tularensis* in environmental or biological samples. Here, we evaluated its performance for detecting *F. tularensis* in comparison to previously validated qPCR assays. The Biotoxis qPCR was positive for 87/87 *F. tularensis* subsp. *holarctica* (type B) strains but also for *F. tularensis* subsp. *novicida*. It was negative for *Francisella philomiragia* and 24/24 strains belonging to other bacterial species. For 31 tularemia clinical specimens, the Biotoxis qPCR displayed a sensitivity between 90.32% and 96.55%, compared to qPCR tests targeting ISFtu2 or a type B-specific DNA sequence, respectively. All 30 nontularemia clinical specimens were Biotoxis qPCR negative. For water samples, the Biotoxis qPCR limit of detection was 1,000 CFU/liter of *F. tularensis*. For 57 environmental water samples collected in France, the Biotoxis qPCR was positive for 6/15 samples positive for ISFtu2 qPCR and 4/4 positive for type B qPCR. In conclusion, the Biotoxis qPCR detection kit demonstrated good performances for *F. tularensis* detection in various biological and environmental samples, although cross-amplification of *F. tularensis* subsp. *novicida* must be considered. This plate format assay could be useful to test a large number of clinical or environmental specimens, especially in the context of natural or intentional tularemia outbreaks.

KEYWORDS *Francisella tularensis*, diagnosis, real-time PCR, tularemia

Francisella tularensis is a small Gram-negative bacterium causing the zoonotic disease tularemia (1, 2). More strictly, among the four subspecies of *F. tularensis*, only two are currently associated with human and animal tularemia cases: *Francisella tularensis* subsp. *tularensis* (type A strains), in North America, and *Francisella tularensis* subsp. *holarctica* (type B strains) in the whole Northern Hemisphere and southern Australia (1–3). *Francisella tularensis* subsp. *mediasiatica* has been detected in arthropods and rodents in central Asia and Russia (1) but never associated with human infections. *Francisella tularensis* subsp. *novicida* (also referred to as *F. novicida*) is an aquatic bacterium, rarely responsible for opportunistic human diseases (4). Other aquatic *Francisella* species have been described, including *Francisella philomiragia*, another rare opportunistic human pathogen (4).

F. tularensis can infect a wide range of vertebrate species (especially lagomorphs and small rodents) and arthropods (including *Ixodidae* ticks and mosquitoes) (1, 2). This bacterium is also able to survive for prolonged periods in the hydrotelluric environment (1, 2, 4). *F. tularensis* can contaminate humans through contact with infected animals, arthropod bites, exposure to contaminated environments, or ingestion of contami-

Citation Hennebique A, Gas F, Batina H, De Araujo C, Bizet K, Maurin M. 2021. Evaluation of the Biotoxis qPCR detection kit for *Francisella tularensis* detection in clinical and environmental samples. J Clin Microbiol 59:e01434-20. https://doi.org/10.1128/JCM.01434-20.

Editor Brad Fenwick, University of Tennessee at Knoxville

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Received 8 June 2020
Returned for modification 9 July 2020
Accepted 16 October 2020
Accepted manuscript posted online 28 October 2020
Published 17 December 2020

nated food or water (1, 2). Several clinical forms of tularemia exist, mainly depending on the portal of entry of the bacteria. The ulceroglandular and glandular forms correspond to localized lymphadenopathy with or without a skin ulcer, respectively. The oropharyngeal form combines pharyngitis and cervical lymphadenopathy. The oculoglandular form usually corresponds to conjunctivitis with periauricular lymphadenopathy. The pneumonic form (pneumonia or pleuropneumonia) usually occurs through the inhalation of contaminated aerosols. The typhoidal form is severe sepsis, often with confusion, but with no inoculation lesion or lymphadenopathy (1, 2). Because of its ability to be spread by aerosols, its low infectious dose, and high virulence, *F. tularensis* is classified in category A of potential biological threat agents by the U.S. Centers for Disease Control and Prevention (CDC) (5). Pneumonic tularemia cases caused by the most virulent type A strains are associated with up to 30% mortality rates (1, 2, 5).

Tularemia diagnosis is currently based on serological methods, and *F. tularensis* detection is performed by culture or PCR-based methods (2). However, this fastidious and slow-growing bacterium is usually isolated from less than 10% of tularemia patients (2). Serological methods can detect significant antibody titers only 2 to 3 weeks after disease onset (2, 6). Therefore, real-time PCR tests are currently the most effective method for detecting *F. tularensis* in human, animal, or environmental samples (2). Although many in-house qPCR tests have been developed, a user-friendly commercial test allowing rapid, accurate, and standardized detection of *F. tularensis* in various types of samples is a high priority.

The Biotoxis quantitative PCR (qPCR) detection kit is a plate-format TaqMan probe-based real-time PCR assay designed for the combined detection of *Bacillus anthracis*, *Yersinia pestis*, and *F. tularensis* DNA in various sample types. In the present study, we evaluated this kit's performance for detection and identification of *F. tularensis* in DNA extracts from bacterial strains, clinical samples, and environmental water samples, in comparison to previously validated PCR tests used routinely in our laboratory.

MATERIALS AND METHODS

Biotoxis qPCR assay. The Biotoxis qPCR detection kit (Bertin Bioreagent, Montigny-le-Bretonneux, France) was carried out according to the manufacturer's instructions. Briefly, each of the PCR mixtures (25 μl) contained 12.5 μl of the qPCR mix, 3.75 μl of primer and probe mix, 3.75 μl of water, and 5 μl of DNA sample (at various concentrations according to sample type; see below). The PCR was performed on a LightCycler 480 instrument II (Roche) with an initial enzyme activation step of 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 30 s. The results were analyzed at the endpoint.

***Francisella tularensis* reference qPCR assays.** We compared results obtained with the Biotoxis qPCR detection kit with those obtained with three previously validated qPCR methods, which are routinely used at the French National Reference Center for *Francisella* (French NRCF) (Grenoble University Hospital, Grenoble, France). One qPCR test (referred to as ISFtu2 qPCR) targets the ISFtu2 insertion sequence present in multiple copies in the *F. tularensis* genome (7). The second one (Tul4 qPCR) targets the gene encoding the single-copy Tul4 surface protein-encoding gene (7). The last one (type B qPCR) targets a DNA fragment located between ISFtu2 and a flanking 3′ region and is specific for *F. tularensis* subsp. *holarctica* (8).

These three qPCR tests were performed using the same protocol. Each of the PCR mixtures (20 μl) contained 10 μl of 2× TaqMan Fast Advanced PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania), 0.4 μl of each primer at 10 μM, 0.4 μl of 2 μM probe, 3.8 μl of water, and 5 μl of DNA sample (at various concentrations according to sample type; see below). The PCRs were run on a LightCycler 480 instrument II (Roche) with an initial enzyme activation step of 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s. The results were analyzed at the endpoint.

DNA extraction methods. For bacterial strains and clinical samples, DNA extraction was obtained using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France), according to the manufacturer's recommendations.

For artificial and environmental water samples, 1 liter of water was filtered through 0.22-μm or 0.45-μm filters, and DNA was extracted from the filter using the NucleoMag DNA/RNA water kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's recommendations.

Concentrations of bacterial DNA extracts and environmental water DNA extracts were then assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

Bacterial strains. We tested 90 *Francisella* sp. strains, including 86 clinical strains of *F. tularensis* subsp. *holarctica*, one clinical strain of *F. philomiragia*, and the reference strains *F. philomiragia* ATCC 25015, *F. novicida* CIP 56.12, and *F. tularensis* subsp. *holarctica* LVS NCTC 10857 (Table 1). Clinical strains

TABLE 1 Bacterial strains used in this study

Species	Strain
F. tularensis subsp. *holarctica*	Clinical strains (86 strains, including Ft5 to -46, Ft48 to -65, Ft67 to -74, Ft76 to -80, Ft83 to -89, Ft91, and Ft92 to -96)
	LVS NCTC 10857
F. novicida	CIP 56.12
F. philomiragia	Clinical strain Ft47
	ATCC 25015
Corynebacterium jeikeium	CIP 8251
Staphylococcus epidermidis	CIP 103627
Staphylococcus aureus	ATCC 6538
Streptococcus agalactiae	ATCC 12400
Streptococcus pneumoniae	ATCC 49619
Streptococcus uberis	ATCC 9727
Streptococcus salivarius	Clinical strain
Streptococcus mitis	CIP 103335
Streptococcus oralis	Clinical strain
Enterococcus faecalis	ATCC 29212
Enterococcus faecium	CIP 5432
Bacillus subtilis	ATCC 6633
Haemophilus influenzae	ATCC 49766
Acinetobacter baumannii	ATCC 19606
Escherichia coli	ATCC 25922
Enterobacter cloacae	ATCC 13047
Klebsiella pneumoniae	ATCC 35657
Staphylococcus sciuri	ATCC 29061
Stenotrophomonas maltophilia	ATCC 17666
Pseudomonas aeruginosa	CIP 5933
Streptococcus pyogenes	CIP 104226
Neisseria elongata	Clinical strain
Moraxella catarrhalis	Clinical strain
Serratia marcescens	CIP 103551

of *Francisella* sp. were previously identified at the French NRCF. *F. philomiragia* was identified by whole 16S rRNA gene sequencing (9). *F. tularensis* subsp. *holarctica* was identified at the subspecies level by combining a positive ISFtu2 or Tul4 qPCR test and either a positive type B qPCR or PCR amplification and sequencing of the expected 16S-23S intergenic region (10). The French NRCF owns all the above strains, and specific authorizations were obtained from the Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM; authorization number ADE-103892019-7). In addition, 24 reference or clinical strains belonging to bacterial species other than *Francisella* sp. were used (Table 1).

Bacteria were grown on chocolate agar medium supplemented with PolyViteX (bioMérieux, Marcy-l'Etoile, France) or on sheep blood agar medium (bioMérieux, Marcy-l'Etoile, France) at 35°C in a 5% CO_2-enriched atmosphere for 1 or 2 days. *Francisella tularensis* strains were grown in a biosafety level 3 laboratory.

DNA extracts from *Francisella* sp. strains were prepared at 10 ng/μl for analysis with the Biotoxis qPCR detection kit. DNA extracts from four clinical strains of *F. tularensis* subsp. *holarctica*, one clinical strain of *F. philomiragia* (*F. philomiragia* ATCC 25015), and *F. novicida* CIP 56.12 were used to compare cycle threshold (C_T) values obtained with the Biotoxis qPCR to those of the ISFtu2, Tul4, and type B qPCR tests (Table 2). For non-*Francisella* strains, three DNA extract concentrations were used (0.1, 1, and 10 ng/μl).

Clinical specimens. For sensitivity purposes, we tested 31 clinical samples (mainly lymph node and respiratory samples) collected for routine medical care in 30 tularemia patients, including 29 confirmed cases and one probable case (Table 3). These samples were received at the French NRCF between 2018

TABLE 2 Biotoxis qPCR testing of bacterial strains

	Result (C_T) in qPCR			
Strain[a]	Biotoxis	ISFtu2	Tul4	Type B
F. tularensis type B Ft41	Positive (20)	Positive (14)	Positive (19)	Positive (19)
F. tularensis type B Ft54	Positive (20)	Positive (13)	Positive (17)	Positive (18)
F. tularensis type B Ft72	Positive (19)	Positive (13)	Positive (17)	Positive (18)
F. tularensis type B Ft92	Positive (18)	Positive (12)	Positive (15)	Positive (16)
F. novicida CIP 56.12	Positive (18)	Positive (12)	Positive (16)	Negative
F. philomiragia Ft47	Negative	Positive (26)	Negative	Negative
F. philomiragia ATCC 25015	Negative	Positive (36)	Negative	Negative

[a]DNA extracts were standardized at 10 ng/μl.

TABLE 3 Biotoxis qPCR testing of 31 clinical samples from 30 tularemia patients[a]

Sample	Type	Clinical form	Result of:			
				qPCR (C_T)[b]		
			Serology/culture	ISFtu2	Type B	Biotoxis
S1	LN	GL	POS/NA	POS (27.97)	POS (31.96)	POS (34.50)
S2[c]	Peritoneal tissue	INT	POS/NEG	POS (29.35)	POS (35.06)	POS (32.59)
S3[c]	Peritoneal liquid	INT	POS/NEG	POS (28.22)	POS (32.57)	POS (35.05)
S4	LN	GL	POS/NEG	POS, 1/100 (31.98)	POS, 1/100 (39.71)	POS, 1/100 (38.06)
S5	LN	PNE	POS/NEG	POS (20.47)	POS (28.89)	POS (27.37)
S6	BAL	PNE	POS/NA	POS (30.48)	POS (34.65)	POS (37.29)
S7	BAL	PNE	POS/NA	POS (28.64)	POS (33.06)	POS (35.66)
S8	LN	GL	POS/NEG	POS (27.35)	POS (34.82)	POS (32.60)
S9	LN	PNE	POS/NEG	POS (30.62)	POS (35.17)	POS (39.06)
S10	Tracheal aspirate	PNE	NA/NEG	POS (30.88)	POS (37.02)	POS (37.63)
S11	LN	GL	NA/NEG	POS (30.55)	POS (34.66)	POS (36.23)
S12	LN	GL	POS/NEG	POS (23.56)	POS (32.92)	POS (30.22)
S13	LN	GL	NEG/NEG	POS (32.14)	POS (36.43)	POS (38.76)
S14	LN	GL	SCV/NEG	POS (33.85)	POS (38.25)	NEG
S15	LN	GL	SCV/NEG	POS (30.56)	POS (35.02)	POS (34.90)
S16	Sputum	PNE	NA/NA	POS (29.64)	POS (36.09)	POS (36.04)
S17	LN	UG	POS/NEG	POS (27.10)	POS (33.76)	POS (32.23)
S18	LN	GL	NA/NEG	POS (26.24)	POS (30.64)	POS (30.09)
S19	LN	GL	POS/NEG	POS (28.50)	POS (34.76)	POS (31.90)
S20	LN	PNE	POS/NA	POS (36.61)	NEG	NEG
S21	Knee prosthesis	OA	POS/POS	POS (30.94)	POS (35.89)	POS (37.19)
S22	LN	UG	POS/NEG	POS (23.82)	POS (29.68)	POS (30.79)
S23	LN	UG	NA/NEG	POS (25.29)	POS (29.53)	POS (28.71)
S24	LN	OG	NA/NEG	POS (28.70)	POS (35.76)	POS (35.80)
S25	LN	OP	POS/NEG	POS (24.18)	POS (30.14)	POS (31.69)
S26	LN	UG	POS/NEG	POS (30.11)	POS (35.57)	POS (35.77)
S27	LN	GL	FFR/NEG	POS (34.85)	NEG	NEG
S28	LN	GL	POS/NEG	POS (25.50)	POS (30.33)	POS (31.91)
S29	LN	GL	POS/NEG	POS (29.46)	POS (33.56)	POS (35.86)
S30	LN	PNE	NA/NEG	POS (32.82)	POS (35.83)	POS (37.96)
S31	LN	UG	NA/NEG	POS (24.72)	POS (31.98)	POS (31.25)

[a]UG, ulceroglandular; GL, glandular; PNE, pneumonic; OG, oculoglandular; OP, oropharyngeal; INT, intestinal; OA, osteoarticular; LN, lymph node; BAL, bronchoalveolar lavage; NA, not available; NEG, negative; POS, positive; SCV, seroconversion; FFR, a fourfold rise in antibody titers.
[b]DNA extracts were tested pure or at the specified dilution.
[c]Samples from the same patient.

and 2019 for tularemia diagnostic confirmation. A tularemia confirmed case was defined as an instance of clinically compatible disease with detection of *F. tularensis* from any clinical specimen by culture or PCR (ISFtu2 or type B qPCR, $C_T \leq 35$), seroconversion, or a 4-fold (or greater) change in titers of serum antibody to *F. tularensis* antigen between acute- and convalescent-phase sera. A probable case was defined as an instance of a clinically compatible disease with a single positive serum sample. According to our laboratory's routine procedure, all clinical samples were first tested using ISFtu2 qPCR for *F. tularensis* detection (7). Positive samples were further tested using type B qPCR for the identification of subspecies *F. tularensis* subsp. *holarctica* (8). When possible, *F. tularensis* culture was also performed.

These samples were analyzed by the Biotoxis qPCR detection kit retrospectively. DNA extracts were analyzed pure and diluted to 1/10 and 1/100. Samples were considered positive if at least one of the three DNA dilutions tested was positive. Negative results were duplicated for confirmation.

For specificity purposes, we also tested 30 clinical samples from patients for which the French NRCF excluded tularemia diagnosis because of negative diagnostic tests for *F. tularensis*, including eight samples positive for *Bartonella henselae* by PCR. All these samples were collected as part of routine patient care and analyzed retrospectively using the Biotoxis qPCR detection kit.

Environmental samples. We first determined the limit of detection (LOD) of the Biotoxis qPCR using *F. tularensis*-spiked water samples. For this purpose, a water sample collected in the environment was first sterilized by filtration on a 0.22-μm filter. Several aliquots were then inoculated with *F. tularensis* subsp. *holarctica* LVS NCTC 10857 strain at concentrations ranging from 0.1 to 10,000 CFU/liter. One liter of each aliquot was filtered through a 0.22-μm filter, and DNA was extracted from the filter. The DNA extracts were tested using the Biotoxis qPCR detection kit (in triplicate) and by ISFtu2, Tul4, and type B qPCRs, which were used as controls.

We then tested 57 surface water samples collected in France in 2019 from various aquatic environments. One liter of each sample was passed through a 0.45- or 0.22-μm filter, from which DNA was extracted. DNA extracts were then adjusted to 10 ng/μl and tested in duplicate by the ISFtu2, Tul4, and type B qPCR tests. These qPCR tests were considered positive only when both duplicate tests displayed a C_T of $\leq$36 for ISFtu2 qPCR or a C_T of $<$40 for Tul4 and type B qPCRs. However, the presence of *F.*

tularensis DNA was considered confirmed only for water samples with a positive type B qPCR test, which can specifically detect *F. tularensis* subsp. *holarctica* strains found in France. Water samples were then tested using the Biotoxis qPCR detection kit, and results were compared to those of the three previous qPCR tests.

RESULTS

Bacterial strains. The Biotoxis qPCR gave a strongly positive signal (C_T values between 15 and 22) for the 86 clinical strains of *F. tularensis* subsp. *holarctica* and for the reference strain *F. tularensis* subsp. *holarctica* LVS NCTC 10857, corresponding to a 100% sensitivity for the tested type B tularemia strains. DNA extracts from four clinical strains of *F. tularensis* subsp. *holarctica* were tested to determine the analytical sensitivity of the Biotoxis qPCR, compared to those of the ISFtu2, Tul4, and type B qPCR tests. The C_T ranges for these four samples were 18 to 20 for the Biotoxis qPCR, 12 to 14 for ISFtu2 qPCR, 15 to 19 for Tul4 qPCR, and 16 to 19 for type B qPCR (Table 2).

The Biotoxis qPCR also gave a strongly positive signal for *F. novicida* CIP 56.12, like the ISFtu2 and Tul4 qPCR tests (7). As expected, type B qPCR was negative for this strain (Table 2). Biotoxis qPCR did not amplify the two *F. philomiragia* strains tested. The ISFtu2 qPCR gave a weak signal for this species, while Tul4 and type B qPCRs were negative (Table 2) (7).

The Biotoxis qPCR was negative for 21 non-*Francisella* strains but gave a weak signal (C_T between 34 and 37 at 10 ng/μl of DNA) for *Streptococcus salivarius*, *Pseudomonas aeruginosa* CIP 5933, and *Neisseria elongata* strains. For these three strains, testing lower DNA concentrations (0.1 to 1 ng/μl) abolished the amplification signal. Overall, the Biotoxis qPCR displayed a 96.30% specificity for the 27 non-type B strains tested owing to the cross-amplification of *F. novicida*.

Clinical samples. The 31 clinical samples collected from 30 tularemia patients included 24 lymph nodes, four respiratory samples, two peritoneal samples, and one osteoarticular sample (Table 3).

Tests were positive for 31/31 clinical samples for ISFtu2 qPCR, 29/31 for type B qPCR, and 28/31 for Biotoxis qPCR (Table 3). Therefore, the Biotoxis qPCR test's sensitivity could be evaluated at 90.32% and 96.55% compared to ISFtu2 and type B qPCR tests, respectively. The C_T ranged from 20.47 to 36.61 for ISFtu2 qPCR (with 30/31 tests with a C_T of $\leq$35), 28.89 to 39.71 (20/31 tests with a C_T of $\leq$35) for type B qPCR, and 27.37 to 39.06 (15/31 tests with a C_T of $\leq$35) for Biotoxis qPCR (Table 3).

The Biotoxis qPCR was positive for 27/31 pure DNA extracts (with C_T values ranging from 27.37 to 39.06), 19/31 DNA extracts diluted 1/10 (C_T, 29.85 to 39.43), and 13/31 DNA extracts diluted 1/100 (C_T, 33.26 to 39.26). Interestingly, sample S4 was positive by ISFtu2 qPCR, type B qPCR, and Biotoxis qPCR only when diluted 1/100, suggesting the presence of PCR inhibitors.

The 30 clinical samples from patients for which tularemia diagnosis was excluded corresponded to 14 lymph nodes, six blood samples, five abscess samples, one respiratory sample, and four biopsy samples. The Biotoxis qPCR gave negative results for these 30 clinical samples, including the eight patients with bartonellosis, corresponding to a 100% specificity.

Environmental samples. Water samples artificially inoculated with *F. tularensis* subsp. *holarctica* LVS NCTC 10857 from 0.1 to 10,000 CFU/liter were analyzed by Biotoxis qPCR and by ISFtu2, Tul4, and type B qPCRs. Biotoxis qPCR detected *F. tularensis* from 1,000 CFU/liter (with a C_T at 34.47). It was less sensitive than type B qPCR (positive from 100 CFU/liter with a C_T at 38.23), Tul4 qPCR (positive from 10 CFU/liter with a C_T at 36.96), and ISFtu2 qPCR (positive from 0.1 CFU/liter with a C_T at 37.57).

The 57 environmental water samples collected in France were previously tested using the ISFtu2, Tul4, and type B qPCR tests. Fifteen were positive by ISFtu2 qPCR, nine were positive by both ISFtu2 qPCR and Tul4 qPCR, and four were positive by ISFtu2 qPCR, Tul4 qPCR, and type B qPCRs. The Biotoxis qPCR was positive for six (40%) of the 15 samples that were positive by ISFtu2 qPCR, five of the nine samples that were positive by both ISFtu2 and Tul4 qPCR, and the four samples that were positive by

TABLE 4 Biotoxis qPCR testing of 57 environmental water samples

Result of qPCR				No. of water samples
ISFtu2	Tul4	Type B	Biotoxis (C_T)	
Positive	Positive	Positive	Positive (35.52–39.53)	4
Positive	Positive	Negative	Positive (38.23)	1
Positive	Negative	Negative	Positive (39.58)	1
Positive	Positive	Negative	Negative	4
Positive	Negative	Negative	Negative	5
Negative	Negative	Negative	Negative	42

ISFtu2, Tul4, and type B qPCRs (Table 4). The Biotoxis qPCR was negative for all 42 samples that were negative by the ISFtu2, Tul4, and type B qPCRs (Table 4).

DISCUSSION

In this study, we evaluated the commercial Biotoxis qPCR detection kit's performances for the detection of *F. tularensis* in clinical and water samples, compared to previously validated qPCR tests recommended by the World Health Organization for tularemia diagnosis (11).

We first evaluated this kit's ability to detect clinical and reference strains of *F. tularensis* previously identified in our laboratory. The 86 clinical strains and the reference LVS strain of *F. tularensis* subsp. *holarctica* gave strong amplification signals with the Biotoxis qPCR (C_T between 15 and 22 for a DNA extract standardized at 10 ng/μl). Similar C_T values were obtained with the qPCR tests targeting the single-copy Tul4 or type B-specific DNA sequences. In contrast, lower C_T values were obtained with the multicopy ISFtu2 target (26 to 30 copies in *F. tularensis* subsp. *holarctica* genome [7]) (Table 2). To evaluate the specificity of the Biotoxis qPCR, we tested two *F. philomiragia* strains, one *F. novicida* strain, and 24 strains not belonging to *Francisella* species. The Biotoxis qPCR gave a strongly positive signal for the reference strain of *F. novicida*. This result was not unexpected, since *F. novicida* (also referred to as *F. tularensis* subsp. *novicida*) has $\geq$97.7% similarity at the genome level to *F. tularensis* (12). The same cross-amplification has been described for the ISFtu2 and Tul4 qPCR tests (7). Differentiating these two closely related microorganisms using a qPCR test remains highly challenging. However, such cross-amplification currently has little impact on tularemia diagnosis, because human infections with *F. novicida* are rare and associated with clinical and epidemiological contexts different from those of tularemia (4). Additional diagnostic tests are usually performed for tularemia diagnostic confirmation and differentiation of type A and type B infections (11). Because *F. novicida* is an aquatic bacterium, the situation is different when environmental water samples are tested. In this case, the Biotoxis qPCR test is not sufficiently discriminating. A more specific test must be performed for accurate differentiation between *F. novicida* and *F. tularensis*. Therefore, in the context of bioterrorism, the Biotoxis qPCR kit is currently not specific enough for rapid and accurate confirmation of the presence of *F. tularensis* in environmental samples.

The Biotoxis qPCR was negative for *F. philomiragia*, confirming the absence of cross-amplification of this other aquatic bacterium. The Biotoxis qPCR was also negative for 21 non-*Francisella* species tested, representing common human pathogens. When testing high DNA concentrations (10 ng/μl), we observed a weak qPCR signal ($C_T \geq 34$) for the *S. salivarius*, *P. aeruginosa*, and *N. elongata* strains. Using lower DNA concentrations could eliminate such nonspecific signals. It should be recommended that DNA concentrations of 0.1 ng/μl be used when DNA extracts from bacterial strains are being tested, to avoid nonspecific amplification.

We did not include *F. tularensis* subsp. *tularensis* (type A) strains because we do not possess such strains in the French NRCF. The genes classically used to target *F. tularensis* (i.e., the ISFtu2 element, the 23-kDa-protein gene, *fopA*, and *tul4*) detect all four *F. tularensis* subspecies (7). Although the identity of the Biotoxis qPCR detection kit's targeted genes is proprietary, the manufacturer validated during the development

process that this test can detect both type A and type B strains. This kit's performance for type A strains in different sample types will have to be checked by further studies.

As for clinical specimens, we tested 31 samples previously collected from 30 tularemia patients. At the time of diagnosis, all samples were positive for the ISFtu2 qPCR (C_T range of 20.47 to 36.61) and 29/31 for type B qPCR (C_T range of 28.89 to 39.71). The Biotoxis qPCR was positive for 28/31 clinical samples, with C_T values ranging from 27.37 to 39.06 (Table 3). Therefore, the Biotoxis qPCR test's sensitivity could be evaluated at 90.32% and 96.55% compared to ISFtu2 and type B qPCR tests, respectively. The *F. tularensis* subsp. *holarctica* genome contains 26 to 30 copies of ISFtu2 (7), explaining the higher sensitivity of the ISFtu2 qPCR compared to the Tul4 and type B qPCR tests, which target a single DNA copy. However, the ISFtu2 insertion sequence is also found in the genomes of other *Francisella* species, including *F. novicida* and *F. philomiragia* (7). The Biotoxis qPCR displayed sensitivity similar to that of the type B qPCR. C_T values higher than 35 were found for 9/29 samples for type B qPCR and 13/28 for Biotoxis qPCR. Such high C_T values likely reflected the low *F. tularensis* inoculum and the presence of PCR inhibitors in the tested clinical samples. However, for the Biotoxis qPCR test, no C_T is specified by the manufacturer. Overall, this test's sensitivity could be considered very satisfactory, taking into account its multiplex nature. In addition, clinical samples were stored at −80°C for 1 to 2 years before Biotoxis qPCR testing, which could have slightly altered DNA quality. Regarding specificity, no amplification was observed for the 30 *F. tularensis*-free clinical specimens with this kit.

When testing water samples artificially contaminated with *F. tularensis* subsp. *holarctica*, we found LODs of 1,000 CFU/liter for the Biotoxis qPCR, 100 CFU/liter for type B qPCR, 10 CFU/liter for Tul4 qPCR, and 0.1 CFU/liter for ISFtu2 qPCR. Here again, the lower LOD of ISFtu2 qPCR could be explained by this target's multicopy nature (7). The higher LOD of the Biotoxis qPCR compared to those of the Tul4 and type B qPCR assays could be related to the multiplex nature of this commercial test. Multiplexed qPCR tests usually display lower analytical sensitivities than their simplex counterparts do (13).

We then tested 57 environmental water samples collected from natural aquatic environments. The Biotoxis qPCR was positive for 6/15 samples that were positive by ISFtu2 qPCR, 5/9 Tul4 qPCR-positive samples, and 4/4 type B qPCR-positive samples (Table 4). As expected, the Biotoxis qPCR did not detect some samples that were positive in the ISFtu2 and Tul4 qPCR tests. However, only samples positive in the highly specific type B qPCR test could be considered genuinely contaminated with *F. tularensis* (type B strain). The same samples tested positive with the Biotoxis qPCR test. This result likely indicates the higher specificity of these two tests to detect *F. tularensis* DNA in aquatic environments. It should be noted that high C_T values were found for most qPCR-positive water samples, reflecting low bacterial loads, as previously reported in other environmental studies (14). A much higher bacterial load would be expected in a bioterrorist attack context. It would have been interesting to culture these environmental water samples to correlate PCR and culture results. However, we did not try to culture them, since it would be tedious because of (i) the fastidious nature of *F. tularensis* and (ii) the high quantity of contaminant bacteria in these samples.

The Biotoxis qPCR detection kit is designed to perform multiple tests in a 96-well-plate format. Unlike other commercial products, such as the BioFire FilmArray Biothreat panel, it is not suitable for unit sample analysis. Consequently, the Biotoxis qPCR detection kit appears to be more appropriate for testing many biological or environmental samples at a moderate cost. In our hands, this kit displayed equivalent specificity but a slightly lower sensitivity compared to *F. tularensis* reference qPCR assays. However, the purpose of the Biotoxis kit is to simultaneously detect the presence of *F. tularensis*, *Y. pestis*, and *B. anthracis* in a bioterrorism context. It is a ready-to-use kit that can be handled in all laboratories equipped with a qPCR apparatus, without need for specific expertise. We did not evaluate the Biotoxis qPCR detection kit's performances for the detection of *B. anthracis* and *Y. pestis*. The kit was validated for all three pathogens by the manufacturer during the development process. However, further studies will be needed to assess the sensitivity and specificity of the Biotoxis kit for

detection of *Y. pestis* and *B. anthracis*. Consequently, our study does not enable us to state the usefulness of the Biotoxis qPCR kit in the context of bioterrorism. Apart from the investigation of a suspected bioterrorist attack, we believe that this test could be useful for rapid exploration of the potential sources of human infections during natural tularemia outbreaks.

In conclusion, the Biotoxis qPCR detection kit displayed good performance for detecting *F. tularensis* in clinical specimens and environmental water samples. However, the cross-amplification of *F. novicida* should be taken into account. This kit was easy to use, and results were available within 60 min. It can be useful for the rapid detection of *F. tularensis* DNA in many clinical samples, especially in the context of a tularemia outbreak. It can also be used to detect *F. tularensis* in a large number of environmental samples. The Biotoxis qPCR detection kit was designed primarily for simultaneous detection of *F. tularensis*, *B. anthracis*, and *Y. pestis* in a bioterrorism context. Further studies are needed to assess the performance of this kit for detection of the two latter pathogens.

ACKNOWLEDGMENTS

We declare that we have no conflicts of interest.

Bertin Bioreagent provided the Biotoxis qPCR detection kit used in this study but was not involved in the study design, result interpretation, or the article content.

We thank the Direction Generale de l'Armement (ANR-17-ASTR-0024) and the Programme Interministériel de R&D NRBC for funding.

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