Evaluation of the Biotoxis qPCR detection® kit for Francisella tularensis detection in clinical and environmental samples.

Aurélie Hennebique, Fabienne Gas, Hélène Batina, Cécilia de Araujo, Karine Bizet, Max Maurin

Evaluation of the Biotoxis qPCR detection® kit for *Francisella tularensis* detection in clinical and environmental samples.

Aurélie Hennebique[a, b], Fabienne Gas[c], Hélène Batina[c], Cécilia De Araujo[d], Karine Bizet[d], Max Maurin[a, b,#]

[a] *Centre National de Référence des Francisella, Institut de Biologie et de Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France.*

[b] *Université Grenoble Alpes, Centre National de la Recherche Scientifique, TIMC-IMAG, UMR5525, Grenoble, France.*

[c] *Université Paris Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SPI, 30200 Bagnols-sur-Cèze, France.*

[d] *Bertin Technologies, Montigny-le-Bretonneux, France.*

[#] Corresponding author: Prof. M. Maurin, Email: mmaurin@chu-grenoble.fr

Running title: Evaluation of the Biotoxis qPCR detection® kit

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 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 *F. 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 the ISFtu2 (ISFtu2-qPCR) or a Type B-specific DNA sequence (Type B-qPCR), respectively. All 30 non-tularemia clinical specimens were Biotoxis qPCR negative. For water samples, the Biotoxis qPCR limit of detection was 1,000 CFU/l 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.

INTRODUCTION

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: *F. tularensis* subsp. *tularensis* (Type A strains), in North America, and *F. tularensis* subsp. *holarctica* (Type B strains) in the whole northern hemisphere and southern Australia (1–3). *F. tularensis* subsp. *mediasiatica* has been detected in arthropods and rodents in central Asia and Russia (1) but never associated with human infections. *F. 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 formerly or recently described, including *F. 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 hydro-telluric environment (1, 2, 4). *F. tularensis* can contaminate humans through contact with infected animals, arthropod bites, exposure to contaminated environments, or ingestion of contaminated food or water (1, 2). Several clinical forms of tularemia exists, mainly depending on the portal of entry of the bacteria. The ulceroglandular and glandular forms correspond to localized lymphadenopathy, respectively, with or without a skin ulcer. 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 as a category A of potential biological threat agents by the US 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 by culture or PCR-based methods (2). However, this fastidious and slow-growing bacterium is usually isolated in 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 friendly commercial test allowing rapid, accurate, and standardized detection of *F. tularensis* in various types of samples is a high priority.

The Biotoxis qPCR detection® kit is a plate format TaqMan probe-based real-time PCR assay designed for the combined detection of *B. anthracis, Y. 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 primers and probes mix, 3.75 µl of water, and 5 µl of DNA sample (at variable concentration 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 sec, and annealing and extension at 60°C for 30 sec. 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 2X TaqMan™ Fast Advanced PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania), 0.4 µl of each 10 µM primers, 0.4 µl of 2µM probe, 3.8 µl of water, and 5 µl of DNA sample (at variable concentration 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 sec, and annealing and extension at 60°C for 30 sec. 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, one 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 strain 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 of *Francisella* sp. were previously identified at the French NRCF. *F. philomiragia* was identified by whole 16S rDNA 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 have been obtained from the Agence Nationale de Sécurité du Médicament et des Produits de santé (ANSM, authorization number ADE-103892019-7). Besides, 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 one or two day(s). *Francisella tularensis* strains were grown in a biosafety level 3 laboratory.

DNA extracts from *Francisella* sp. strains were prepared at 10 ng/µl concentration 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 Ct 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 and 2019 for diagnostic expertise. A tularemia confirmed case was defined as a clinically compatible disease with detection of *F. tularensis* from any clinical specimen by culture or PCR (ISFtu2- or Type B-qPCR, Ct $\leq$ 35), or seroconversion, or a fourfold (or greater) change in serum antibody titers to *F. tularensis* antigen between acute and convalescent-phase sera. A probable case was defined as 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* PCR test. All these samples were collected as part of routine patients' 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/l. 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 ISFtu2-, Tul4- and Type B-qPCR taken 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 $Ct \leq 36$ for ISFtu2-qPCR or a $Ct < 40$ 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 (Ct values between 15 to 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, comparatively to those of the ISFtu2-, Tul4- and Type B-qPCR tests. The Ct ranges for these four samples were 18-20 for the Biotoxis qPCR, 12-14 for ISFtu2-qPCR, 15-19 for Tul4-qPCR, and 16-19 for Type B-qPCR (Table 2).

The Biotoxis qPCR also gave a strongly positive signal for *F. novicida* CIP 56.12 strain, 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 week 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 (Ct between 34 and 37 at 10 ng/µL of DNA concentration) 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 Ct ranged from 20.47 to 36.61 for ISFtu2-qPCR (with 30/31 tests with a Ct $\leq$ 35), 28.89 to 39.71 (20/31 tests with a Ct $\leq$ 35) for Type B-qPCR, and 27.37 to 39.06 (15/31 tests with a Ct $\leq$ 35) for Biotoxis qPCR (Table 3).

The Biotoxis qPCR was positive for 27/31 pure DNA extracts (with Ct ranging from 27.37 to 39.06), 19/31 DNA extracts diluted to the 1/10 (Ct, 29.85-39.43), and 13/31 DNA extracts diluted to the 1/100 (Ct, 33.26-39.26). Interestingly, sample S4 was positive for ISFtu2-qPCR, Type B-qPCR, and Biotoxis qPCR only when diluted to the 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 abscesses, one respiratory sample, and four biopsies. 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 CFU/l to 10,000 CFU/l were analyzed by Biotoxis qPCR and by ISFTu2-, Tul4- and Type B-qPCR. Biotoxis qPCR detected *F. tularensis* from 1,000 CFU/l (with a Ct at 34.47). It was less sensitive than Type B-qPCR (positive from 100 CFU/l with a Ct at 38.23), Tul4-qPCR (positive from 10 CFU/l with a Ct at 36.96), and ISFtu2-qPCR (positive from 0.1 CFU/l with a Ct 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 for the ISFtu2-qPCR, nine were

positive for both ISFtu2-qPCR and Tul4-qPCR, and four were positive for ISFtu2-qPCR, Tul4-qPCR, and Type B-qPCR. The Biotoxis qPCR was positive for six (40%) of the 15 samples positive for ISFtu2-qPCR, five of the nine samples positive for both ISFtu2- and Tul4-qPCRs, and four samples positive for ISFtu2-, Tul4- and Type B-qPCRs (Table 4). The Biotoxis qPCR test was negative for all 42 samples negative for the ISFTu 2-, Tul 4- and Type B-qPCR tests (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, comparatively 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 a strong amplification signal with the Biotoxis qPCR (Ct between 15 and 22 for a DNA extract standardized at 10ng/µl). Similar Ct values were obtained with the qPCR tests targeting the single-copy Tul4 or Type B-specific DNA sequences. In contrast, lower Cts were obtained with the multi-copy 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 ≥ 97.7% similarity at the genome level with *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 testing environmental water samples. 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 (Ct ≥ 34) for the *S. salivarius*, *P. aeruginosa*, and *N. elongata* strains. Using lower DNA concentrations could eliminate such non-specific signals. When working on DNA extracts from bacterial strains, it should be recommended to work with DNA concentrations of 0.1 ng/µl to avoid unspecific 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., IS*Ftu2* element and the *23kDa*, *fopA*, and *tul4* genes) detect all four *tularensis* subspecies (7). Although the Biotoxis qPCR detection® kit's targeted genes are confidential, 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 (Ct ranges of 20.47-36.61) and 29/31 for Type B-qPCR (Ct ranges of 28.89-39.71). The Biotoxis qPCR was positive for 28/31 clinical samples with Ct ranging from 27.37 to 39.06 (Table 3). Therefore, the Biotoxis qPCR tests' 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 the ISFtu2 (7), explaining the higher sensitivity of the ISFtu2-qPCR compared to the Tul4- and Type B-qPCR tests targeting a single DNA copy. However, the ISFtu2 insertion sequence is also found in the genome of other *Francisella* species, including *F. novicida* and *F. philomiragia* (7). The Biotoxis qPCR displayed similar sensitivity to the Type B-qPCR. Ct values higher than 35 were found for 9/29 samples for Type B-qPCR and 13/28 for Biotoxis qPCR. Such high Ct 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 Ct threshold is specified by the manufacturer. Overall, this test's sensitivity could be considered very satisfying, taking into account its multiplex nature. Besides, clinical samples were stored at -80°C for 1-2 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 a LOD of 1,000 CFU/l for the Biotoxis qPCR, 100 CFU/l for Type B-qPCR, 10 CFU/l for Tul4-qPCR, and 0.1 CFU/l for ISFtu2-qPCR. Here again, the lower LOD of ISFTu2-qPCR could be explained by this target's multi-copy 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 positive for 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 positive samples for ISFtu2- and Tul4-qPCR tests. However, only samples positive for 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 highlighted that high Ct 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 attractive 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 1/ the fastidious nature of *F. tularensis* and 2/ the high quantity of contaminant bacteria in these samples.

The Biotoxis qPCR detection® kit is designed to perform multiple tests in a 96 wells plate format. Unlike other commercial products such as the BioFire FilmArray® Biothreat Panel, it is not suitable for unit samples analysis. Consequently, the Biotoxis qPCR detection® kit appears 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 than *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 the 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 its 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 claim the usefulness of the Biotoxis qPCR kit in the context of bioterrorism. Apart the investigation of a suspected bioterrorist attack, we beleave 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 performances 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 has been primarilly designed for simultaneous detection of *F. tularensis*, *B. anthracis*, and *Y. pestis* in a bioterrorislm context. Futher studies are needed to assess the performances of this kit for detection of the two later pathogens.

ACKNOWLEDGMENTS

The authors declare that they have no conflict of interest. Bertin Bioreagent provided the Biotoxis qPCR detection® kit used in this study but was not involved in the study design, result interpretation, and this manuscript's content. Bertin Bioreagent thanks the inter-ministerial program of R&D against CBRNE that financed the test development

REFERENCES

1. Sjöstedt A. 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci 1105:1–29.

2. Maurin M, Gyuranecz M. 2016. Tularaemia: clinical aspects in Europe. Lancet Infect Dis 16:113–124.

3. Jackson J, McGregor A, Cooley L, Ng J, Brown M, Ong CW, Darcy C, Sintchenko V. 2012. Francisella tularensis subspecies holarctica, Tasmania, Australia, 2011. Emerging Infect Dis 18:1484–1486.

4. Hennebique A, Boisset S, Maurin M. 2019. Tularemia as a waterborne disease: a review. Emerg Microbes Infect 8:1027–1042.

5. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K, Working Group on Civilian Biodefense. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763–2773.

6. Yanes H, Hennebique A, Pelloux I, Boisset S, Bicout DJ, Caspar Y, Maurin M. 2018. Evaluation of In-House and Commercial Serological Tests for Diagnosis of Human Tularemia. J Clin Microbiol 56.

7. Versage JL, Severin DDM, Chu MC, Petersen JM. 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of Francisella tularensis in complex specimens. J Clin Microbiol 41:5492–5499.

8. Kugeler KJ, Pappert R, Zhou Y, Petersen JM. 2006. Real-time PCR for Francisella tularensis types A and B. Emerging Infect Dis 12:1799–1801.

9. Kreitmann L, Terriou L, Launay D, Caspar Y, Courcol R, Maurin M, Lemaître N. 2015. Disseminated Infection Caused by Francisella philomiragia, France, 2014. Emerging Infect Dis 21:2260–2261.

10. Maurin M, Pelloux I, Brion JP, Del Banõ J-N, Picard A. 2011. Human tularemia in France, 2006-2010. Clin Infect Dis 53:e133-141.

11. WHO Press, Geneva, Switzerland. 2007. World Health Organization. WHO guidelines on tularaemia.

12. Larsson P, Elfsmark D, Svensson K, Wikström P, Forsman M, Brettin T, Keim P, Johansson A. 2009. Molecular evolutionary consequences of niche restriction in Francisella tularensis, a facultative intracellular pathogen. PLoS Pathog 5:e1000472.

13. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE. 2000. Multiplex PCR: Optimization and Application in Diagnostic Virology. Clin Microbiol Rev 13:559–570.

14. Janse I, van der Plaats RQJ, de Roda Husman AM, van Passel MWJ. 2018. Environmental Surveillance of Zoonotic Francisella tularensis in the Netherlands. Front Cell Infect Microbiol 8:140.

396 **TABLE 1. Bacterial strains used in this study**

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

397

TABLE 2. Biotoxis qPCR testing of bacterial strains

Strains*	**Biotoxis qPCR (Ct)**	**ISFtu2-qPCR (Ct)**	**Tul4-qPCR (Ct)**	**Type B-qPCR (Ct)**
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

*: DNA extracts were standardized at 10 ng/µl

403 **TABLE 3. Biotoxis qPCR testing of 31 clinical samples from 30 tularemia patients**

Samples		Clinical form	Serology / culture	qPCR tests (Ct, DNA extract tested pure or at specified dilution)		
Name	**Nature**			**ISFtu2**	**Type B**	**Biotoxis**
S1	LN	GL	POS/NA	POS (27.97)	POS (31.96)	POS (34.50)
S2*	Peritoneal tissue	INT	POS/NEG	POS (29.35)	POS (35.06)	POS (32.59)
S3*	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)

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; *: samples from the same patient

410 **TABLE 4. Biotoxis qPCR testing of 57 environmental water samples**

411

ISFtu2-qPCR	Tul4-qPCR	Type B-qPCR	Biotoxis qPCR (Ct)	Number of water samples
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

412