



Whole genome sequence of bacteremic *Clostridium tertium* in a World War I soldier, 1914

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ABSTRACT

Background: Dental pulp, encapsulating a blood drop, could be used to diagnose pathogen bacteraemia in archaeological materials using DNA-based techniques. We questioned the viability of such ancient pathogens preserved in ancient dental pulp.

Methods: After meticulous decontamination of 32 teeth collected from 31 World War I soldiers exhumed in Spincourt, France, dental pulps were extracted and cultured under strict anaerobiosis. Colonies were identified by mass spectrometry and whole genome sequencing. Fluorescent in situ hybridisation (FISH) was used for the direct microscopic detection of pathogens of interest in the dental pulp. All the experimental procedures included negative controls, notably sediments in contact with individual SQ517 to ensure that results did not arise from contamination.

Findings: *Clostridium tertium* was detected by FISH in two dental pulp specimens taken from a 1914 soldier. After a two-day incubation period, both dental pulp samples grew colonies identified by mass spectrometry and genome sequencing as *C. tertium*; whereas negative controls remained free of *C. tertium* in all the observations, and no *C. tertium* was founded in sediments. Skeletal remains of this soldier exhibited two notches in the left tibia evocative of a cold steel wound, and a probably fatal unhealed bullet impact in the hip bone.

Interpretation: Data indicated the presence of *C. tertium* in the dental pulp at the time of the death of one World War I soldier, in 1914. This observation diagnosed *C. tertium* bacteraemia, with war wounds as the probable portal of entry for *C. tertium*. Our *C. tertium* strains ante-dated by three years, the princeps description of this deadly opportunistic pathogen.

The soil inhabitant *Clostridium tertium* (*C. tertium*) has been described as an opportunistic pathogen associated with gangrenous wounds in the context of World War I (Henry, 1918). In 1917, Captain Herbert Henry from the British Royal Army Medical Corps reported that a quarter of the 100 wounds he observed in British and French soldiers were infected with *C. tertium* (King et al., 1963). *C. tertium*, a micro-organism further detected in the oral cavity and intestines, has been further associated with necrotising gangrene and bacteraemia (Henry, 1918; Ray et al., 2003; Vanderhofstadt et al., 2010). In this situation, its deadly potential was confirmed after cumulative reports of deadly *C. tertium* bacteraemia, notably in neutropenic patients (Miller et al., 2001; Wazir et al., 2019).

Using an original culture protocol, we diagnosed a *C. tertium* bacteraemia in a French soldier who died in 1914, at the beginning of World War I.

1. Materials and methods

1.1. Archaeological investigations

After war declaration between France and Germany on August 3rd 1914, the first weeks of conflict involved territories close to the village of Spincourt, Great-East France (latitude: 49.3333; longitude: 5.6667), regarded as the heart of the « Border battle ». After German troops

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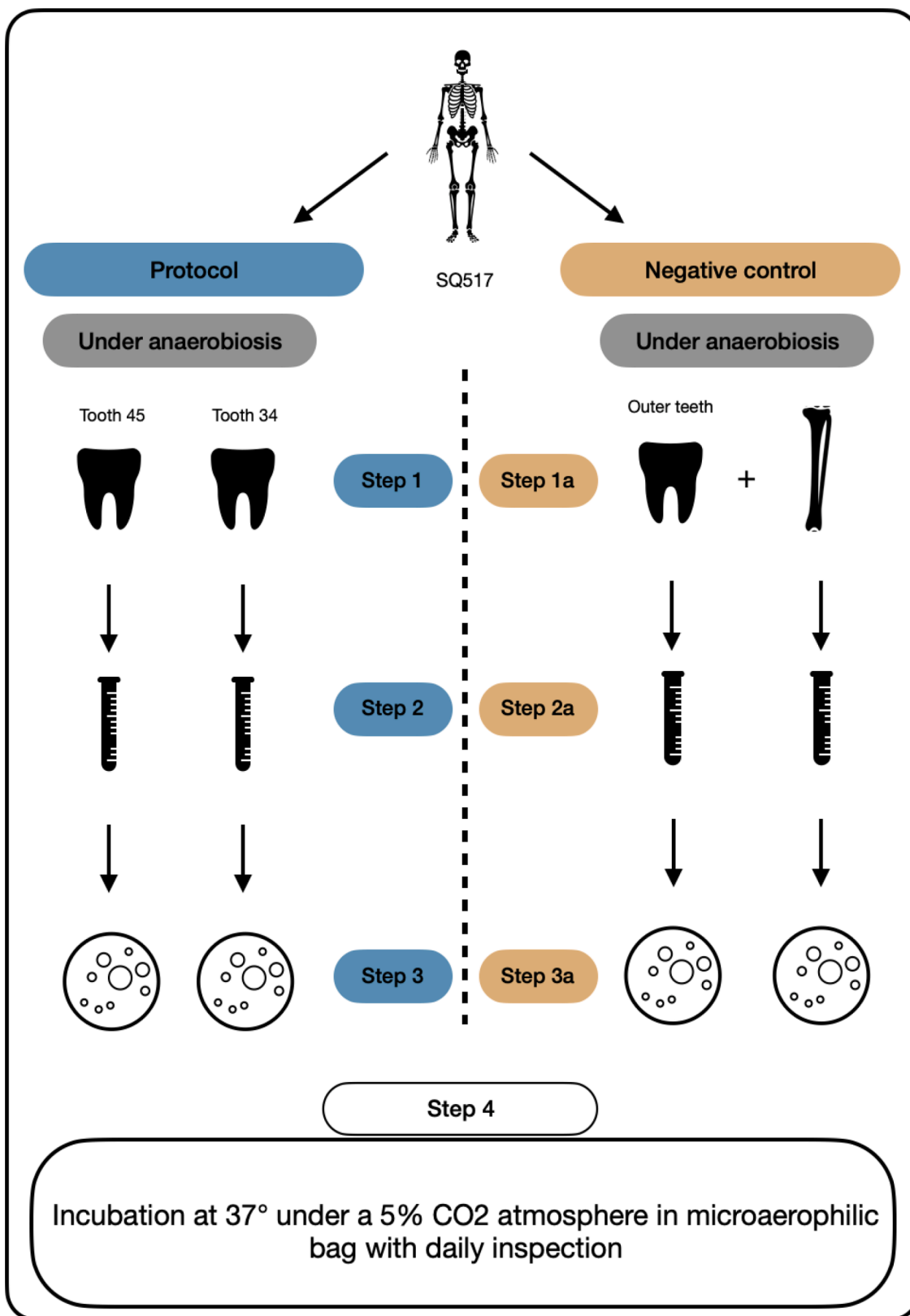


Fig. 1. Workflow summarizing culture of ancient dental pulps: Step 1: Teeth 45 and 34 were disinfected with pure ethanol and bleach. After the teeth were fractured, the dental pulp was extirpated under anaerobiosis; Step 1a: Sediment from the non-disinfected teeth of individual SQ517 and surrounding the tibia were used; Step 2: Dental pulp was mixed with 10uL of PBS; Step 2a: Sediment from the outer teeth and tibia were respectively mixed with 10µl and 150µl of PBS; Step 3: The rehydrated pulp was placed onto an agar plate with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany); Step 3a: Rehydrated sediments were placed onto an agar plate with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany); Step 4: Agar plates were incubated at 37° under a 5% CO₂ atmosphere in a microaerophilic bag and were inspected daily.

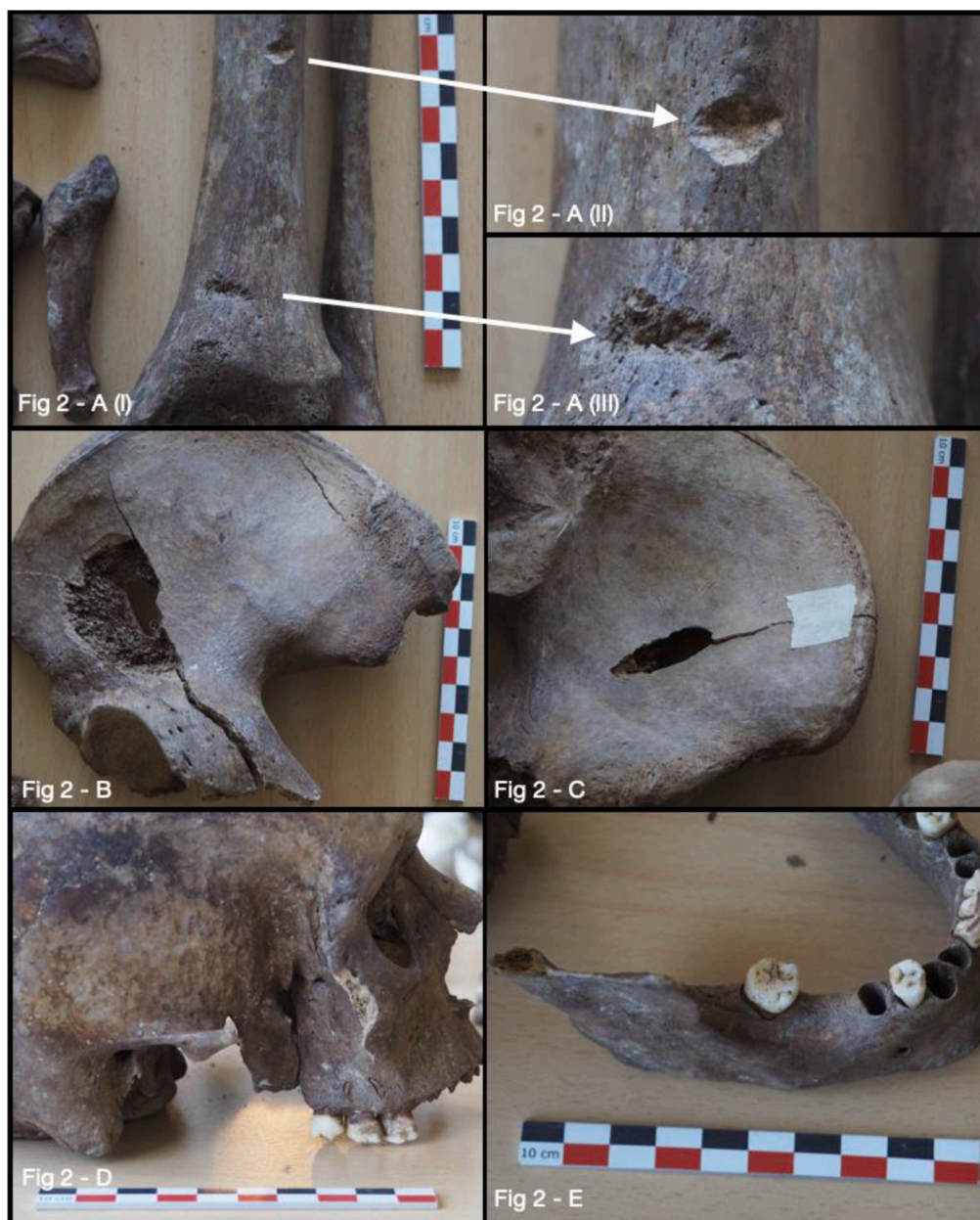


Fig. 2. Osteological traumas on individual SQ517; A (I), (II) and (III): Marks on the left tibia resulting from a cold steel weapon; B: Bullet exit hole on the ilium of the left hip bone; C: Bullet entry hole on the ilium of the left hip bone; D and E: Perimortem fracture to the right zygomatic branch of the skull and to the mandibular condyle.

defeated at Mangiennes, a frontal battle against French troops retreating towards Verdun, took place in Spincourt between August 20th and August 25th. Fights raged and losses were heavy (27,000 deaths only for August 22nd). Fights ended-up with Spincourt firing and its occupation by German troops, until October 1918 (Verna et al., 2020). Few days after August fights, German troops assured the funeral gestion of deads including the French ones into a provisional cemetery. In the aftermath of war during the 1920's, numerous bodies were unearthed from the provisional cemetery to be returned to their families and be reinsulated in military necropolis. Starting February 2017, the provisional military cemetery was investigated by Institut National Recherche Archéologique Préventive, disclosing testifying of an organised gestion of corpses (graves rigorously aligned, coffin inhumation) and 31 remaining skeletons by October 2018 (Verna et al., 2021). Examination of these skeletons testified of the violence of fightings (multiple traumas on skeleton, supernumerary anatomical part in coffin). Skeleton were

examined using the Diagnose Sexuelle Probabiliste v2 (DSP2) software method for sex estimation and Lovejoy's quotation for age (Lovejoy et al., 1985; Brůžek et al., 2017). A total of 32 teeth were collected from the 31 skeletons for palaeomicrobiological investigations.

1.2. Paleomicrobiological investigations

Thirty-two teeth were investigated for palaeomicrobiology, one tooth for each one of the 31 soldiers and two teeth (45 and 34) for individual SQ517. The external surface of each tooth was cleaned with sterile gauze soaked with pure ethanol and bleach for 30 s (Fig. 1). Pre-opening, fracture and pulp extirpation were all carried out under anaerobiosis to avoid any direct exposure of the dental pulp to atmospheric oxygen. Accordingly, all instruments necessary for opening the teeth and extracting the pulp, including a small circular diamond saw and its motor or excavator, were placed under an anaerobic hood (Don

Whitley, Bingley, UK) before the manipulation. Dental pulps were extracted with specific tools (an excavator) following a previously described protocol (Drancourt et al., 1998). Pulps were rehydrated for one minute with 10 μ L sterile phosphate buffered saline (PBS) and 0.5 μ L of rehydrated dental pulp were inoculated onto a 5% sheep blood agar Petri plate (Becton Dickinson GmbH, Heidelberg, Germany) (Fig. 1). A negative control culture medium was opened in the anaerobiosis hood at the beginning of the manipulation to assess the hood's sterility. A piece of sterile gauze soaked with sterile water was placed under each culture plate to keep humidity, and plates were placed into a microaerophilic bag (BD GasPak, EZ Pouch Systems, Becton-Dickinson, Franklin Lakes, NJ, US) incubated at 37 °C under a 5% CO₂ atmosphere (Fig. 1). Each microaerophilic bag also contained one negative control inoculate with 10 μ L sterile PBS. Sediments in direct contacts with individual SQ517's tooth 45, tooth 34 and left tibia were put in culture before decontamination, following the same protocol described above.

1.3. *C. tertium* identification

Colonies observed by daily naked eye inspection were stained by Gram-staining (bioMérieux, Craponne, France) and further identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (Seng et al., 2009).

The minimum inhibitory concentration (MIC) of amoxicillin-clavulanic acid (30 mg/L), erythromycin (15 mg/L), metronidazole (4 mg/L), and clindamycin (2 mg/L) (Vanderhofstadt et al., 2010; Salvador et al., 2013) was determined for each *C. tertium* isolate by Scan 1200® (Interscience, Saint-Nom-la-Bretèche, France).

The *C. tertium* isolate Q5690 was further investigated by whole genome sequencing (WGS) as previously described (Muñoz et al., 2019). Briefly, DNA was extracted using the standard EZ1 protocol from isolate Q5690, from strain SP2622 initially isolated in 1923 (Johnson and Francis, 1975) (DSMZ 2485, Leibniz Institute, Braunschweig, Germany), and from a previous *C. tertium* (Q6181) cultivated in our laboratory. DNAs were sequenced on Illumina Miseq and sequencing reads were assembled using Spades software (Bankevich et al., 2012). In silico DNA-DNA hybridization (DDH) was used to delineate bacterial species (Rossello-Mora, 2006) and *C. tertium* Q5690 genomic sequence was compared to those retrieved in public databases including *C. tertium* MGYG HGUT 01328 reference genome. Mugsy software was used for genome alignments (Angiuoli and Salzberg, 2011) and a phylogenetic tree was done using Raxml software (Stamatakis, 2014) incorporating the maximum likelihood method (ML) algorithm, with 1,00 bootstrap replicates. Genes related to pathogenicity and virulence factors were searched in the literature and sequences available in the Virulence Factor Database (VFDB) (Chen, 2004). Antibiotic resistance profiling was achieved by using Abricate pipeline comparison with CARD (McArthur et al., 2013), ARG-ANNOT (Gupta et al., 2014), Restfinder (Zankari et al., 2012) and Bacterial Antimicrobial Resistance Reference Gene Database. SNPs analyses were performed with SNP-sites on (Angiuoli and Salzberg, 2011; Page et al., 2016).

1.4. Fluorescence in situ hybridization (FISH)

C. tertium was specifically detected in the dental pulp specimen by fluorescent in situ hybridisation (FISH), incorporating probe 3'-CTCCAACCCTAGTAAACCCCT-5' labelled with Alexa fluor-488 and targeting the specific *C. tertium* gene *fur* coding for a transcriptional regulator following the MGYG HGUT 01328 reference genome annotation on Dfast software. The gene sequence was blasted on NCBI against the *Clostridium* genus (taxid 1485) database yielded 100% identity and 87% coverage with *C. tertium* and 99.76% identity and 87% coverage with *Clostridium perfringens*. Then, a specific probe was designed from this gene using NCBI primers and yielded 100% identity and coverage only with *C. tertium* on NCBI blast against the *Clostridium* genus (taxid 1485) database. Briefly, the dental pulp of individual SQ517 was fixed



Fig. 3. *C. tertium* colonies yield from SQ517 individual after three days of incubation.

for three hours in Sandison's rehydration solution (aqueous formaldehyde 1%, 96% ethanol and 5% aqueous sodium carbonate) (Collini et al., 2014) and smeared on microscopic slide using Cytospin (ThermoFisher, Illkirch, France). In situ hybridization was performed with a hybridiser (Dako, Les Ulis, France) at 65 °C for 10 min and then at 37° overnight, following a previously reported protocol (Millogo et al., 2020). Dental pulp of the individual SQ536, which remained negative in culture for *C. tertium* was used as a negative control to assess the probe specificity.

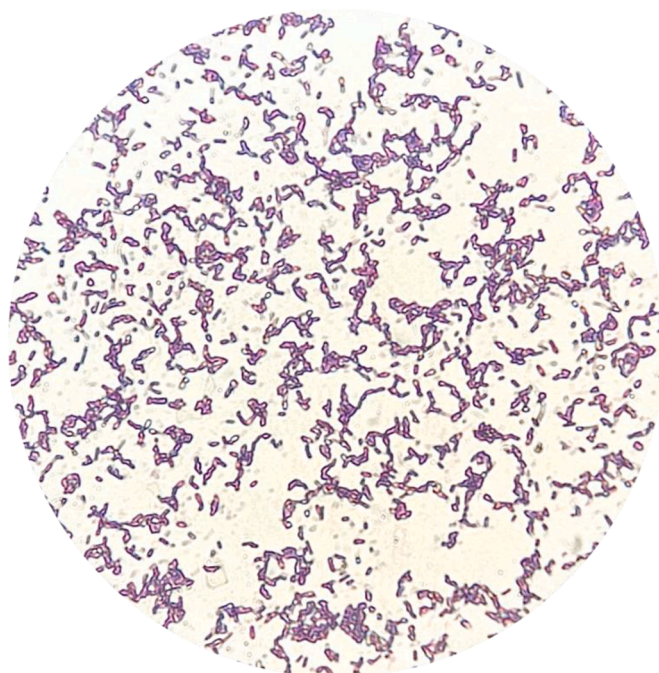


Fig. 4. Gram staining from Q5690 *C. tertium* strain under 100x optical microscopic lenses.

Table 1

Bacteria founded in negative control cultures: Bacterial species isolated from the sediment samples around SQ517, from the anaerobic hood and from the culture media inoculated with PBS.

Species	Score Value 1 (MALDI-TOF)	Score Value 2 (MALDI-TOF)
<i>Clostridium sordellii</i>	2.28	2.31
<i>Clostridium sporogène</i>	2.00	2.22
<i>Clostridium novyi</i>	2.00	1.92
<i>Bacillus megaterium</i>	2.15	2.24
<i>Bacillus cereus</i>	2.28	2.39
<i>Bacillus simplex</i>	2.00	1.76
<i>Bacillus licheniformis</i>	2.42	2.30
<i>Bacillus pumilus</i>	2.15	2.24
<i>Paenibacillus glucanolyticus</i>	2.13	2.21
<i>Clostridium tetani</i>	2.14	2.13
<i>Bacillus toyonensis</i>	2.19	2.09
<i>Staphylococcus homini</i>	2.19	2.19

2. Results

2.1. Archaeological investigations

After meticulous inspection and bacterial identification of the 32 dental pulps, only teeth from the individual SQ517 yielded *C. tertium* as reported below. He was a man aged 30–35 years at the time of his death. The anthropological examination of the skeleton of individual SQ517 revealed two notches in the left tibia, evocative of the impact of a cold steel weapon (knife or bayonet), surrounded by a periosteal reaction indicating that the wounds occurred before death; a fracture to the right zygomatic branch and the right condyle of the mandible; left and right rib fractures which may have occurred at the time of death or post-mortem. A bullet impact was also observed with an entry point in the anterior part of the ilium and the exit point in the posterior part with significant bone loss and no sign of healing (Fig. 2).

2.2. Culture of ancient dental pulp

The negative control culture medium remained free of any visible colonies for five days, but the plate inoculated with the dental pulp of the second right premolar tooth (45) and first left premolar tooth (34), collected directly from individual SQ517's mandible, yielded colonies after three days of incubation (Fig. 3). These colonies were stained purple by Gram staining and were identified as *C. tertium* by MALDI-TOF mass spectrometry with index of 2.18 for tooth 45 and 2.45 for tooth 34 (Fig. 4). Both *C. tertium* isolates were deposited in the Collection de Souches de l'Unité des Rickettsies (IHU Méditerranée Infection, Marseille, France) under reference numbers, CSUR Q5690 and CSUR Q5873, respectively. No *C. tertium* was cultured from sample sediments collected around the individual SQ517, neither from the negative controls manipulated under the anaerobic hood and Phosphate Buffered Saline (PBS) (Table 1). Likewise, no *C. tertium* isolates were retrieved from any of the 30 dental pulp samples. The antibiogram indicated *C. tertium* strains CSUR Q5690 and Q5873 have in-vitro a MIC inferior to 30 mg/L for amoxicillin-clavulanic acid with an inhibitory zone of 30 mm, and inferior to 15 mg/L for erythromycin with an inhibitory zone of 20,2 mm. The strains also have a MIC superior to 2 mg/L for clindamycin and superior to 4 mg/L for metronidazole with both of their inhibitory zone at 0,0 mm (Fig. 5).

2.3. *C. tertium* identification

Isolate Q5690 genome sequence (obtained with a 13.7 X coverage for a 3776,523 base pairs (bp) size) yielded DDH values ranging from 19.5% with *Clostridium celatum* and *Clostridium saudiense*, 19.9% with *Clostridium gasigenes*, 28.8% with *Clostridium septicum* and *Clostridium chauvoei* up to 89.4% with *C. tertium* strain Q5690 (Table 2). Accordingly, DDH values between isolate strain Q5690 and *C. tertium* MGYG HGUT 01328 reference genome were greater than the 70% threshold acknowledged to delimit bacterial species, are support the strain Q5690 is the same species as *C. tertium* (Wayne, 1988; Tindall et al., 2010). Phylogenic tree indicated that isolate Q5690 was closer *C. tertium* MGYG HGUT 01328 reference genome than to *C. tertium* strain Q6181 previously cultivated in our laboratory (Fig. 6). Noteworthy,

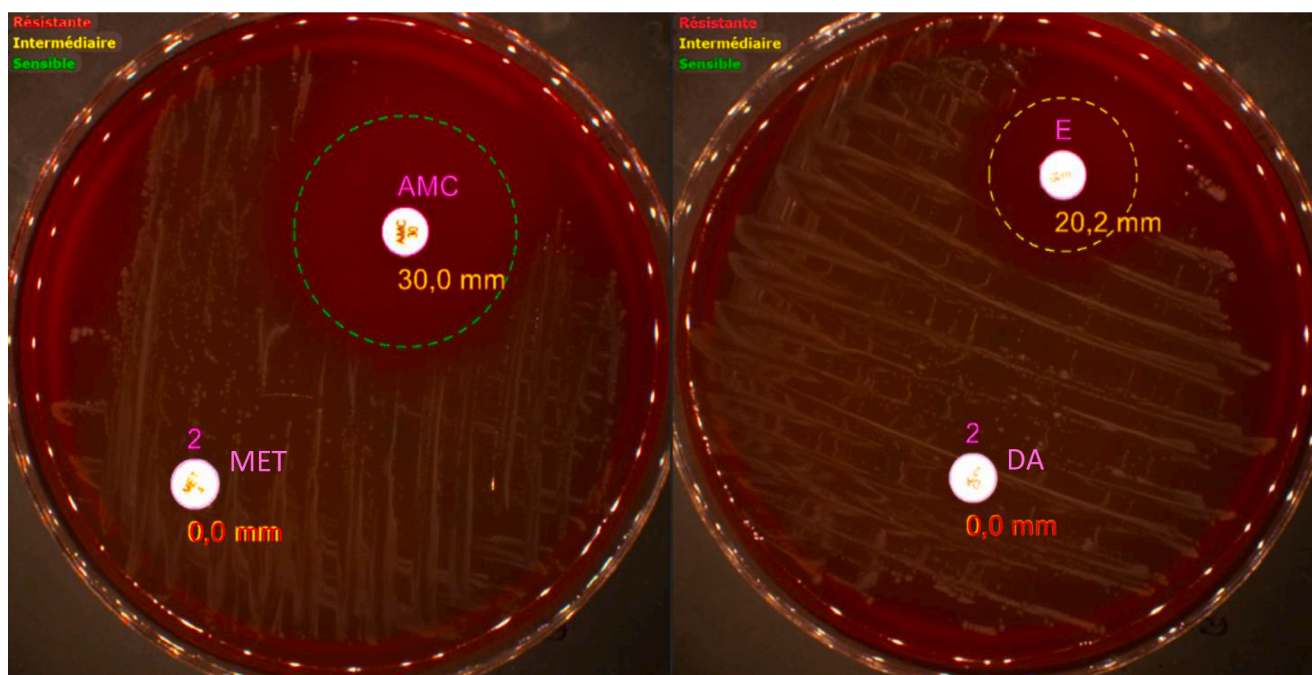


Fig. 5. Antibiogram: amoxicillin-clavulanic acid (AMC) 30 mg/L, erythromycin (E) 15 mg/L, metronidazole (MET) 4 mg/L and clindamycin (DA) 2 mg/L on Q5690. - Scan 1200®.

Table 2 Pairwise genomic comparison of strain Q5690 with other species using the GGDC software, formula 2 (dDDH estimates based on identities over HSP length). The confidence intervals indicate the inherent uncertainty in estimating dDDH values from intergenomic distances based on models derived from empirical test data sets.

	Q5690	Clostridium tertium	Clostridium chauvoei	Clostridium isatidis	Clostridium sartagoforme	Clostridium septicum	Clostridium jeddahitimonense	Clostridium saudiense	Clostridium celatum	Clostridium disporicum	Clostridium gasigenes
Q5690	100%										
Clostridium tertium	89.4 [87 - 92]	100%									
Clostridium chauvoei	21.7 [19.4-24]	100%									
Clostridium isatidis	21.6 [19.4-24]	21 [18.8-23]	100%								
Clostridium sartagoforme	23.8 [21.5-26]	23.6 [21.3-26]	21.6 [19.4-24]	21 [18.8-24]	100%						
Clostridium septicum	21.9 [19-24]	21.8 [19.6-24]	28.8 [26.4-31]	21.1 [18.9-24]	21.9 [19.6-24]	100%					
Clostridium jeddahitimonense	20.5 [18.2-23]	20.7 [18.4-23]	20.3 [18.1-23]	21.1 [18.9-24]	21.2 [19-24]	100%					
Clostridium saudiense	20.4 [18.2-23]	20.4 [18.2-23]	20.7 [18.4-23]	20.3 [18.1-23]	21.2 [19-24]	21.8 [19.5-24]	100%				
Clostridium celatum	20 [18.2-25]	21.2 [18.9-24]	20.6 [18.4-23]	21.2 [19-24]	20.7 [18.5-23]	21 [18.7-23]	19.5 [17.3-22]	100%			
Clostridium disporicum	20 [17.8-22]	20.3 [18.1-23]	20.3 [18.1-23]	20.1 [17.9-23]	20.4 [18.2-23]	20.7 [18.5-23]	22.2 [30.7-36]	21.9 [19.9-25]	21.9 [19.7-24]	100%	
Clostridium gasigenes	19.9 [17.7-22]	20.7 [18.5-23]	21.7 [19.5-24]	21.6 [19.3-24]	21.2 [19-24]	21.6 [19.3-24]	20.7 [18.7-23]	20.7 [18.4-23]	20.6 [18.4-23]	20.6 [18.4-23]	100%

isolate Q5690 genome sequence was closer to that of strain SP2622 isolated in 1923, than to recent isolate genome sequence (Fig. 7). However, no specific difference in the repertoire of virulence and resistance genes was observed between *C. tertium* strain Q5690 and all the other *C. tertium* genomes (Supplementary tables A-D). Regarding SNPs analysis, we founded 18,107 SNPs between isolate Q5690 and isolate Q6181, and 15,657 SNPs between isolate Q5690 and *C. tertium* reference genome MGYG HGUT 01328. Further analysis on SNP profile indicated 36, 615 SNPs differences between isolates Q5690 and SP2622, and 37, 402 SNPs difference between SP2622 and the reference genome MGYG HGUT 01328.

2.4. Microscopy

FISH investigation showed that the SQ517 dental pulp was positive for *C. tertium* fur gene, whereas the negative control (the SQ536 dental pulp) remained negative (Fig. 8).

3. Discussion

We interpreted that *C. tertium* was encapsulated in the dental pulp of soldier SQ517 at the time of his death after highly vascularized dental pulp of soldier SQ517 has preserved a drop of blood (Barbieri et al., 2020). The analyse of such blood drop allowed detection of *C. tertium* in two dental pulp specimens collected from soldier SQ517, suggesting individual SQ517 suffered *C. tertium* bacteraemia. This condition is associated with a fatal outcome in modern literature (Valtonen et al., 1990), even if its pathogenicity mechanism still be misunderstood due to its lack of exotoxin (Muñoz et al., 2019). Nevertheless, nagH gene encoded by *C. tertium* genome and present in isolate Q5690 (Supplementary Table C), could act on connective tissue of gas gangrene, as it was proposed for *C. perfringens* (Muñoz et al., 2019). This gene was detected in all investigated genomes sequences including the one isolate from 1914 (Q5690) at the exception of the strain Q6181 previously isolated in our laboratory. Indeed, even if 100 years form a long time to execute a bacterial isolation from dental pulp, regarding bacterial evolution one century may be too short to observe any differences between the archaeological sample here investigated and modern genome sequences as illustrated by a lack of significant differences between ancient and modern genomes.

The detection in isolate Q5690 of MLS VatB gene and cdeA gene (Supplementary Table D) conferring respectively resistance to streptogramin A and fluoroquinolones (Dridi et al., 2004; Mayers et al., 2017), is one more example of antibiotic resistance predating the medical use of antibiotics.

Historical sources indicated that soldier SQ517 died between 20 and 30 August 1914. He was, therefore, the index case of *C. tertium* bacteraemia, as this microorganism had only previously been isolated in 1917 (Henry, 1918). Our present study took us three years back in time from the first discovery and isolation of *C. tertium*.

We are reporting on the first ever culture-based paleomicrobiological diagnosis from the dental pulp of an individual from 1914 who was suffering from a *C. tertium* bacteraemia. More than that, it is also the first time that ancient dental pulp was used for bacterial culture and led to bacterial isolation. Previous paleomicrobiological detections of pathogens have indeed all been based upon the detection of specific biomolecules culminating in the recovery of entire genome sequences, yet no such pathogen has ever been isolated and cultivated from human remains. Only sporulated *Bacillus* genus microorganisms have been reported from soil, the record being *Bacillus massiliiglaeii* which was retrieved by culture from Siberian permafrost specimens dating from, on average, 10 million years ago (Afouda et al., 2016). There has also been report of a 1917 Prussian spy carrying a piece of sugar hiding anthrax spores (Redmond et al., 1998). Such isolates could be explained by the spore forming capacity of *Clostridium* and *Bacillus* (Kennedy et al., 1994). The spores are known to allow bacteria to survive in extreme

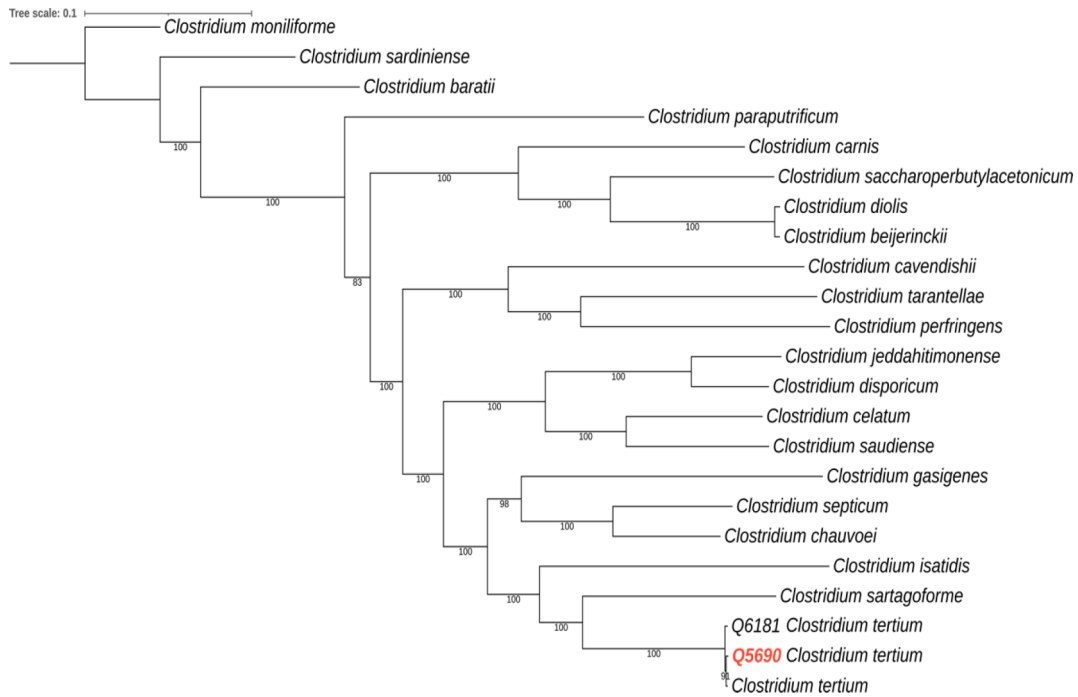


Fig. 6. Phylogenetic tree of 20 *Clostridium* species: The tree underlines the proximity between 22 genomes from different *Clostridium* species. Q5690 appears closer to the reference genome MGYG-HGUT-01,328 than from Q6181 (modern diagnostic strain). The closest relative to *C. tertium* is *Clostridium sartagoforme*. All genomes can be found on NCBI.

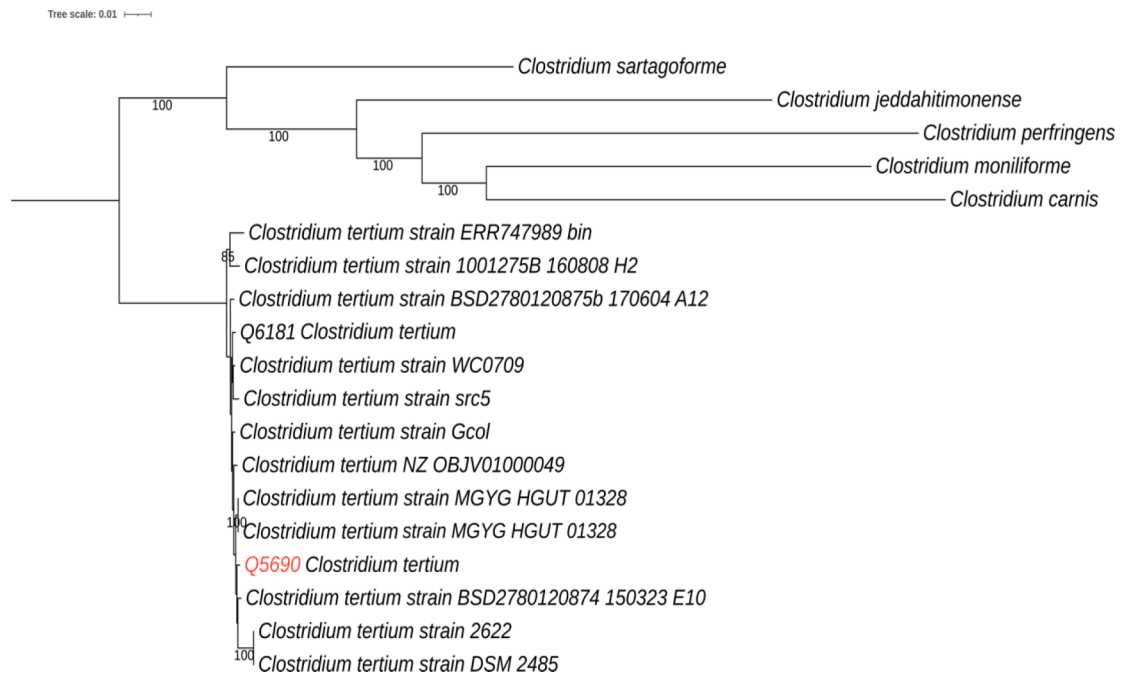


Fig. 7. Phylogenetic tree including all *C. tertium* genomes available on NCBI database: The tree underlines the proximity between several genomes from different *C. tertium* strain. Q5690 appears closer to the reference strain from 1923 (SP2622) than from the reference genome MGYG-HGUT-01,328. The closest relative to *C. tertium* Q5690 is strain BSD2780120874_150,323_E10. All genomes can be found on NCBI.

environmental conditions, such as cold, heat or anaerobiosis (Roszac and Colwell, 1987; Kennedy et al., 1994). In the present study, we can see that *Clostridium tertium* remains alive through its spores, even 107 years later.

The present case report illustrates the effectiveness of an original culture protocol incorporating anaerobiosis, for the isolation by culture of ancient pathogens, opening the door to the possibility of ancient

dental pulp.

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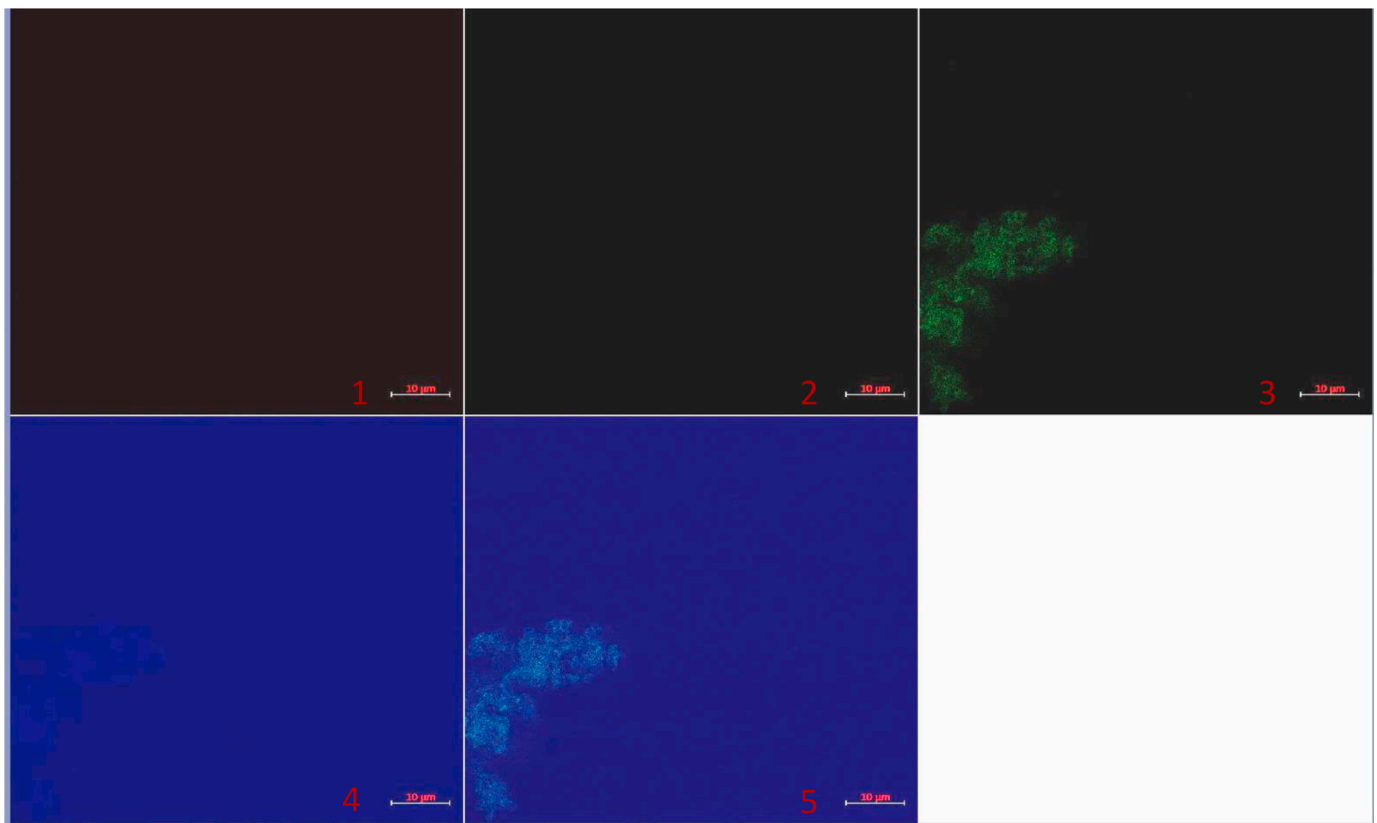


Fig. 8. FISH on SQ517 dental pulp: 1: filter AF555; 2: filter AF640; 3: Filter AF488; 4: DAPI; 5: MERCH.

Agency for Research) (reference: Méditerranée Infection 612 10-IAHU-03).

Data available

Raw sequencing read have been deposited on NCBI (Accession number: PRJNA770891)

Contributors

MM, RB, MD and GA conceived and designed the study. MM performed the experiments. MM, CC, MS, FA, EV, RB, MD, MB and GA analysed the material and the data. MM, MD, RB, MB and GA wrote the manuscript. MB did the bioinformatical analysis. All authors contributed to data interpretation, critically reviewed the manuscript, and approved the final manuscript for submission.

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Validation; All authors contributed to data interpretation, critically reviewed the manuscript, and approved the final manuscript for submission.

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Declaration of Competing Interest

All authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.crmicr.2021.100089](https://doi.org/10.1016/j.crmicr.2021.100089).

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