New Microbes and New Infections 59 (2024) 101242

Contents lists available at ScienceDirect

New Microbes and New Infections

journal homepage: www.journals.elsevier.com/new-microbes-and-new-infections

Original Article

Insights from genomic analysis of a novel *Coxiella burnetii* strain isolated in Israel

Inbar Cohen-Gihon [a,1], Ofir Israeli [a,1], Gal Bilinsky [a], Barak Vasker [b], Shirley Lazar [a], Adi Beth-Din [a], Anat Zvi [a], Nesrin Ghanem-Zoubi [c,**], Yafit Atiya-Nasagi [b,*]

[a] Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness Ziona, Israel
[b] Department of Infectious Diseases, Israel Institute for Biological Research, Ness Ziona, Israel
[c] Infectious Diseases Institute, Rambam Health Care Campus, Haifa, Israel

ARTICLE INFO

Handling Editor: Patricia Schlagenhauf

Keywords:
Coxiella burnetii
Q fever
Culture isolation
Molecular sequencing
Genotyping
High throughput sequencing
Hybrid assembly

ABSTRACT

The diagnosis of Q fever is challenging due to nonspecific symptoms and negative standard blood culture results. Serological testing through immunofluorescence assay (IFA) is the most commonly used method for diagnosing this disease. Polymerase chain reaction (PCR) tests can also be used to detect bacterial DNA if taken at an appropriate time. Once the presence of bacteria is confirmed in a sample, an enrichment step is required before characterizing it through sequencing. Cultivating *C. burnetii* is challenging as it can only be isolated by inoculation into cell culture, embryonated eggs, or animals. In this article, we describe the isolation of *C. burnetii* from a valve specimen in Vero cells. We conducted genome sequencing and taxonomy profiling of this isolate and were able to determine its taxonomic affiliation. Furthermore, Multispacer sequence typing (MST) analysis suggests that the infection originated from a local strain of *C. burnetii* found around northern Israel and Lebanon. This novel strain belongs to a previously described genotype MST6, harboring the QpRS plasmid, never reported in Israel.

1. Introduction

Q fever is caused by the highly infectious *Coxiella burnetii*, an obligate intracellular bacterium. *C. burnetii* has been found to infect both wild and domestic animals, such as reptiles, birds, and arthropods (particularly ticks). Goats, sheep, and cattle are considered to be the primary reservoirs of this bacterium. *C. burnetii* is shed in large numbers in the birth products of infected animals and can also be found in milk during parturition [1]. People can become infected through inhalation of contaminated air that contains infectious organisms [2–4]. Human Q fever has a polymorphic clinical presentation [5–7] with two stages that are distinct in their antigenic phases of antibody response: acute and chronic stages. Symptoms of acute Q fever can range from asymptomatic to a flu-like self-limited illness or more severe symptoms such as pneumonia [8], granulomatous hepatitis, and, in rare cases, cardiac involvement [9]. The chronic disease (which occurs in a minority (<5%) of the cases) may manifest months to years after an acute infection and can be lethal if not treated appropriately. Chronic Q fever typically manifests as endocarditis caused by *C. burnetii*, primarily affecting the aortic and mitral valves as well as valvular prostheses [10]. Vascular infections are the second most common, persistent focalized infection after endocarditis [6,10,11]. It has been proposed that the clinical presentation, host type, and severity of Q fever may differ depending on the *C. burnetii* strain involved [12–15]. The first *C. burnetii* genome sequenced was in 2003 from the Nine Mile Phase I RSA493 strain [16]. In recent years, additional 14 genomes of *C. burnetii* strains were sequenced, generating data that facilitated comparative genomic analyses of this pathogen. Previously, it was thought that no pathotype specific gene was associated with *Coxiella burnetii* [17]. However, a recent publication demonstrates that there is a significant association between presence of plasmids and the clinical presentation outcome. Strains containing the QpRS plasmid were associated with persistent focalized infections (chronic Q fever) and were never found in acute Q fever [18]. In this paper, we present the diagnosis of *C. burnetii* in a

* Corresponding author.
** Corresponding author.
E-mail addresses: n_ghanem@rambam.health.gov.il (N. Ghanem-Zoubi), yafita@iibr.gov.il (Y. Atiya-Nasagi).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.nmni.2024.101242
Received 28 June 2023; Received in revised form 25 March 2024; Accepted 25 March 2024
Available online 26 March 2024

42-year-old male patient, who was admitted to the emergency room with a stroke. He was diagnosed with aortic valve endocarditis. The patient's condition deteriorated rapidly, and he underwent open-heart surgery. Despite medical intervention, the patient passed away 18 days after hospitalization.

1.1. Clinical case

A 42-year-old male with a congenital bicuspid aortic valve was admitted to several hospital emergency rooms (ER) presenting different symptoms such as rash, blurred vision, and myalgia. Due to a heart murmur, he had three sets of blood cultures, which were negative, therefore leading to his discharge. In his last admission, he presented a stroke and a fever. Echocardiography showed large vegetation on the aortic valve with moderate stenosis, moderate aortic regurgitation, and abscess formation. Serological tests by enzyme-linked immunosorbent assay (ELISA) showed positive anti-phase 2 IgM and IgG and anti-phase 1 IgG. This result was confirmed by an indirect fluorescence assay (IFA) showing: Phase 2 IgM positive, Phase 2 IgG-3200, Phase 1 IgM positive, and Phase 1 IgG-3200. Following these results, the empiric treatment was directed with the administration of intravenous doxycycline and ciprofloxacin. On his sixth day of hospitalization, the patient underwent open-heart surgery. The aortic valve was inspected to be severely damaged with calcified vegetations and an abscess on the membranous septum. He underwent aortic valve replacement with a bioprosthetic valve and replacement of the root of the aorta. A sample of the aortic valve, as well as his blood sample, were tested by PCR for the presence of *C. burnetii* and were found to be positive (data not shown). The bacterium was isolated from the positive valve sample. On day 21 post-isolation, the infected culture was harvested and homogenized. DNA was extracted for PCR analysis of *C. burnetii*-specific genes. PCR results were positive for *hpt*A and IS1111 specific genes of *C. burnetii*. The cultured-grown bacterium was sequenced by Illumina short reads technology combined with ONT long reads technology to perform whole genome hybrid assembly.

2. Methods

2.1. Serologic diagnosis

The serum sample was tested for IgG and IgM antibodies against both phase I and phase II antigens using an indirect immunofluorescence assay (IFA) as previously described [19].

2.2. C. burnetii isolate

Blood and aortic valve tissue samples were collected shortly after the initiation of antibiotic treatment from a patient who had undergone heart valve replacement. The samples were sent to the Rickettsioses reference laboratory at the Israeli Institute for Biological Research for diagnosis of *C. burnetii*. After confirming as positive by PCR, pieces of the valve tissue were minced with 2%FCS-DMEM and inoculated into African green monkey kidney fibroblasts (Vero, American Type Culture Collection, ATCC) which are routinely used for culturing of *C. burnetii*. Bacterial growth was monitored using an *in-house* IFA method and lasted about three weeks. *C. burnetii* were purified from host cells using differential centrifugation and stored at −80 °C. Bacterial isolation was carried out in a biosecurity level 3 facility according to the guidelines of the Israel Institute for Biological Research.

2.3. DNA extraction for C. burnetii

DNA was extracted from 200 µl of blood specimen using the QIAamp Blood Kit (Qiagen, Hilden, Germany), as described by the manufacturer.

2.4. Whole genome sequencing

Illumina short read sequencing: 1 ng of DNA was transferred to the library preparation step utilizing the Nextera XT DNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). The standard manufacturer protocol was modified to optimize library preparation from clinical samples. For each sample, the 20 µl tagmentation (combined DNA fragmentation and tagging) reaction contained 5 µl of amplicon tagment mix (ATM), which includes the enzyme used for tagmentation, 10 µl of TD buffer, 1 ng of input DNA and 1 µl of 20 mg/ml bovine serum albumin (BSA). The tagmentation reactions were incubated in a thermal cycler at 50 °C for 5 min. Subsequently, the tagmented DNA was amplified via limited-cycle PCR. The quality and quantity of the purified libraries were assessed using the high-sensitivity (HS) DNA kit on TapeStation (Agilent). The libraries were sequenced on an Illumina MiSeq,150 bp paired-end.

Oxford nanopore technologies (ONT) long reads: 100 ng of DNA was transferred to the library preparation step of the Rapid sequencing kit (SQK-RAD004, ONT). The libraries were processed according to the manufacturer's protocol and sequencing was conducted using an R9 flow cell on a MK1B apparatus (ONT).

2.5. Genome assembly and genotyping of Coxiella burnetii

2.5.1. Reads quality control and host removal

Reads from both Illumina and ONT sequencing were subjected to quality control and host removal. Reads that mapped to a reference of Vero cells (GCF_000409795.2) with over 90% were removed, as well as reads shorter than 50 bases, contained 10 "N"s or more, and had a fraction of mono-/di-nucleotide sequence > 0.85 of the read length. Unpaired Illumina reads were also discarded. Read mapping was performed using BWA MEM [20]. Finally, reads were trimmed using FaQCs [21], with a quality threshold of $q = 20$ for Illumina reads and $q = 7$ for ONT reads.

2.5.2. Genome assembly

The filtered reads were used to build a hybrid assembly via Unicycler [22], utilizing default parameters. Essentially, Unicycler initially assembles short reads using SPAdes [23], subsequently employing long ONT reads to bridge gaps, a strategy that results in fewer contigs and a more comprehensive assembly. To verify the assembly's integrity, resequencing was performed on all filtered reads against the assembled genome using bowtie2 [24]. A total of 99.85% of the reads were mapped to the assembly, with an average coverage of 45x. This Whole Genome Shotgun project was named CBI_2022 and has been deposited at DDBJ/ENA/GenBank under the accession JASNNV000000000. The version described in this paper is version JASNNV010000000.

2.5.3. Phylogenetic analysis

C. burnetii assemblies were downloaded from NCBI RefSeq [25] Core genome alignment and phylogeny of the assemblies were performed using Parsnp [26] with default parameters, with respect to reference genome *C. burnetii* RSA 493 strain (GCF_000007765.2). Pairwise genome similarity was calculated with the average nucleotide identity (ANI) index [27].

2.5.4. Multispacer sequence typing (MST) analysis

MST analysis of CBI_2022 was performed using the CoxBase database [28]. This platform included information on MST of 328 samples from 29 countries, as of February 2023.

3. Results

A total of 85% of the reads were mapped to the Vero reference. The remaining 588,416 Illumina and 1845 ONT good quality reads were assembled *de novo* using the hybrid strategy, resulting in 47 contigs, with

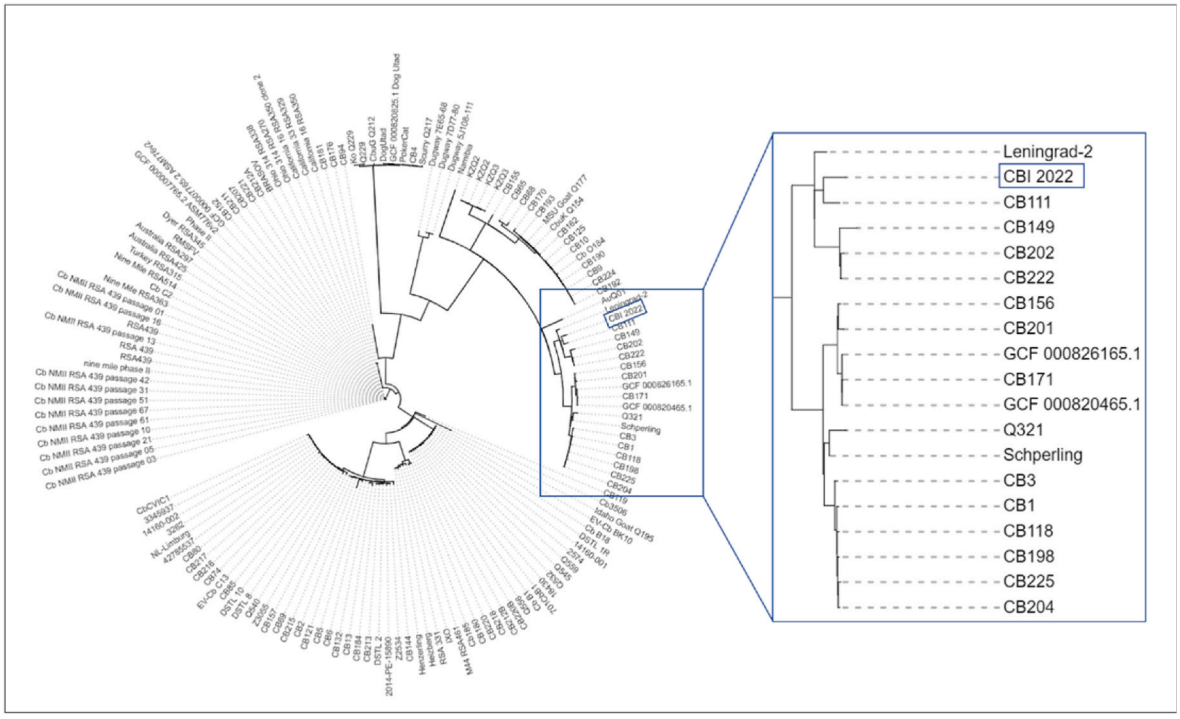

Fig. 1. A phylogenetic tree constructed from all assemblies available at NCBI, and the location of CBI_2022 on it.

N50 at 98,349 bp, a maximum contig size of 308,761 bp, and a total assembly size of 2,058,687 bp. The mean GC content was 43.65%, closely resembling the values of the validated RefSeq assembly of the RSA 493 strain (GCF_000007765.2). Aiming at profiling the taxonomic relation of the CBI_2022 strain, a total of 147 *Coxiella burnetii* assemblies were downloaded from NCBI RefSeq [25], including the curated reference RSA493 strain (GCF_000007765). Following core genome alignments and phylogeny analysis of all assemblies, a phylogenetic tree was constructed using the Parsnp tool, which extracts the core SNP information derived from the alignments (Fig. 1). The closest strains to the CBI_2022 were found to be: CB111, CB1498, CB202, CB222, Leningrad-2. The average nucleotide identity (ANI) was calculated for each strain and a maximum ANI of 99.95% was found for the closest strain CB111, which was identified as originating from a human sample in France in 2013 (Supplementary Table 1).

While a comprehensive phylogenetic analysis allows for the resolution of evolutionary relationships and comparison of genetic diversity, MST which is based on intergenic region sequencing subjected to lower selection pressure compared to adjacent genes, offers a standardized typing scheme, epidemiological surveillance capabilities and is widely used in epidemiological studies to investigate the spread and transmission of microbial pathogens. Such regions enable epidemiologic tracking of strains with close geographic origin, disease manifestations, or hosts. This methodology was implemented using the CoxBase platform, for typing *Coxiella burnetii* strains, the MST profile of CBI_2022 was determined for the following 10 intergenic regions: Cox2 [4], Cox5 [6], Cox18 [3], Cox20 [5], Cox22 [6], Cox37 [5], Cox51 [8], Cox56 [2], Cox57 (new), Cox61 [6]. A list of the closest MST profiles was compiled, including any sample harboring a profile with up to three different sites, yielding 22 samples in the database (Supplementary Table S2). Out of these, 14 samples were obtained from cattle, sheep, and goats in southern Lebanon. The extent of samples from Lebanon identified in the CBI_2022 sample is 64%, over 12-fold higher than its overall representation in the database (5%), reflecting a significant enrichment in the CBI_2022 sample. These findings may suggest that the infection originated from a local strain of *Coxiella burnetii* found around northern Israel and Lebanon. According to all these findings, the CBI_2022 strain corresponds to the MST6 genotype with the QpRS plasmid, thus associated with persistent focalized infections (endocarditis, vascular infection and miscarriage [18]). Altogether, this suggests that MST6 is a geotype (a specific genotype that is associated with geography) endemic from Senegal to Saudi Arabia (all North Africa and Middle East), where it could be found in persistent focalized infection. The present report confirms that MST6 is not associated with acute Q fever (the patient did not report a symptomatic primary infection).

4. Conclusions

In this study, we successfully isolated *C. burnetii* from the aortic valve of a patient with chronic Q fever in Israel and confirmed the pathogen presence through PCR and sequence analysis. We sequenced and assembled it to a draft genome level and performed a phylogenetic analysis. This novel strain, which has never been reported in Israel, belongs to the previously identified genotype MST6, carrying the QpRS plasmid. This previously uncharacterized local strain may contribute to comparative pan-genomic analysis with worldwide *C. burnetii* strains. While genomic analysis provides valuable insights into *C. burnetii* epidemiology and correlation with clinical outcomes, further studies utilizing a wider range of clinical samples are needed to achieve a more comprehensive understanding, especially in severe and fatal cases.

CRediT authorship contribution statement

Inbar Cohen-Gihon: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Ofir Israeli:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Gal Bilinsky:** Data curation, Formal analysis, Writing – original draft. **Barak Vasker:** Data curation. **Shirley Lazar:** Data curation. **Adi Beth-Din:** Supervision. **Anat Zvi:** Supervision. **Nesrin Ghanem-Zoubi:** Data curation, Methodology, Writing – original draft. **Yafit Atiya-Nasagi:** Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None declared

Funding

The authors declare that no specific funding was used for this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2024.101242.

References

[1] Arricau-Bouvery N, Rodolakis A. Is Q fever an emerging or re-emerging zoonosis? Vet Res 2005;36(3):327–49.
[2] Hawker JI, Ayres JG, Blair I, Evans MR, Smith DL, Smith EG, et al. A large outbreak of Q fever in the West Midlands: windborne spread into a metropolitan area? Comm Dis Publ Health 1998;1(3):180–7.
[3] Tissot-Dupont H, Amadei MA, Nezri M, Raoult D. Wind in november, Q fever in december. Emerg Infect Dis 2004;10(7):1264–9.
[4] Guatteo R, Seegers H, Taurel AF, Joly A, Beaudeau F. Prevalence of Coxiella burnetii infection in domestic ruminants: a critical review. Vet Microbiol 2011;149(1–2):1–16.
[5] Raoult D, Fournier PE, Eremeeva M, Graves S, Kelly PJ, Oteo JA, et al. Naming of Rickettsiae and rickettsial diseases. Ann N Y Acad Sci 2005;1063:1–12.
[6] Eldin C, Melenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, et al. From Q fever to Coxiella burnetii infection: a paradigm change. Clin Microbiol Rev 2017;30(1):115–90.
[7] Melenotte C, Million M, Raoult D. New insights in Coxiella burnetii infection: diagnosis and therapeutic update. Expert Rev Anti Infect Ther 2020;18(1):75–86.
[8] Caron F, Meurice JC, Ingrand P, Bourgoin A, Masson P, Roblot P, et al. Acute Q fever pneumonia: a review of 80 hospitalized patients. Chest 1998;114(3):808–13.
[9] Fournier PE, Etienne J, Harle JR, Habib G, Raoult D. Myocarditis, a rare but severe manifestation of Q fever: report of 8 cases and review of the literature. Clin Infect Dis 2001;32(10):1440–7.
[10] Angelakis E, Raoult D. Q fever. Vet Microbiol 2010;140(3–4):297–309.
[11] O'Donnell ME, Manshani N, McCaughey C, Soong C, Lee B. Coxiella burnetii infection of an aortic graft with multiple vertebral body erosion. J Vasc Surg 2007;45(2):399–403.
[12] Glazunova O, Roux V, Freylikman O, Sekeyova Z, Fournous G, Tyczka J, et al. Coxiella burnetii genotyping. Emerg Infect Dis 2005;11(8):1211–7.
[13] Roest HI, Ruuls RC, Tilburg JJ, Nabuurs-Franssen MH, Klaassen CH, Vellema P, et al. Molecular epidemiology of Coxiella burnetii from ruminants in Q fever outbreak, The Netherlands. Emerg Infect Dis 2011;17(4):668–75.
[14] de Bruin A, van Alphen PT, van der Plaats RQ, de Heer LN, Reusken CB, van Rotterdam BJ, et al. Molecular typing of Coxiella burnetii from animal and environmental matrices during Q fever epidemics in The Netherlands. BMC Vet Res 2012;8:165.
[15] Angelakis E, Million M, D'Amato F, Rouli L, Richet H, Stein A, et al. Q fever and pregnancy: disease, prevention, and strain specificity. Eur J Clin Microbiol Infect Dis 2013;32(3):361–8.
[16] Seshadri R, Paulsen IT, Eisen JA, Read TD, Nelson KE, Nelson WC, et al. Complete genome sequence of the Q-fever pathogen Coxiella burnetii. Proc Natl Acad Sci U S A 2003;100(9):5455–60.
[17] Stein ARD. Lack of pathotype specific gene in human Coxiella burnetii isolates. Microb Pathog 1993;15(3).
[18] Rita Abou Abdallah MM, Delerce Jeremy, Hussein Anani, Diop Awa, Caputo Aurelia, Zgheib Rita, Rousset Elodie, Karim Sidi Boumedine, Raoult Didier, Fournier Pierre-Edouard. Pangenomic analysis of Coxiella burnetii unveils new traits in genome architecture. Front Microbiol 2022;21(13):1022356.
[19] Siegman-Igra Y, Kaufman O, Keysary A, Rzotkiewicz S, Shalit I. Q fever endocarditis in Israel and a worldwide review. Scand J Infect Dis 1997;29(1):41–9.
[20] Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25(14):1754–60.
[21] Lo CC, Chain PS. Rapid evaluation and quality control of next generation sequencing data with FaQCs. BMC Bioinf 2014;15(1):366.
[22] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol.13(6): e1005595. .
[23] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19(5):455–77.
[24] Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012;9(4):357–9.
[25] O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res 2016;44(D1):D733–45.
[26] Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 2014;15(11):524.
[27] Figueras MJ, Beaz-Hidalgo R, Hossain MJ, Liles MR. Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. Genome Announc 2014;2(6).
[28] Fasemore AM, Helbich A, Walter MC, Dandekar T, Vergnaud G, Forstner KU, et al. CoxBase: an online platform for epidemiological surveillance, visualization, analysis, and typing of Coxiella burnetii genomic sequences. mSystems 2021;6(6): e0040321.