A new TaqMan real-time PCR assay to detect Parachlamydia acanthamoebae and to monitor its co-existence with SARS-COV-2 among COVID-19 patients

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Abstract
Human respiratory infections caused by a large variety of microbial pathogens are the most common diseases responsible for hospitalization, morbidity and mortality. Parachlamydia acanthamoebae, a Chlamydia-related bacterium, has been found to be potentially associated with these diseases. An early and accurate diagnosis of this pathogen could be useful to avoid the potential respiratory complications linked especially to COVID-19 patients and to set suitable outbreak control measures. A TaqMan-PCR assay was developed to detect and quantify Parachlamydia acanthamoebae in environmental and clinical samples from patients of all ages with COVID-19. The selected hydrolysis probe displayed no cross-reaction with the closely related Chlamydia or the other tested pathogens. This q-PCR achieved good reproducibility and repeatability with a detection limit of about 5 DNA copies per reaction. Using this q-PCR assay, Parachlamydia acanthamoebae was detected in 2/78 respiratory specimens and 9/47 water samples. Only one case (1.3%) of Parachlamydia acanthamoebae and SARS-COV-2 co-infection was noticed. To our knowledge, the combination of these two respiratory pathogens has not been described yet. This new TaqMan-PCR assay represents an efficient diagnostic tool to survey Parachlamydia acanthamoebae on a large-scale screening programs and also during outbreaks.

Keywords Pneumonia · Parachlamydia · SARS-COV-2 · PCR · COVID

Introduction

Community-acquired pneumonia (CAP) is considered as the main infectious cause of death among children and the elderly. This fatal pulmonary disease might be engendered by viruses, bacteria and fungi. The co-existence of these pathogens may play a crucial role in its severity and mortality (Bakare et al. 2020). Since the start of the corona pandemic, death rates due to pneumonia worldwide are growing at an alarming increase. Recently, many studies described the co-infection between SARS-cov2 and other atypical pathogens like Mycoplasma and Chlamydia (Kim et al. 2020). Therefore, it is of great importance to identify the causative agent in order to establish the adequate antibiotic therapy.

In major cases of patients with pneumonia, the available and routinely diagnosis methods fail to identify the etiological agents. This can be explained by the fact that pneumonia in this situation is due to another atypical or emerging pathogenic agents. Among the unknown agents are the
Chlamydia-related bacteria (Garin et al. 2015) such as Sim-kia negevensis, Waddlia chondrophila and Parachlamydia acanthamoebae, which were documented to be associated with CAP. Parachlamydia acanthamoebae (P. acanthamoebae) is recognized as an emerging pulmonary agent, being responsible for 0.5–2% of CAP (Lamoth and Greub 2010). Epidemiological, serological and molecular studies have demonstrated the closely involvement of P. acanthamoebae to pneumonia diseases (Marrie et al. 2001; Greub 2009; Pilloux et al. 2015; Hokynar et al. 2019). Indeed, it was proved to penetrate, replicate and lyse pulmonary pneumocytes and fibroblasts (Casson et al. 2006). Animal models of lung infection supported its potential role in pneumonia and suggested that human exposure to P. acanthamoebae can lead to bronchitis and pneumonia (Casson et al. 2008a; Lohr et al. 2015; Pilloux et al. 2015).

As all the members of Chlamydia-related bacteria, P. acanthamoebae is an obligate intracellular bacterium that survive within free-living amoebae which act as a widespread reservoir enabling its resistance to many environmental stresses (Kebbi-Beghdadi and Greub 2014; Balczan and Scheid 2017). It has been recognized as a worldwide distributed bacteria (Corsaro and Venditti 2006), which lives in aquatic and terrestrial environments such as rivers, drinking and wastewaters (Horn and Wagner 2001; Thomas et al. 2006), soil (Kaplan and Kitts 2004), and activated sludge (Collingro et al. 2005). Till now, no studies reporting the existence of P. acanthamoebae in Africa are available.

Detection of P. acanthamoebae by classical culture methods remains difficult because it cannot grow in axenic media. In addition, coculture with amoeba and serological diagnosis are time-consuming and inconclusive (Greub et al. 2003; Rames 2019). That is why, molecular techniques have been increasingly used for the detection of Chlamydia-related bacteria in various types of samples. Many studies have proved the high specificity, sensitivity, and effectiveness of the molecular diagnostic tools to rapidly and directly identify these pathogens (Goy et al. 2009; Lienard et al. 2011; Barkallah et al. 2013; Baccari et al. 2020). Different effective PCR-based assays for detection of P. acanthamoebae, including classical and quantitative PCR, have been described (Corsaro et al. 2002; Greub et al. 2003; Corsaro and Greub 2006; Thomas et al. 2006; Casson et al. 2008b; Fukumoto et al. 2010; Hokynar et al. 2019) in which DNA was recovered from amoeba co-culture, swab and nasal samples. Yet, no real-time TaqMan PCR has been designed to detect and quantify P. acanthamoebae in both clinical and environmental samples.

In this context, we aimed to develop a new TaqMan-PCR assay for the quick detection and absolute quantification of P. acanthamoebae in the hospital-environment interface. This study would contribute to enhance our knowledge on the coronavirus and P. acanthamoebae co-infection.

### Materials and methods

#### Microbial species

Thirty bacterial cultures, four amoeba species and one virus were used to assess the analytical specificity of primers and probe (Table 1).

#### Water samples

This study was conducted between 2017 and 2021 in six governorates of Tunisia: Sfax, Gabes, Gafsa, Medenine, Tozeur, Jendouba, Sousse and Nabeul. A total of 53 water samples were collected in 50-ml sterilized tubes and concentrated by two successive filtrations through membranes with porosities of 70 and 0.45 μm.

#### Clinical samples

Two batches of clinical samples were collected between 2017 and 2021 in the Clinical Microbiology Laboratory of Habib Bourguiba University Hospital of Sfax, Tunisia. The first batch consisted of specimen nasopharyngeal swabs recently collected from COVID-19 patients. The second batch consisted of sputum samples from adults with suspected pneumonia or bronchial superinfections and nasopharyngeal aspirates from children. Each sample was collected in a 2-ml Eppendorf tube and homogenized with an equal volume of dithiothreitol (Digest-EUR® Eurobio, Courtabœuf, France) to reduce its viscosity.

#### DNA isolation

Genomic DNA extraction from the reference bacterial and amoeba strains were done using an automatic extractor (MagNA Pure LC system, Roche, Rotkreuz, Switzerland) and DNA isolation kit (MagNA Pure LC, Roche) according to the manufacturer’s instructions. For the environmental samples and the second batch of clinical samples, genomic DNA were extracted using a commercially available extraction kits known as “PureLink ™ Genomic DNA Mini Kit”, as recommended by the manufacturer (Invitrogen (Abdelkafi et al. 2005; Fendri et al. 2010; Miladi et al. 2018).

#### Viral RNA extraction and reverse transcription

RNA was extracted from the nasopharyngeal swabs of COVID-19 patients using the alphaPrep™ Viral DNA/ RNA Extraction Mini-Kit (Cat. No. VID-C110, VID-C120; AlphaGene, Seongnam-si, Republic of Korea). RNA was eluted in a final volume of 20 μl. The detection of
the 2019-nCoV (COVID-19) virus was realized using the Wondfo RT-PCR assay kit (Guangzhou Wondfo Biotech, China). Nucleic acids were then stored at −20 °C until further use.

**Primers and probe**

A pair of primers PChlam-F-5′-GTTCTGGAGCCGAAGCGAAA-3′ and PChlam-R-5′- CAAAGGCTTTTTCAACGGCAAC-3′ and a hydrolysis probe PChlam-P-5′- JOE-CCATGACCAGGTTAAGCAAGGGT BHQ1-3′ were selected using Primer Express software version 3.0. They were complementary to a region of 146 bp of the gene encoding the large subunit LSU rRNA of *P. acanthamoebae* strain Y07551.1. The newly designed primers and probe were optimized in silico, using IDT Oligoanalyzer tools available online, to avoid the dimer hairpin and secondary structures formation. The NCBI Blast tool was also used to verify the specificity of primers and probe against the targeted sequence of genomic DNA.

| Microbial species | Source/strain | TaqMan real-time PCR detection |
|-------------------|---------------|-------------------------------|
| *Parachlamydia acanthamoebae* | ATCC VR-1476 | D |
| *Simkania negevensis* | ATCC VR-1471/2 | ND |
| *Listeria monocytogenes* | ATCC 19,115 | ND |
| *Listeria grayi* | ATCC 25,401 | ND |
| *Listeria ivanovii* | ATCC 19,115 | ND |
| *Listeria innocua* | ATCC 43,547 | ND |
| *Listeria seeligeri* | ATCC 35,967 | ND |
| *Salmonella typhimurium* | Bovine milk sample (Tunisia) | ND |
| *Salmonella enterica Enteritidis* | Chicken (Tunisia) | ND |
| *Coxiella burnetii* | Clinical sample (Tunisia) | ND |
| *Waddlia chondrophila* | ATCC VR-1470 | ND |
| *Chlamydia suis* | S45 | ND |
| *Chlamydia abortus* | S26 | ND |
| *Chlamydia pecorum* | W73 | ND |
| *Chlamydia psittaci* | T49 | ND |
| *Chlamydia trachomatis* | Clinical sample (Swizerland) | ND |
| *Legionella pneumophila* | Clinical sample (Swizerland) | ND |
| *Klebsiella pneumonia* | Clinical sample (Swizerland) | ND |
| *Brucella abortus* | S19 | ND |
| *Brucella melitensis* | Bovine milk sample (Tunisia) | ND |
| *Escherchia coli* | ATCC 8739 | ND |
| *Staphylococcus aureus* | ATCC 25,923 | ND |
| *Neisseria ovis* | Ovine occular sample (Swizerland) | ND |
| *Pseudomonas aeruginosa* | ATCC 27,853 | ND |
| *Streptococcus bovis* | ATCC 33,317 | ND |
| *Campylobacter jejuni* | Ovine vaginal sample (Tunisia) | ND |
| *Campylobacter coli* | Chicken (Tunisia) | ND |
| *Bacillus subtilis* | Bovine milk sample (Tunisia) | ND |
| *Bacillus cereus* | Bovine milk sample (Tunisia) | ND |
| *Vibrio parahaemolyticus* | Clam sample (Tunisia) | ND |
| *Acanthamoebae castellanii* | ATCC 30,010 | ND |
| *Acanthamoebae comandoni* | ATCC 30,135 | ND |
| *Dictyostelium discoideum* | ATCC 25,697 | ND |
| *Hartmannella vermiformis* | ATCC 50,237 | ND |
| SARS-CoV-2 | Clinical sample (Tunisia) | ND |

*D detection, ND no detection*
Real-time PCR assay

All reactions were assessed using a StepOnePlus™ PCR cycler (Applied Biosystems, Foster City, USA) as previously reported by Barkallah et al. (2020) and Elleuch et al. (2021). Plasmid copy numbers were calculated using the following equation: \( C_n = \frac{(P_c \times A_c)}{(M_w \times P_l)} \), where \( C_n \) is the copy number, \( P_c \) is the plasmid concentration (g/µl), \( A_c \) is Avogadro’s constant \( = 6.022 \times 10^{23} \) mol\(^{-1} \), \( M_w \) is the base pair mean molecular weight \( = 660 \) Da and \( P_l \) is the plasmid length containing the cloned sequence (bp).

A set of optimizations has been generated by amplification of tenfold serial dilutions \( 10^5 \) to \( 10^3 \) of standard plasmid in search of best conditions. For this purpose, primers were evaluated in different concentrations ranging from 0.05 to 0.2 µM and probes were varied from 0.1 to 0.4 µM. The thermal cycling conditions were determined by setting an annealing temperature gradient (between 57 and 62 °C) during 20 and 30 s testing two different cyclic denaturation times (1 s and 5 s). The best reaction conditions were determined based on a PCR efficiency of 90 to 120%, a lower quantification cycle (Cq) value and a higher fluorescence intensity for each dilution. Therefore, the optimized PCR program was as follows: an initial 20-s denaturation step at 95 °C, followed by 40 cycles at 95 °C for 1 s, 60 °C for 20 s. The q-PCR assays were performed on a reaction final volume of 20 µl that consists of the following: 10 µl “Premix Ex Taq™” (TaKaRa), 0.1 µM of primer, 0.2 µM of probe, 0.4 µl of ROX (1 × final), 3.8 µl of ultra-pure distilled water and 5 µl of DNA sample. Data were analyzed using the step one software version 2.3. The threshold cycle number (Ct) was performed by setting the instrument’s threshold line at 0.08 ΔRn unit.

Sensitivity, specificity and reproducibility

The specificity of amplification was measured using agarose gel electrophoresis to verify the size amplicon. The limit of detection was estimated by testing a sextuplicate of the diluted plasmids at 50, 25, 12.5 and 5 copies per reaction. The analytical specificity of real-time PCR was estimated by screening DNA extracts from the bacterial and amoebal strains mentioned in Table 1. To measure inter-assay variability, six different concentrations of plasmid DNA ranging from 5 to \( 5 \times 10^6 \) copies per reaction were assayed in duplicate in five separate runs that were carried out on different days.

Intra-assay variability was assessed by performing a single q-PCR assay using five replicates of each of the following plasmid copy numbers per reaction: \( 5 \times 10^2 \), \( 5 \times 10^3 \), \( 5 \times 10^4 \), \( 5 \times 10^5 \), \( 5 \times 10^6 \). Analysis of the results was carried out based on the mean of Ct values, coefficient of variation (CV) and the standard deviation. Coefficient of variation is equal to the standard deviation divided by the mean of Ct. Each conducted run contained negative controls. A standard curve was developed through tenfold serial dilutions of plasmid DNA (\( 5 \times 10^5 \)–5 copies/reaction).

Validation

Once standardized, the q-PCR assay was used for detection as well as quantification of \( P. \) acanthamoebae in environmental and clinical samples. The corresponding Ct values were collected and converted to \( \log_{10} \) copy number/µl. The obtained amplicons were then subjected to agarose gel migration and checked by sequencing using a taq DyeDeoxy terminator cycle Sequencing kit and a 3700 ABI Prism DNA sequencer (Applied Biosystems, Foster City, CA, USA). PCR inhibitors were tested by adding an internal control (1 µl of \( 10^2 \) copies/µl of plasmid DNA + 1 µl of negative sample) to some negative samples and PCR amplification.

Statistical analyses

SPSS 17.0 software was used for statistical analysis. Ct data were normalized by \( \log_{10} \) transformation. Results were presented as the mean ± SEM. A two-tailed Student’s \( t \)-test was assigned in order to determine the significance of the difference between Ct values. An ANOVA was used to compare the slope of linear regressions.

Results

Optimization of \( P. \) acanthamoebae-specific q-PCR assay

The plasmid DNA of \( P. \) acanthamoebae was successfully detected by the q-PCR assay using the designed oligonucleotides with Ct value of 22.5 when \( 5 \times 10^5 \) of DNA copies/reaction. The size of the obtained PCR product was checked on an agarose gel and it corresponded to the expected size of ~ 146 bp.

Specificity, sensitivity and reproducibility

Blast nucleotide analysis of the amplicon, primers and probe resulted in 100% identity with the partial sequence of LSU gene of \( P. \) acanthamoebae, which proved a high theoretical specificity of q-PCR. The experimental specificity was high, as no amplification was obtained for the untargeted pathogens listed in Table 1. Thus, the specificity of the probe ensured a high degree of discrimination between the amplicon of \( P. \) acanthamoebae and those of the other tested microorganisms. The analytical sensitivity of our q-PCR revealed about 5 copies of linear plasmid DNA per reaction.
as all replicates were positive at a concentration of 5 copies with a Ct value of about 37.11 ± 0.45 as showed in Table 2.

A standard curve was developed through a tenfold serial dilution of plasmid DNA ranging from 5 x 10^5 to 50 copies/reaction. As shown in Fig. 1, it exhibited an acceptable amplification efficiency, slope and correlation coefficient: R^2 = 0.99, S = -3.158, E value = 107%, indicating that the designed primers and probe were efficient. The repeatability and reproducibility of q-PCR assays were evaluated using intra- and inter-assay variation, respectively. Coefficients of variation between and within runs are ranged from 0.4 to 1.7 for each dilution factor across reactions, demonstrating the repeatability and the reproducibility of the assays (Table 2). The inter- and intra-run reproducibility of the q-PCR assays were also evaluated using statistical tests as shown in Fig. 2.

Table 2 Intra- and inter-run standard deviation (SD) and coefficient of variation (CV) of mean threshold cycle (Ct) for gene real-time PCR performed on 5 replicates in a single run or in ten different runs

| Reproducibility setting | Control plasmid (copies/reaction) | N  | Ct mean | SD (±) | CV (%) |
|------------------------|----------------------------------|----|---------|--------|--------|
| Inter-run              | 500,000                          | 5  | 22.18   | 0.18   | 0.84   |
|                        | 50,000                           | 5  | 26.10   | 0.10   | 0.42   |
|                        | 5000                             | 5  | 29.20   | 0.18   | 0.64   |
|                        | 500                              | 5  | 32.71   | 0.25   | 0.79   |
|                        | 50                               | 5  | 34.69   | 0.12   | 0.37   |
|                        | 5                                | 5  | 37.11   | 0.45   | 1.21   |
|                        | 0                                | ND | ND      | ND     | ND     |
| Intra-run              | 500,000                          | 5  | 22.44   | 0.10   | 0.44   |
|                        | 50,000                           | 5  | 25.60   | 0.07   | 0.29   |
|                        | 5000                             | 5  | 29.00   | 0.13   | 0.47   |
|                        | 500                              | 5  | 32.29   | 0.43   | 1.33   |
|                        | 50                               | 5  | 34.18   | 0.27   | 0.80   |
|                        | 5                                | 5  | 37.38   | 0.63   | 1.70   |
|                        | 0                                | ND | ND      | ND     | ND     |

Intra-run reproducibility was confirmed. The Ct of both duplicates were relatively similar with a correlation coefficient R^2 of 0.995 (panel A). The Bland–Altman graph (panel C) demonstrated that the 95% confidence interval is obtained when the difference in Ct between duplicates is 0.71. The inter-run variability is shown in panel B; the inter-run variability varied inversely with the amount of plasmid DNA: when the plasmid DNA concentration decreases, the inter-run variability increases, but it remains low (at a concentration of 5 plasmid copies/reaction Ct = 37.11 ± 0.45). The efficiency was evaluated with 6 replicates of four different linear plasmid control concentrations (50, 25, 12.5 and 5 DNA copies/reaction). As presented in panel D, the PCR showed 100% detection for 50, 25 and 12.5 DNA copies per reaction and 80% detection for 5 DNA copies/reaction. Moreover, by using probit analysis, we found out that the detection limit per reaction with a 95% confidence interval was 10 copies of linear recombinant plasmid per reaction.

Application of the designed q-PCR assay

When testing for false negative results, there were no PCR inhibitors on the specimens. When applying this new q-PCR, *P. acanthamoebae* was detected in 9 and 3 of environmental and clinical samples, respectively (Table 3). The contaminated samples from environment those collected from wastewater treatment plants were located in Sfax, Gafsa and Tozeur and swimming pools of Sfax. The estimated *P. acanthamoebae* DNA copy numbers in the aquatic-contaminated samples ranged from 3.58 to 71.29 copies/µl of collected water. As for clinical samples, DNA of *P. acanthamoebae* was covered from nasopharyngeal swab and sputum samples of child and adult patients with pneumonia, respectively. Among the 100 COVID-19-positive patients, only one patient was co-infected with *P. acanthamoebae*. The patient was a woman of 36 years old from the region of Sfax. When

![Standard curve](https://example.com/standard_curve.png)

\[
R^2 = 0.994 \\
\text{Slope} = -3.158 \\
E = 107.346\%
\]
the nasopharyngeal swab sample of this patient was tested for coronavirus. Ct values of the SARS-CoV-2-specific targets were ORF:Ct = 37 and N:Ct = 34.

Discussion

To the best of our knowledge, the specific q-PCR newly developed test permits to investigate the presence of *P. acanthamoebae* in both aquatic and clinical samples for the first time in Tunisia. Furthermore, our current paper is the first study that raises concern about *P. acanthamoebae* and SARS-COVID-19 co-infection and enables to report the first case of co-infection worldwide.

The developed q-PCR incorporated amplification of the gene encoding for the LSU rRNA. LSU is part of the rRNA gene, which is highly conserved in bacterial strains and its usefulness as a target gene for molecular techniques including PCR techniques (Kemp et al. 2010; Gaibani et al. 2013). The designed primers and probe, under the optimized conditions, allow a rapid and reliable method for detection and quantification of *P. acanthamoebae*, with high qualities in terms of specificity, sensitivity and reproducibility. Indeed, the sensitivity of this PCR essay is higher than that of other TaqMan PCR method, in which the lowest amount of DNA that can be detected is 10 copies/reaction (Casson et al. 2008b). Our q-PCR is also more sensitive than the pan-Chlamydiales-specific PCR used by Thomas et al.
(2006). The inter- and intra-assay variability was low for all plasmid copy numbers proving the performance consistency of the designed oligonucleotides during different runs. All these technical characteristics make it a reliable and suitable method for the detection and quantification of *P. acanthamoebae* in different types of samples known for their richness in PCR inhibitors (Brunk et al. 2002; Zhang et al. 2010). Moreover, the proposed q-PCR proved less time-consuming than traditional techniques, such as culture-based methods. This is particularly interesting in the environmental practice, epidemiological surveys and surveillance programs, which requires the analysis of a large number of samples and is helpful when an antibiotherapy has to be established.

Given the growing body of evidence that supports the ability of *P. acanthamoebae* to survive in environmental habitats (Corsaro and Venditti 2006) and its potential role as an etiological agent of human respiratory infections (Greub 2009; Hokynar et al. 2019), we applied TaqMan PCR in aquatic and clinical samples. The use of the developed method resulted in 17% of water samples collected from wastewater treatment plants. The results are not impressive in comparison with other studies, obtaining similar results in terms of frequency of detection. Thomas et al. (2006) documented the presence of *P. acanthamoebae* in 12.5% of water samples taken from the Seine in France using both amoeba co-culture and pan-Chlamydiaceae-specific PCR. The contamination of reclaimed post-treatment wastewater could be explained by the fact that secondary treatments applied in most Tunisian wastewater treatment plants have no effect on pathogenic microorganisms (Varela et al. 2018). Yet, what is seriously more dangerous was the presence of *P. acanthamoebae* in 3 tested recreational water samples (swimming pools).

While testing the clinical samples, DNA of *P. acanthamoebae* was recovered from 1.685% of tested samples which is in agreement with several previously described studies. Indeed, Corsaro et al. (2002) have documented the presence of *P. acanthamoebae* in 1% (2/178) of human respiratory samples detected by a pan-Chlamydiaceae 16S rDNA PCR. Using the same q-PCR, DNA of *P. acanthamoebae* was detected in 6.2% (26/422) and 5.8% (16/278) of nasopharyngeal aspirates obtained from children with respiratory syncytial virus-negative bronchiolitis were positive for *P. acanthamoebae* when analyzed by q-PCR (Casson et al. 2008b). In fact, the imperfection, the choice of sampling strategy and the changing of techniques used for chlamydiosis screening between studies are important factors that contribute to the variability of results among studies (Casson et al. 2008b; Lienard et al. 2011; Hokynar et al. 2019).

Among 100 analyzed samples of patients confirmed with COVID-19 infection, only 1% of them were co-infected with *P. acanthamoebae*. Retrospective studies conducted in Italy (Oliva et al. 2020), the USA (Gayam et al. 2020) and Spain (Blasco et al. 2020), based on serology, documented low rates of co-infection with *Mycoplasma pneumoniae* accounting for 1.1%, 1.7% and 0.97% of SARS-COVID-19-infected subjects, respectively. In the UK, Easom et al. (2020) also reported the presence of *Mycoplasma pneumoniae* in (1/67) 1.49% of patients diagnosed with SARS-COVID-19 by applying multiplex PCR assays of nasopharyngeal swabs. In India, Chaudhry et al. (2022) used a real-time PCR targeting the CARDS toxin gene for

### Table 3 Screening of environmental and clinical samples positive for *P. acanthamoebae*

| Sample sources | Latitude | Longitude | Sample type | Sample ID | Ct | DNA quantity copies/µl |
|----------------|----------|-----------|-------------|-----------|----|-----------------------|
| Sfax           | 34° 30’ 32.89″ N 10° 27’ 20.25″ E | Wastewater treatment plants | S.M4 | 34.715 | 5.32 |
|                | 34° 49’ 58.95″ N 10° 51’ 1226″ E | | S.J7 | 33.142 | 16.75 |
|                | 34° 42’ 48.24″ N 10° 32’ 48.56″ E | | S.A10 | 31.619 | 50.82 |
|                | 34° 41’ 24.04″ N 10° 43’ 56.86″ E | | S.S20 | 35.258 | 3.58 |
| Sfax           | 34° 44’ 06.42″ N 10° 44’ 35.89″ E | Swimming pool | S.P23 | 33.945 | 9.33 |
|                | 34° 44’ 00.04″ N 10° 44’ 25.47″ E | | S.P24 | 32.569 | 25.47 |
|                | 34° 43’ 30.24″ N 10° 43’ 25.79″ E | | S.P25 | 35.156 | 3.86 |
| Gafsa          | 34° 22’ 52.51″ N 8° 45’ 26.35″ E | Waste water treatment plants | G32 | 33.395 | 13.92 |
|                | 33° 55’ 53.43″ N 8° 09’ 33.32″ E | | T38 | 31.156 | 71.29 |
| Tozeur         | 33° 55’ 53.43″ N 8° 09’ 33.32″ E | Nasopharyngeal swab from an adult with COVID-19 | A.Cov.22 | 35.347 | 3.35 |
| University Hospital Habib Bourguiba, Sfax, Tunisia | | Nasopharyngeal aspiration from a child with pneumonia | E10 | 35.063 | 4.13 |

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diagnosing *Mycoplasma pneumoniae* and likewise noted a low percentage of about 5.2% of co-infection in SARS-COVID-19-infected patients. *Legionella pneumophila* has been also reported only in one patient (1/191) with SARS-COVID-19 by using a broad-range respiratory PCR (Sreenath et al. 2021). Lansbury et al. (2020) emphasized, in their meta-analysis study, that *Chlamydia pneumoniae* was the most common bacteria detected in patients with COVID-19 who had respiratory bacterial co-infections, followed by *Pseudomonas aeruginosa, Haemophilus influenzae, Klebsiella pneumoniae* and *Chlamydophila spp.*, suggesting that in addition to co-infection by atypical bacteria, other respiratory bacteria were also the candidates of co-infections in COVID-19 patients.

**Conclusion**

The optimized TaqMan real-time PCR assay provided an appropriate and fast cultivation-independent process for the quantification of the pathogenic bacterium *P. acanthamoebae* at an early infection stage. The application of this method to various clinical and environmental samples containing many bacterial cells confirmed its high specificity and sensitivity. These characteristics proved that the optimized q-PCR could be a convenient tool for routine diagnosis and large-scale screening programs during outbreaks.

**Author contribution**  O.B., M.B., and S.A conceived and designed the present study. O.B., N.B.A., A.C., H.K.H., and A.H. were responsible of data and sample collection. O.B., M.B., and A.C carried out experiments. O.B., M.B., and J.E performed the statistical analysis and the data acquisition. M.B., P.M., I.F., and S.A contributed to manuscript revisions.

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**Data availability** All data generated or analyzed during this study are included in this published article.

**Declarations**

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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**Author contribution**  O.B., M.B., and S.A conceived and designed the present study. O.B., N.B.A., A.C., H.K.H., and A.H. were responsible of data and sample collection. O.B., M.B., and A.C carried out experiments. O.B., M.B., and J.E performed the statistical analysis and the data acquisition. M.B., P.M., I.F., and S.A contributed to manuscript revisions.

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**Declarations**

**Ethics approval and consent to participate** Not applicable.

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