Identification of *Trueperella bernardiae* isolated from peking ducks (*Anas platyrhynchos domesticus*) by phenotypical and genotypical investigations and by a newly developed loop-mediated isothermal amplification (LAMP) assay

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Abstract

*Trueperella* (*T.*) *bernardiae* is a well-known bacterial pathogen in infections of humans, rarely in animals. In the present study, five *T. bernardiae* isolates, isolated from five Peking ducks of four different farms, were identified by phenotypic properties, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, and genotypically by sequencing the 16S ribosomal RNA (rRNA) gene, the superoxide dismutase A encoding gene *sodA*, and the glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap*. In addition, the *T. bernardiae* isolates could be identified with a newly developed loop-mediated isothermal amplification (LAMP) assay based on the gyrase encoding housekeeping gene *gyrA*. All these tests clearly identified the *T. bernardiae* isolates to the species level. However, the detection of the specific gene *gyrA* with the newly designed LAMP assay appeared with a high sensitivity and specificity, and could help to identify this bacterial species in human and animal infections in future. The importance of the *T. bernardiae* isolates for the clinical condition of the ducks and for the problems at farm level remains unclear.

Introduction

*Trueperella* (*T.*) *bernardiae* is a gram-positive, non-motile, and facultatively anaerobic coccobacillus which was originally identified as a coryneform group 2 bacterium (Na’Was et al. 1987). Eight years later, this bacterium was recognized as *Actinomyces bernardiae* (Funke et al. 1995) and then reclassified as part of the genus *Arcanobacterium* as *Arcanobacterium bernardiae* (Ramos et al. 1997). According to a proposal of Yassin et al. (2011), *Arcanobacterium bernardiae* was finally classified to the newly described genus *Trueperella* as *T. bernardiae*, together with *T. pyogenes*, *T. bialowiezensis*, *T. bonasi*, and *T. abortisuis*. In humans, *T. bernardiae* was first described as an opportunistic pathogen and later could be identified alone or in combination with other bacteria as causing joint infections (Gilarranz et al. 2016; Gowe et al. 2018), urinary tract infections (Lepargneur et al. 1998), abscesses (Parha et al. 2015; VanGorder et al. 2016; Calatrava et al. 2019; Pan et al. 2019), wound infections (Weitzel et al. 2011; Rattes et al. 2016; Cobo et al. 2017), a diabetic foot infection (Schneider et al. 2015), bacteremia (Otto et al. 2013; Roh et al. 2019), and septic thrombophlebitis (Lawrence et al. 2018).

In humans, *T. bernardiae* was first described as an opportunistic pathogen and later could be identified alone or in combination with other bacteria as causing joint infections (Gilaranz et al. 2016; Gowe et al. 2018), urinary tract infections (Lepargneur et al. 1998), abscesses (Parha et al. 2015; VanGorder et al. 2016; Calatrava et al. 2019; Pan et al. 2019), wound infections (Weitzel et al. 2011; Rattes et al. 2016; Cobo et al. 2017), a diabetic foot infection (Schneider et al. 2015), bacteremia (Otto et al. 2013; Roh et al. 2019), and septic thrombophlebitis (Lawrence et al. 2018).

The first characterization of *T. bernardiae* of animal origin (3-day-old piglet) was made by Hijazin et al. (2012c). In a second case, Arnafia et al. (2017) described a *T. bernardiae* strain recovered from a purulent thelitis of a 12-year-old male dog.
These previously isolated *T. bernardiae* isolates of animal origin were identified and further characterized by matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and by sequencing various genomic targets (Hijazin et al. 2012c; Arnafia et al. 2017).

At present, no further data are available concerning the isolation of *T. bernardiae* from animals or animal infections.

In the present investigation, five *T. bernardiae* isolates recovered from post mortem samples of Peking ducks (*Anas platyrhynchos domesticus*) could be identified phenotypically and genotypically, and with a newly described loop-mediated isothermal amplification (LAMP) assay based on the housekeeping gene *gyrA*.

### Material and methods

#### Bacterial strains

The five *T. bernardiae* isolates investigated in the present study (*T. bernardiae* D12-0613-1-4-3, *T. bernardiae* D13-1622-5-3-2, *T. bernardiae* D14-1577-4-8-1, *T. bernardiae* D14-1481-2029-1-1) were isolated from five Peking ducks (*Anas platyrhynchos domesticus*) from three different farms in Germany (*n* = 3) and one in Thailand (*n* = 2). The Geman samples were collected from the heart, lung, and joint, while the Thailand samples were delivered as swabs after post-mortem examination; unfortunately, the source organs were not defined. All five isolates were collected during post-mortem examination in the period between 2012 and 2014. Further details about the five isolates are given in Table 1.

The bacterial cultivation and a preliminary identification of the bacteria were performed at Ripac-Labor GmbH, Potsdam, Germany. The bacterial culturing of the *T. bernardiae* isolates was carried out on sheep blood agar plates (Oxoid GmbH, Wesel, Germany) for 48 h at 37 °C under a microaerophilic gas atmosphere using a candle jar.

#### Phenotypic identification

A phenotypic identification was performed using conventional cultural and biochemical assays as previously shown (Hassan et al. 2009; Ülbegi-Mohyla et al. 2009) and with the API-Coryne test system (BioMérieux Deutschland GmbH, Nürtingen, Germany) in accordance with the manufacturer’s instructions. Furthermore, the bacterial isolates were identified by MALDI-TOF MS using a Microflex LT (Bruker Daltonik GmbH, Bremen, Germany) instrument following the manufacturer’s instructions using the direct transfer method. Briefly, one microbial colony was first smeared in duplicate onto spots of the MALDI MSP 96 target plate (MicroScout Target plate; Bruker Daltonik GmbH) with sterile toothpicks. The air-dried bacteria were overlaid with 1µL of an α-cyan 4-hydroxycinnamic acid matrix solution (HCCA, in 50% acetonitrile and 2.5% trifluoroacetic acid in pure water), followed by drying and loading into the mass spectrometer. The analysis of the spectra was carried out by MBT Compass Explorer 4.1 software (Bruker Daltonik GmbH).

#### DNA extraction

The genomic DNA of the five isolates, type strain *T. bernardiae* DSM 9152T, and various other strains of genus *Trueperella* and genus *Arcanobacterium* (A.) were extracted using the DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany), in conformance with the manufacturer’s instructions. The concentration and purity of DNA were measured by means of a NanoDrop spectrophotometer (ND1000; Thermo Fisher Scientific GmbH, Dreieich, Germany).

### Table 1 Data on the five *T. bernardiae* isolates recovered from Peking ducks investigated in the present study

| Strain code          | Farm/country | Sample drawing | Sample source                                          | Further information                                           |
|----------------------|--------------|----------------|--------------------------------------------------------|---------------------------------------------------------------|
| *T. bernardiae* D12-0613-1-4-3 | A/G          | 07/05/2012     | Post-mortem/heart; no pathological findings            | Accompanying bacteria: *Corynebacterium* spp., *Aerococcus viridans*, *Escherichia (E.) coli* |
| *T. bernardiae* D13-1622-5-3-2 | B/G          | 02/10/2013     | Post-mortem/lung edema                                 | Accompanying bacteria: *Aspergillus fumigatus*, *Corynebacterium* spp., *Coenonia anatina*; increased mortality and joint infections at farm level |
| *T. bernardiae* D13-1772-748-1-2 | C/T          | 25/10/2013     | Post-mortem                                            | Accompanying bacteria: *Globicatella sulfidifaciens*           |
| *T. bernardiae* D14-1481-2029-1-1 | C/T          | 20/08/2014     | Post-mortem                                            | Accompanying bacteria: *Trueperella pyogenes*, *E. coli*; Increased mortality at farm level |
| *T. bernardiae* D14-1577-4-8-1 | D/G          | 05/09/2014     | Post-mortem/joint infection                            |                                                               |

G Germany, T Thailand, n.d. no data available
Sequencing the molecular targets

The five *T. bernardiae* isolates were also investigated by sequencing the following molecular targets: 16S rRNA gene, superoxide dismutase A encoding gene *soda*, and glycer-aldehyde-3-phosphate dehydrogenase encoding gene *gap*. The sequences of the oligonucleotide primers and the PCR conditions were used as previously described for 16S rRNA gene (Hassan et al. 2009), *soda* (Hijazin et al. 2011, 2012c), and gene *gap* (Wickhorst et al. 2019). The PCR products were purified and sequenced by Eurofins Genomics Germany GmbH (Ebersberg, Germany). The obtained sequences of the different genes of the *T. bernardiae* isolates were aligned and further analyzed using the clustal w method of the MegAlign program version 15 (DNASTAR, Inc., Madison, WI, USA) and compared with the nucleotide sequences of the targets 16S rRNA gene, *soda*, and *gap* of type strain *T. bernardiae* DSM 9152\(^T\), and type strain of *T. pyogenes* DSM 20630\(^T\) obtained from the NCBI GenBank, and for control purposes from *A. haemolyticum* DSM 20595\(^T\) also obtained from the NCBI GenBank.

**LAMP assay**

Design of oligonucleotide primers for LAMP assay

Oligonucleotide primers for the *T. bernardiae*-specific LAMP assay were developed using the gyrase subunit A encoding gene *gyrA* of *T. bernardiae* (LNIZ01000002).

The LAMP primers (forward outer primer *gyrA*-F3, backward outer primer *gyrA*-B3, forward inner primer *gyrA*-FIP, backward inner primer *gyrA*-BIP, forward loop primer *gyrA*-LoopF, and backward loop primer *gyrA*-LoopB) were designed using the LAMP designer software (PREMIER Biosoft, San Francisco, CA, USA) (Table 2). The oligonucleotide primers were synthesized by Eurofins Genomics.

### Table 2  Oligonucleotide primer sequences of gyrase subunit A encoding gene *gyrA* used for development of the *T. bernardiae* LAMP assay

| Designation | Sequences 5′-3′ | Primer length (bp) | Melting temperature (°C) |
|-------------|----------------|-------------------|------------------------|
| *gyrA*-F3   | CACCAAGGTAGAGTC ATCA | 19 | 56.7 |
| *gyrA*-B3   | TCCTCGACGATCTTC TGC | 18 | 56.0 |
| *gyrA*-FIP  | GCCGGATGAGGGGCAA TGAGAAGAAGCCTC ATGATC | 36 | 75 |
| *gyrA*-BIP  | CGGTCATCGACACT GCTCGAATGGCGGAG GATATG | 36 | 75 |
| *gyrA*-LoopF| CTGCAGCGATGTC GAG | 18 | 58.2 |
| *gyrA*-LoopB| CGCGATCAACGAGAT CCA | 18 | 56.0 |

**LAMP reaction and amplification conditions**

In accordance with the manufacturer’s instructions, the LAMP assay based on gene *gyrA* was carried out with the five *T. bernardiae* isolates, type strain *T. bernardiae* DSM 9152\(^T\), and closely related genus *Arcanobacterium*. A total volume of 25 µL for each reaction included 15 µL GspSSD isothermal master mix (ISO-001) (OptiGene Ltd., Horsham, UK) and 2.5 µL primer mix (ISO-001; OptiGene Ltd.), *gyrA*-F3 primer, and *gyrA*-B3 primer with a final concentration equivalent to 0.2 µmol/L, *gyrA*-FIP primer, and *gyrA*-BIP primer with final concentration equivalent to 0.8 µmol/L and *gyrA*-LoopF Primer and *gyrA*-LoopB Primer with a final concentration equivalent to 0.4 µmol/L. Subsequently, 5 µL DNA was added as a template. The LAMP assay was run at 70 °C for 20 min with a melting curve analysis step (annealing curve 98 to 80 °C ramping at 0.05 °C/s) in a real-time fluorometer GenieII® (OptiGene Ltd.).

**Analytical sensitivity and specificity of the LAMP assay**

Determination of the analytic sensitivity of the LAMP assay was performed seven times using a serially diluted DNA (10\(^{-1}\)–10\(^{-6}\)) isolated from type strain *T. bernardiae* DSM 9152\(^T\) in AE buffer (10 mM Tris–Cl, 0.5 mM EDTA; pH 9.0) with the conditions mentioned above. DNA isolation and concentration were performed as stated above. The amount of DNA ranged from 3.0 ng/µL (10\(^{-6}\)) to 3.0 fg/µL (10\(^{-6}\)) bacterial DNA. The colony-forming unit (cfu/mL) was subsequently estimated.

The specificity of the LAMP assay was determined using the DNA of *T. bernardiae* DSM 9152\(^T\) and closely related species of genus *Trueperella* and *Arcanobacterium*. These included *T. pyogenes* DSM 20630\(^T\), *T. pyogenes* DSM 20594, *T. pyogenes* 59/11, *T. abortisuis* DSM 19515\(^T\), *T. bialowiezensis* DSM 17162\(^T\), *T. bonasi* DSM 17163\(^T\), *A. hippocoleae* DSM 15539\(^T\), *A. pluranimilium* DSM 13483\(^T\), and *A. phocae* DSM 10002\(^T\). The LAMP assay was performed with the optimized LAMP protocol with a run-time of 20 min.

**Results and discussion**

The phenotypic properties of the five *T. bernardiae* isolates of duck origin investigated in the present study were almost identical to those of type strain *T. bernardiae* DSM 9152\(^T\),
Table 3  Biochemical properties of the five *T. bernardiae* isolates investigated in the present study and *T. bernardiae* DSM 9152<sup>T</sup>

| Biochemical properties          | *T. bernardiae* D12-0613–1-4–3 | *T. bernardiae* D13-1622–5-3–2 | *T. bernardiae* D13-1772–748–1–2 | *T. bernardiae* D14-1481–2029–1–1 | *T. bernardiae* D14-1577–4–8–1 | *T. bernardiae* DSM 9152<sup>T</sup> |
|--------------------------------|--------------------------------|--------------------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------|
| Nitrate reduction              | −                              | −                              | −                               | −                               | −                              | −                              |
| Pyrazinamidase                 | +                              | +                              | -                               | +                               | +                              | +                              |
| Pyrrolidonyl Arylamidase       | +                              | +                              | +                               | +                               | +                              | +                              |
| Alkaline phosphatase           | −                              | −                              | −                               | −                               | −                              | −                              |
| α-Glucuronidase                | −                              | −                              | −                               | −                               | −                              | −                              |
| β-Galactosidase                | −                              | −                              | −                               | −                               | −                              | −                              |
| β-Glucosidase                  | +                              | +                              | +                               | +                               | +                              | +                              |
| N-Acetyl-β-glucosaminidase     | −                              | −                              | −                               | −                               | −                              | −                              |
| Esculin                        | −                              | −                              | −                               | −                               | −                              | −                              |
| Urease                         | −                              | −                              | −                               | −                               | −                              | −                              |
| Gelatine                       | −                              | −                              | −                               | −                               | −                              | −                              |

**Fermentation**

| Sugar                        | *T. bernardiae* D12-0613–1-4–3 | *T. bernardiae* D13-1622–5-3–2 | *T. bernardiae* D13-1772–748–1–2 | *T. bernardiae* D14-1481–2029–1–1 | *T. bernardiae* D14-1577–4–8–1 | *T. bernardiae* DSM 9152<sup>T</sup> |
|------------------------------|--------------------------------|--------------------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------|
| Glucose                      | +                              | +                              | +                               | −                               | +                              | −                              |
| Ribose                       | +                              | +                              | +                               | +                               | +                              | +                              |
| Xylose                       | −                              | −                              | −                               | −                               | −                              | −                              |
| Mannitol                     | −                              | −                              | −                               | −                               | −                              | −                              |
| Maltose                      | +                              | +                              | +                               | +                               | +                              | +                              |
| Lactose                      | −                              | −                              | −                               | −                               | −                              | −                              |
| Saccharose                   | −                              | −                              | −                               | −                               | −                              | −                              |
| Glycogen                     | +                              | +                              | +                               | +                               | +                              | +                              |
| Catalase                     | −                              | −                              | −                               | −                               | −                              | −                              |

**T. bernardiae** identification (%) according to Api-Coryne test system

|                                | 99.7  | 99.7  | 99.7  | 99.9  | 99.7  | 99.9  |
|--------------------------------|-------|-------|-------|-------|-------|-------|

+ positive reaction, – negative reaction, \(^T\) type strain

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**Fig. 1** Phylogenetic analysis based on nucleotide sequences of 16S rRNA gene of the five investigated *T. bernardiae* isolates isolated from Peking ducks, type strain *T. bernardiae* DSM 9152<sup>T</sup>, and closely related *T. pyogenes* DSM 20630<sup>T</sup> and *A. haemolyticum* DSM 20595<sup>T</sup> obtained from NCBI GenBank.
and to previously characterized *T. bernardiae* strains of pig and dog origin (Hijazin et al. 2012c; Arnafia et al. 2017). All *T. bernardiae* isolates gave positive reactions for pyrazinamidase, pyrrolidonyl arylamidase, and α-glucosidase, and reacted negatively in nitrate reduction and for alkaline phosphatase, β-glucuronidase, β-galactosidase, and N-acetyl-β-glucosaminidase. Also, all isolates did not hydrolyze esculin, urea, and gelatine. The isolates also fermented D-glucose, except *T. bernardiae* D14-1481-2029-1-1 and type strain *T. bernardiae* DSM 9152T, D-ribose, D-maltose, and glycogen, but not D-xylose, D-mannitol, D-lactose, and D-saccharose. In addition, all isolates showed a negative catalase reaction (Table 3).

With the additionally performed MALDI-TOF MS analysis, all five isolates were identified to the species level as *T. bernardiae* with log-score values varying between 1.87 and 2.2 (data not shown). MALDI-TOF MS appeared to be a fast, accurate, and less expensive tool for microbial identification of bacteria, viruses, and fungi (Singhal et al. 2015), also including *T. bernardiae* (Hijazin et al. 2012a) and various other species of genera *Trueperella* and *Arcanobacterium* (Hijazin et al. 2012a, b).

The five *T. bernardiae* isolates in the current study were additionally identified genotypically by amplification and sequencing of the 16S rRNA gene. The nucleotide sequence of *T. bernardiae* D12-0613-1-4-3 (GenBank accession number: MT364890), *T. bernardiae* D13-1622-5-3-2 (MT364891), *T. bernardiae* D13-1772-748-1-2 (MT364892), *T. bernardiae* D14-1481-2029-1-1 (MT364893), and *T. bernardiae* D14-1577-4-8-1 (MT364894) were compared with type strain *T. bernardiae* DSM 9152T (HE653979), *T. pyogenes* DSM

Fig. 2   Dendrogram analysis of superoxide dismutase A encoding gene sodA (a) and glyceraldehyde-3-phosphate dehydrogenase encoding gene gap (b) of the five *T. bernardiae* isolates isolated from Peking ducks, type strain *T. bernardiae* DSM 9152T, and closely related *T. pyogenes* DSM 20630T and *A. haemolyticum* DSM 20595T obtained from NCBI GenBank
Table 4  Specificity of the T. bernardiae LAMP assay based on gene gyrA for T. bernardiae DSM 9152^T, the five T. bernardiae isolates of duck origin, and other closely related species of genus Trueperella and Arcanobacterium

| Species and strain number | Detection time mm:ss | Melting temperature (°C) |
|---------------------------|----------------------|--------------------------|
| T. bernardiae DSM 9152^T  | 11:45                | 91.9                     |
| T. bernardiae D12-0613–1–4–3 | 14:15                | 92.0                     |
| T. bernardiae D13–1622–5–3–2 | 13:15                | 92.3                     |
| T. bernardiae D13-1772–748–1–2 | 13:30                | 92.3                     |
| T. bernardiae D14-1481-2029–1–1 | 13:00                | 92.4                     |
| T. bernardiae D14-1577–4–8–1 | 13:15                | 92.4                     |
| T. pyogenes DSM 20630^T     | –                    | –                        |
| T. pyogenes DSM 20594       | –                    | –                        |
| T. pyogenes 59/11           | –                    | –                        |
| T. abortisuis DSM 19515 T   | –                    | –                        |
| T. bialowiezensis DSM 17162 T | –                    | –                        |
| T. bonasi DSM 17163 T       | –                    | –                        |
| A. hippocoleae DSM 15539 T  | –                    | –                        |
| A. pluranimalium DSM 13483^T | –                    | –                        |
| A. phocae DSM 10002 T       | –                    | –                        |

DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen

20630^T (X79225), and Arcanobacterium (A.) haemolyticum DSM 20595^T (AJ234059). The nucleotide sequence data of T. bernardiae D12-0613–1–4–3, T. bernardiae D13-1622–5–3–2, T. bernardiae D13–1772–748–1–2, T. bernardiae D14–1481–2029–1–1, and T. bernardiae D14–1577–4–8–1 revealed a sequence homology of 99.7, 99.9, 99.2, 99.0, and 99.7% with type strain T. bernardiae DSM 9152^T, respectively (Fig. 1). The 16S rRNA gene sequence similarities of the five T. bernardiae isolates showed a clear difference to the control strains T. pyogenes DSM 20630^T (AM949566 and HF947285, respectively) and A. haemolyticum DSM 20595^T (AM983534 and CP002045, respectively) (Fig. 2a, b). All three mentioned genomic targets were already used to characterize various species of genus Trueperella, also including T. bernardiae (Hassan et al. 2009; Hijazin et al. 2011, 2012c; Arnafia et al. 2017).

The additionally used T. bernardiae gyrA-specific LAMP assay could successfully be used to identify the species-specific gene gyrA of all five T. bernardiae isolates in the present investigation. This newly established assay demonstrated a specificity for T. bernardiae DSM 9152^T with an annealing temperature between 91.9 and 92.4 °C. No cross-reactivity with any other related species of genus Trueperella or genus Arcanobacterium could be observed (Table 4).

The developed LAMP assay provided an analytic sensitivity of 30 fg/μL, with a mean detection time between 00:08:58 (3.0 ng/μL) and 00:18:35 min (30 fg/μL) (Table 5). For the DNA concentration at 30 fg/μL (10^-5), the assay gave positive results in six of seven replicates (85.7%), whereas for DNA concentration at 3.0 fg/μL (10^-6), the positive assay resulted in two of seven replicates (28.6%). The results of the T. bernardiae gyrA LAMP assay are shown in Fig. 3 and Table 4.

The application of LAMP assays as being a rapid and reliable method for detecting species of genus Trueperella and Arcanobacterium has been previously published. In 2013, Zhang et al. developed a LAMP assay using the gene encoding pyolysin, the plo gene, for a specific identification
Positive LAMP assay of the five *T. bernardiae* isolates *T. bernardiae* D12-0613–1–4–3, *T. bernardiae* D13-1622–5–3–2, *T. bernardiae* D13-1772–748–1–2, *T. bernardiae* D14-1481–2029–1–1, *T. bernardiae* D14-1577–4–8–1 obtained from Peking ducks, *T. bernardiae* DSM 9152, and as LAMP negative control *T. pyogenes* DSM 20630 and nuclease free water as negative control.

Declarations

Ethics approval and consent to participate This study did not require official or institutional ethical approval. The material was collected post-mortem and/or during routine diagnosis. According to competent authorities, this kind of research does not require ethics approval or general approval with respect to German law.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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Author contribution M.F.E.A, M.A., C.L., A.A., and M.P. contributed to the design of the study, collected, and analyzed the data. B.K., M.M. performed the initial examination of the isolates. M.F.E.A and A.A. drafted the manuscript. C.L. M.M. and M.P. review and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials The data that support the findings of this study are available on NCBI’s Genbank and are accessible through the accession numbers listed in the manuscript.

Fig. 3 Positive LAMP assay of the five *T. bernardiae* isolates *T. bernardiae* D12-0613–1–4–3, *T. bernardiae* D13-1622–5–3–2, *T. bernardiae* D13-1772–748–1–2, *T. bernardiae* D14-1481–2029–1–1, *T. bernardiae* D14-1577–4–8–1 obtained from Peking ducks, *T. bernardiae* DSM 9152, and as LAMP negative control *T. pyogenes* DSM 20630 and nuclease free water as negative control.
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