United States Patent	12391998
Kind Code	B2
Date of Patent	August 19, 2025
Inventor(s)	Feng; Zhixin et al.

Composition and kit for detecting mycoplasma

Abstract

A composition and a kit for detecting *mycoplasma* are provided. The composition for detecting *mycoplasma* is an aqueous solution including a primer M-F, a primer M-R, and a probe M-P. A sequence of the M-F is shown in SEQ ID NO: 1. A sequence of the M-R is shown in SEQ ID NO: 2. A nucleotide sequence of the probe M-P is shown in SEQ ID NO: 3, and includes a fluorophore FAM linked at a 5′ terminus and a quencher BHQ1 linked at a 3′ terminus. The composition exhibits high sensitivity, strong specificity, and a wide detection range when used in the detection of *mycoplasma.*

Inventors: **Feng; Zhixin (Nanjing, CN), Yu; Yanfei (Nanjing, CN), Wang; Jia (Nanjing, CN), Wei; Yanna (Nanjing, CN), Shao; Guoqing (Nanjing, CN), Wu; Yuzi (Nanjing, CN), Liu; Maojun (Nanjing, CN), Xiong; Qiyan (Nanjing, CN), Wang; Li (Nanjing, CN), Liu; Beibei (Nanjing, CN)**

Applicant: **JIANGSU ACADEMY OF AGRICULTURAL SCIENCES** (Nanjing, CN); **GUOTAI (TAIZHOU) CENTER OF TECHNOLOGY INNOVATION FOR VETERINARY BIOLOGICALS, TAIZHOU** (Taizhou, CN)

Family ID: **1000008764643**

Assignee: **JIANGSU ACADEMY OF AGRICULTURAL SCIENCES (Nanjing, CN); GUOTAI (TAIZHOU) CENTER OF TECHNOLOGY INNOVATION FOR VETERINARY BIOLOGICALS, TAIZHOU (Taizhou, CN)**

Appl. No.: **18/911297**

Filed: **October 10, 2024**

Prior Publication Data

Document Identifier	Publication Date
US 20250051860 A1	Feb. 13, 2025

Foreign Application Priority Data

CN	202310719274.5	Jun. 16, 2023

Related U.S. Application Data

continuation parent-doc WO PCT/CN2023/109885 20230906 PENDING child-doc US 18911297

Publication Classification

Int. Cl.: **C12Q1/68** (20180101); **C12Q1/689** (20180101)

U.S. Cl.:

CPC **C12Q1/689** (20130101); C12Q2600/156 (20130101)

Field of Classification Search

USPC: None

References Cited

U.S. PATENT DOCUMENTS

Patent No.	Issued Date	Patentee Name	U.S. Cl.	CPC
10640834	12/2019	Shimizu	N/A	C12Q 1/689
2003/0050470	12/2002	An	435/6.14	C07H 21/00
2004/0023207	12/2003	Polansky	435/456	A61K 48/005
2007/0117120	12/2006	Anderson et al.	N/A	N/A
2017/0240959	12/2016	Shimizu	N/A	C12Q 1/68

FOREIGN PATENT DOCUMENTS

Patent No.	Application Date	Country	CPC
105420379	12/2015	CN	N/A
110343777	12/2018	CN	N/A
110894534	12/2019	CN	N/A
2004305207	12/2003	JP	N/A
2013128334	12/2012	KR	C12N 15/11

OTHER PUBLICATIONS

Dreolini et al., 2020. A rapid and sensitive nucleic acid amplification technique for mycoplasma screening of cell therapy products. Molecular Therapy—Methods & Clinical Development, 17, pp. 393-399. (Year: 2020). cited by examiner

Fourour et al., 2018. A new multiplex real-time TaqMan® PCR for quantification of *Mycoplasma hyopneumoniae*, M. hyorhinis and M. flocculare. Journal of applied microbiology, 125(2), pp. 345-355 (Year: 2018). cited by examiner

English Translation of KR2013-0128334A, pub Nov. 11, 26, 2013. (Year: 2013). cited by examiner

Gadberry MD, Malcomber ST, Doust AN, Kellogg EA. Primaclade—a flexible tool to find conserved PCR primers across multiple species. Bioinformatics. Apr. 1, 2005; 21(7):1263-4. Epub Nov. 11, 2004. (Year: 2004). cited by examiner

Genbank Accession No. AF443616—Mycoplasma hominis DNA, complete cds, submitted Jul. 26,

2006, retrieved on Jan. 25, 2025 from http://www.ncbi.nlm.nih.gov/nuccore/AF443616). (Year: 2006). cited by examiner
Lee, J.I. and Kim, I.S., 2014. TaqMan probe real-time PCR for quantitative detection of mycoplasma during manufacture of biologics. KSBB Journal, 29(5), pp. 361-371. (Year: 2014). cited by examiner
Molla Kazemiha et al., 2016. Real-time PCR assay is superior to other methods for the detection of mycoplasma contamination in the cell lines of the National Cell Bank of Iran. Cytotechnology, 68, pp. 1063-1080. (Year: 2016). cited by examiner
Salling et al., 2016. Multi-primer qPCR assay capable of highly efficient and specific detection of the vast majority of all known Mycoplasma. Biologicals, 44(3), pp. 129-138. (Year: 2016). cited by examiner
SantaLucia Jr., John. Physical principles and visual-OMP software for optimal PCR design. PCR Primer Design. Humana Press, 2007: pp. 3-33. (Year: 2007). cited by examiner
Siegl et al., May 19, 2023. A PCR protocol to establish standards for routine mycoplasma testing that by design detects over ninety percent of all known mycoplasma species. Iscience, 26(5) 10672413, pp. 1-13. (Year: 2023). cited by examiner
Stormer et al., 2009. Broad-range real-time PCR assay for the rapid identification of cell-line contaminants and clinically important mollicute species. International journal of medical microbiology, 299(4), pp. 291-300. (Year: 2009). cited by examiner
Sugita et al., 2021. Detection of mycoplasma contamination in transplanted retinal cells by rapid and sensitive polymerase chain reaction Test. International Journal of Molecular Sciences, 22, 12555, p. 1-13 (Year: 2021). cited by examiner
Sung et al., 2020. A highly sensitive internally-controlled real-time PCR assay for mycoplasma detection in cell cultures. Biologicals, 64, pp. 58-72. (Year: 2020). cited by examiner
Svenstrup et al., 2005. Development of a quantitative real-time PCR assay for detection of Mycoplasma genitalium. Journal of Clinical Microbiology, 43(7), pp. 3121-3128. (Year: 2005). cited by examiner
Wambulawaye, P.P., Dec. 2022. Development of a multiplex real-time PCR to distinguish between Mycoplasma species found in South African poultry (Master's thesis, University of Pretoria (South Africa)) pp. 1-149 (Year: 2022). cited by examiner
Boonyayatra et al., 2012: A PCR assay and PCR-restriction fragment length polymorphism combination identifying the primary Mycoplasma species causing mastitis J Dairy Sci 95: 196-205. (Year: 2012). cited by examiner
Liang Zhe, et al., Effect of the Endogenous Spinal Cord Substances on the Survival of the Neurons of Spinal Cord in Vitro, 1996, pp. 134-139, vol. 18 No. 3. cited by applicant
Huo Xin-Yang, et al., Establishment and Application of Multiplex Quantitative PCR Method for Detection of Mycoplasma, Progress in Modern Biomedicine, 2020, pp. 1259-1262, vol. 20 No. 7. cited by applicant
M. E. Pollock, et al., Isolation and Elimination of Pleuropneumonia-Like Organisms From Mammalian Cell Cultures, PPLO Elimination from Cell Cultures, 1960, pp. 10-15. cited by applicant
Lucili.E B. Robinson, et al., Contamination of Human Cell Cultures by Pleuropneumonialike Organisms, Science, 1956, pp. 1147-1148, vol. 124. cited by applicant
Commission Of Chinese Veterinary Pharmacopoeia, Veterinary Pharmacopoeia of the People's Republic of China, 2020, pp. 3301-3302, ISBN: 9787109275867. cited by applicant
Liu Jiang, et al., Detection of Mycoplasma Contamination in Cell Cultures by Polymerase Chain Reaction, Journal of Cell Biology, pp. 134-139, vol. 18 No. 3. cited by applicant

---

Primary Examiner: Benzion; Gary

Assistant Examiner: Oyeyemi; Olayinka A

Attorney, Agent or Firm: Bayramoglu Law Offices LLC

Background/Summary

CROSS REFERENCE TO THE RELATED APPLICATIONS (1) This application is a continuation application of International Application No. PCT/CN2023/109885, filed on Sep. 6, 2023, which is based upon and claims priority to Chinese Patent Application No. 202310719274.5, filed on Jun. 16, 2023, the entire contents of which are incorporated herein by reference.

SEQUENCE LISTING
(1) The instant application contains a Sequence Listing which has been submitted in XML format via EFS-Web and is hereby incorporated by reference in its entirety. Said XML copy is named GBHS014-PKG_Sequence_Listing_20241023.xml, created on Oct. 23, 2024, and is 8,906 bytes in size.
TECHNICAL FIELD
(2) The present disclosure belongs to the field of biotechnologies, and specifically relates to a composition and kit for detecting *mycoplasma.*
BACKGROUND
(3) *Mycoplasma* contamination is one of the major challenges for cell culture. In 1956, researchers at Johns Hopkins reported the *mycoplasma* contamination of HeLa cells used in the laboratory, and it was the first time *mycoplasma* was detected in a cell culture. *Mycoplasma*-contaminated cells can undergo weakened metabolism and slowed proliferation. However, due to the non-lethality of *mycoplasma* contamination for cells, *mycoplasma* often coexists with cells for a long time and generally does not cause a significant morphological change in cells. At an early stage of *mycoplasma* contamination, the medium does not become turbid, which makes it difficult to determine whether the cell culture undergoes *mycoplasma* contamination with naked eyes. However, *mycoplasma*-contaminated cells may undergo a series of biological changes, such as a change in composition of the cell membrane, chromosomal abnormalities, a change in the enzyme system, and a change in the viral load, which can mislead scientific research tremendously and seriously interfere with experimental results.
(4) The main sources of *mycoplasma* as a contaminant for cell culture are animal serum, trypsin, and aerosols. *Acholeplasma laidlawii* (*A. laidlawii*) (one of the most common contaminants) can also come from soil and other inanimate sources. Since the trypsin commonly on the market is acquired from commercially available porcine pancreases, *Mycoplasma* hyorhinis (*M. hyorhinis*) can also enter the cell culture through this reagent. As early as 1960, Pollock et al. found that 57% of 166 mammalian cell lines and sublines were contaminated with *mycoplasma*. Studies have shown that, in terms of the in vitro growth of mammalian cells, a *mycoplasma*-contaminated cell culture undergoes slowed growth and a shortened logarithmic growth phase.
(5) The “Veterinary Pharmacopoeia of the People's Republic of China” stipulates the following two methods for detecting *mycoplasma*: the cultivation method and the DNA fluorescent staining method. However, when the conventional cultivation method is used to detect *mycoplasma*, there are disadvantages such as a heavy workload and a long cycle time. Some *mycoplasma* individuals with strict nutritional requirements may be missed, and there may be false positives of contamination due to the large time span during cultivation. The DNA fluorescent staining method has high sensitivity, but the result is not easy to determine and is easily affected by the subjective determination of the detector. The DNA fluorescent staining method takes about 1 week, which is slightly shorter than the time required by the cultivation method. There are many other limiting

factors for the application of the DNA fluorescent staining method in scientific research. There is a lack of *mycoplasma* detection methods with high sensitivity, strong specificity, and a wide detection range in the art.

SUMMARY

(6) An objective of the present disclosure is to provide a composition for detecting *mycoplasma*, with high sensitivity, strong specificity, and wide detection range.

(7) The objective of the present disclosure is allowed through the following technical solutions:

(8) The present disclosure provides a composition for detecting *mycoplasma*, where the composition is an aqueous solution including a primer M-F, a primer M-R, and a probe M-P; a sequence of the M-F is shown in SEQ ID NO: 1; a sequence of the M-R is shown in SEQ ID NO: 2; and a nucleotide sequence of the probe M-P is shown in SEQ ID NO: 3, and includes a fluorophore carboxyfluorescein (FAM) linked at a 5′ terminus and a quencher black hole quencher 1 (BHQ1) linked at a 3′ terminus.

(9) In the present disclosure, the primer M-F, the primer M-R, and the probe M-P are in a molar concentration ratio of 1:(0.8-1.2): (0.8-1.2).

(10) The present disclosure also provides a kit for detecting *mycoplasma*, including the composition.

(11) In the present disclosure, the primer M-F, the primer M-R, and the probe M-P in the kit are in a molar concentration ratio of 1:(0.8-1.2): (0.8-1.2).

(12) In the present disclosure, the kit further includes a positive plasmid, and the positive plasmid is obtained by inserting a fragment with a sequence shown in SEQ ID NO: 4 into a pUC57 plasmid vector.

(13) The present disclosure also provides a method for detecting *mycoplasma* using the composition for a non-diagnostic purpose, including the following steps: (1) extracting DNA from a sample; (2) with the DNA of the sample as a template, conducting quantitative polymerase chain reaction (qPCR) detection using the primer M-F, the primer M-R, and the probe M-P; and (3) when a cycle threshold (Ct) value of the qPCR detection for the DNA of the sample is smaller than 38 and there is a typical S-type amplification curve, determining as positive, indicating that there is *mycoplasma* in the sample; and when the Ct value of the qPCR detection for the DNA of the sample is larger than or equal to 38 or there is no Ct value or there is no typical S-type amplification curve, determining as negative, indicating that there is no *mycoplasma* in the sample.

(14) In the present disclosure, a reaction system for the qPCR detection includes: 12.5 μL of a fluorescent polymerase chain reaction (PCR) solution, 1 μL of the DNA of the sample, 3 μL of the composition, and 8.5 μL of double distilled water (ddH.sub.2O).

(15) In the present disclosure, a procedure for the qPCR is as follows: 95° C. for 3 min; 95° C. for 15 sec, and 60° C. for 30 sec, with 40 amplification cycles in total.

(16) The composition of the present disclosure exhibits high sensitivity, strong specificity, and a wide detection range when used in the detection of *mycoplasma*. A total of 106 random cell samples from different laboratories in different regions are collected for testing. Positive samples detected by the composition of the present disclosure have a coincidence rate of 100% with position samples detected by the cultivation method, and a detection time is significantly shortened.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

(1) FIGS. **1**A-**1**B show detection results of the qPCR method in Example 1, where FIG. **1**A shows qPCR detection results of 15 *mycoplasma* species and FIG. **1**B shows qPCR detection results of cells, bacteria, and viruses.

(2) FIG. **2** is an electropherogram illustrating detection results of *mycoplasma* by a commercial

nested *mycoplasma* detection PCR kit, where M: DL2000 DNA Marker; 1: *Mycoplasma gallisepticum* (MG); 2: *Mycoplasma hyosynoviae* (Mhs); 3: *Mycoplasma pneumoniae* (Mp); 4: *Mycoplasma orale* (*M. orale*); 5*: M. hyorhinis;* 6*: A. laidlawii;* 7: *Mycoplasma fermentans* (*M. fermentans*); 8: *Mycoplasma synoviae* (MS); 9*: Spiroplasma citri* (*S. citri*); 10: *Mycoplasma flocculare* (Mf); 11: *Mycoplasma ovipneumoniae* (MO); 12: *Mycoplasma hominis* (Mh); 13: negative control; 14: positive control; 15: *Mycoplasma bovis* (Mb); 16: *Mycoplasma arginini* (*M. arginini*); and 17: *Mycoplasma pirum* (*M. pirum*);

(3) FIGS. **3**A-**3**C show detection results of *mycoplasma* by a commercial qPCR kit, where FIG. **3**A shows amplification curves of 15 *mycoplasma* samples, FIG. **3**B shows an amplification curve of *M. pirum*, and FIG. **3**C shows an amplification curve of *A. laidlawii;*

(4) FIGS. **4**A-**4**B show amplification curves of 106 cell samples detected by the qPCR method in Example 1; and

(5) FIGS. **5**A-**5**B show amplification curves of 106 cell samples detected by a commercial qPCR kit.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Example 1 Composition, Kit, and Method for Detecting *Mycoplasma*

(6) 1. Composition for Detecting *Mycoplasma*

(7) In order to find a highly-sensitive and universal qPCR method for detecting *mycoplasma*, the applicants conducted genome-wide alignment analysis for 143 *mycoplasma* sequences published in an NCBI database, and designed dozens of pairs of primers and probes. It was found that only one pair of primers (M-F and M-R) and a probe M-P could detect the tested 15 *mycoplasma* species with high sensitivity.

(8) A sequence (SEQ ID NO: 1) of the M-F was as follows: 5′-ATCCATCCCCACGTTCTCGT-3′. A sequence (SEQ ID NO: 2) of the M-R was as follows: 5′-TGCGGTGAATACGTTCTCGGG-3′. A nucleotide sequence (SEQ ID NO: 3) of the probe M-P was as follows: 5′-ACGGGCGGTGTGTACA-3′, with a fluorophore FAM (carboxyfluorescein) linked at a 5′ terminus and a quencher BHQ1 (succinimide ester) linked at a 3′ terminus.

(9) The composition for detecting *mycoplasma* was an aqueous solution including 10 μM of the M-F, 10 μM of the M-R, and 10 μM of the probe M-P.

(10) 2. qPCR Method for Detecting *Mycoplasma*

(11) The qPCR method for detecting *mycoplasma* included the following steps: (1) DNA was extracted from a sample. (2) qPCR detection:

(12) With the DNA of the sample as a template, qPCR was conducted. A total reaction system for the qPCR was of 25 μL, including: 12.5 μL of a fluorescent PCR solution (Vazyme, Item No. Q112-AA), 1 μL of the DNA of the sample, 3 μL of the composition for detecting *mycoplasma*, and 8.5 μL of ddH.sub.2O. The reaction system was specifically shown in Table 1. A PCR tube with the total reaction system for qPCR was placed in a detection hole of an ABI fluorescence PCR instrument. An FAM channel was selected for detection (quencher: BHQ-1), a reaction system was set to 25 μL, and cycle parameters were set as follows: 95° C. for 3 min, 95° C. for 15 sec, and 60° C. for 30 sec, with 40 amplification cycles in total. At the end of annealing in each cycle, an FAM fluorescence signal was acquired.

(13) In addition, a negative control and a positive control were set. The negative control and the positive control were the same as the qPCR detection method except that the DNA of the sample was replaced with ddH.sub.2O in the negative control and the DNA of the sample was replaced with a positive plasmid DNA in the positive control. The positive plasmid DNA used in the positive control was a positive plasmid obtained by ligating a gene fragment Spiroplasma (with a sequence shown in SEQ ID NO: 4) from *S. citri* to a pUC57 plasmid vector through two enzyme cleavage sites of BamHI and Xhol. The positive plasmid was chemically transformed into a competent *Escherichia coli* (*E. coli*) strain XL10 for proliferation.

(14) TABLE-US-00001 TABLE 1 qPCR system Component System (μL) Fluorescent PCR

solution 12.5 Composition for detecting mycoplasma 3 Sterile nuclease-free water (ddH.sub.2O) 8.5 DNA of the sample (10 ng/μL) 1 Total 25

(3) Result Determination

(15) When a Ct value of the qPCR detection for the DNA of the sample was smaller than 38 and there was a typical S-type amplification curve, it was determined as positive, indicating that there was *mycoplasma* in the sample. When the Ct value of the qPCR detection for the DNA of the sample was larger than or equal to 38 or there was no Ct value or there was no typical S-type amplification curve, it was determined as negative, indicating that there was no *mycoplasma* in the sample.

Example 2 Specificity and Sensitivity of qPCR

(16) 1. Specificity

(17) (1) 15 *mycoplasma* species, various bacteria, viruses, and different cells each were detected by the qPCR method in Example 1. The 15 *mycoplasma* species were *A. laidlawii, M. fermentans, M. hyorhinis, M. orale, M. arginini*, Mp, MG, MS, *S. citri*, Mhs, Mh, *M. pirum*, Mf, Mb, and MO, respectively. The various bacteria, viral nucleic acids, and different cells included *Salmonella pullorum* (*S. pullorum*), *E. coli, Staphylococcus aureus* (*S. aureus*), *Pseudomonas fragi* (*P. fragi*), Yeast, porcine circovirus type 2 (PCV-2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), African green monkey kidney cells (Vero), porcine kidney cells (PK-15), canine kidney cells (MDCK), human laryngeal epidermoid carcinoma cells (Hep-2), mouse mononuclear macrophage leukemia cells (RAW264.7), or the like.

(18) When the qPCR method in Example 1 was used to detect the above-mentioned common cells, viruses, and bacteria, no peak appeared. When the qPCR method in Example 1 was used to detect DNA of the above 15 *mycoplasma* species, a Ct value was smaller than 38 (Table 2) and there was a typical S-type amplification curve (FIGS. **1**A-**1**B). The above results show that the qPCR method in Example 1 exhibits excellent broad-spectrum activity and specificity when used in the detection of *mycoplasma*.

(19) TABLE-US-00002 TABLE 2 CT values of qPCR detection for the 15 mycoplasma species No. Sample name CT 1 Neg Undet 2 *M. orale* 21.753 3 MS 11.681 4 Mf 27.571 5 Mp 14.406 6 Mb 27.371 7 *M. fermentans* 21.468 8 Mh 19.990 9 *A. laidlawii* 17.394 10 MO 19.585 11 Mhs 14.090 12 *M. arginini* 17.749 13 *S. citri* 19.131 14 MG 22.151 15 *M. pirum* 18.373 16 *M. hyorhinis* 21.559 17 Pos 21.922

(20) Notes: In Table 2, Undet indicates that no CT value is detected, Pos indicates a positive control, and Neg indicates a negative control, the same below.

(21) (2) Commercial Nested PCR Method

(22) The above 15 *mycoplasma* samples in (1) of Title 1 of this example were detected by a commercial nested *mycoplasma* detection PCR kit, GMyc-PCR *Mycoplasma* Test Kit (Yeasen BioTechnologies co., Ltd.).

(23) Operation steps: For a first round of PCR, a reaction system was shown in Table 3 and a reaction procedure was shown in Table 4. After the first round of PCR was completed, an amplification product was collected, diluted 1,000-fold, and then used as a template for a second round of PCR. For the second round of PCR, a reaction system was shown in Table 5 and a reaction procedure was the same as the reaction procedure for the first round of PCR.

(24) TABLE-US-00003 TABLE 3 System for the first round of PCR Experimental Positive Negative Reagent group control control GMyc-1st PCR Mix 25 μL 25 μL 25 μL Template DNA 4 μL 4 μL ddH.sub.2O 21 μL 20 μL 25 μL Positive quality control template 1 μL Total volume 50 μL 50 μL 50 μL

(25) TABLE-US-00004 TABLE 4 Conditions for the first round of PCR Number of reaction PCR conditions Temperature Time cycles Pre-denaturation 94° C. 5 min Denaturation 94° C. 30 sec 30 Annealing 58° C. 30 sec Extension 72° C. 30 sec Re-extension 72° C. 7 min

(26) TABLE-US-00005 TABLE 5 System for the second round of PCR Experimental Positive

Negative Reagent group control control GMyc-2nd PCR Mix 25 μL 25 μL 25 μL ddH.sub.2O 24 μL 24 μL 24 μL Product of the first round 1 μL 1 μL 1 μL of amplification that is diluted 1,000-fold Total volume 50 μL 50 μL 50 μL

(27) The commercial nested *mycoplasma* detection PCR kit was used to detect the 15 *mycoplasma* species, and results were shown in FIG. **2**. Only 12 *mycoplasma* species were detected by the commercial nested *mycoplasma* detection PCR kit. This method required two rounds of PCR and agarose gel electrophoresis, resulting in cumbersome operations. A detection rate of this method was 20% lower than a detection rate of the qPCR method of the present disclosure.

(28) (3) Commercial qPCR Method

(29) The 15 *mycoplasma* samples in (1) of Title 1 of this example were detected by the commercial qPCR kit, MycAway™ *Mycoplasma* Real-time qPCR Detection Kit (Yeasen BioTechnologies co., Ltd.).

(30) Components for the commercial qPCR included 4×qPCR Reaction Buffer, Primer & Probe MIX, positive and negative controls, and sterile nuclease-free water. A qPCR system was shown in Table 6.

(31) FAM was selected as a reporter fluorophore, and MGB was selected as a quenching fluorophore. A reaction system was set to 40 μL. Cycle parameters were set as follows: 95° C. for 5 min, 95° C. for 15 sec, and 62° C. for 30 sec, with 45 amplification cycles in total. At the end of annealing in each cycle, an FAM fluorescence signal was acquired. When Ct was smaller than 40 and there was a clear amplification curve, it was determined as positive. When Ct was greater than or equal to 40 or there was no obvious peak, it was determined as negative.

(32) TABLE-US-00006 TABLE 6 qPCR system Component System (μL) 4 × qPCR Reaction Buffer 10 Primer & Probe MIX 1 Template (10 ng/μL) 20 Sterile nuclease-free water Making up to 40 μL Total 40

(33) Detection results of the commercial qPCR kit: CT values are shown in Table 7. It can be seen from FIGS. **3**A-**3**C that S-type amplification curves of *A. laidlawii* and *M. pirum* are atypical and negative. The qPCR method in Example 1 of the present disclosure has significant advantages over the commercial qPCR kit. The commercial qPCR kit requires 20 μL of a template (10 ng/μL), but the method of the present disclosure only requires 1 μL of sample DNA as a template during detection. The commercial qPCR kit requires 45 cycles, but the method of the present disclosure only requires 40 cycles. The method of the present disclosure can amplify a typical S-type amplification curve for all of the 15 *mycoplasma* species, and allows a stronger fluorescence intensity and a smoother curve than the commercial qPCR kit, making it not prone to mis-determination.

(34) TABLE-US-00007 TABLE 7 CT values of 15 mycoplasma species detected by the commercial qPCR kit No. Sample CT 1 Neg Undet 2 *M. orale* 22.229 3 MS 13.693 4 Mf 22.27 5 Mp 29.855 6 Mb 28.997 7 *M. fermentans* 19.764 8 Mh 21.833 9 *A. laidlawii* 17.9 10 MO 11.621 11 Mhs 13.263 12 *M. arginini* 17.43 13 *S. citri* 11.27 14 MG 33.484 15 *M. pirum* 33.904 16 *M. hyorhinis* 20.428 17 Pos 11.483

2. Sensitivity

(35) The *E. coli* carrying the positive plasmid in Example 1 was allowed to proliferate, the positive plasmid was extracted, and a concentration of the positive plasmid was determined by a spectrophotometer. The plasmid was diluted 10-fold serially to produce plasmid concentrations of 10.sup.9 copies/μL, 10.sup.8 copies/μL, 10.sup.7 copies/μL, 10.sup.6 copies/μL, 10.sup.5 copies/μL, 10.sup.4 copies/μL, 10.sup.3 copies/μL, 10.sup.2 copies/μL, 10.sup.1 copies/μL, 10.sup.0 copies/μL, and 10.sup.−1 copies/μL, respectively. 1 μL of the positive plasmid at each concentration was taken as a template and used for analysis by the qPCR method in Example 1 to investigate the sensitivity of the method. Ten parallel tests were conducted for each concentration.

(36) According to results of the qPCR detection in Example 1 (Table 8): When a concentration of the positive plasmid was 10.sup.−1 copies/μL, a Ct value could not be stably detected in 3 of 10

reactions. When a concentration of the positive plasmid was 10.sup.0 copies/μL, a Ct value could be stably detected, and the Ct value was smaller than 38. When a concentration of the positive plasmid was 10.sup.−1 copies/μL, a Ct value could not stably appear. Therefore, the sensitivity of the qPCR method was determined to be 10.sup.0 copies/μL, and a Ct threshold was 38.

(37) TABLE-US-00008 TABLE 8 Ct values for the positive plasmid at each concentration detected by the qPCR method Plasmid concentration, Ct value Sample copies/ Replicate Replicate Replicate Replicate Replicate Replicate Replicate Replicate Replicate Replicate No. μL 1 2 3 4 5 6 7 8 9 10 1 10.sup.9 7.416 7.646 6.917 7.181 6.922 6.815 6.556 6.459 6.166 6.033 2 10.sup.8 10.043 10.219 10.224 10.652 10.438 10.055 10.089 10.174 10.356 9.917 3 10.sup.7 13.589 13.893 13.928 14.406 13.567 13.699 13.913 13.839 13.750 13.469 4 10.sup.6 17.123 17.048 16.856 17.651 17.216 16.797 17.376 17.221 17.452 17.249 5 10.sup.5 20.982 20.836 21.013 21.814 20.808 21.170 20.442 20.654 20.826 20.489 6 10.sup.4 24.245 23.758 23.759 24.866 24.428 24.446 23.974 23.801 24.213 24.229 7 10.sup.3 27.756 27.071 27.317 27.401 27.636 27.794 27.653 27.598 27.771 27.598 8 10.sup.2 31.096 30.850 31.443 31.425 31.050 30.961 30.795 31.027 30.732 31.038 9 10.sup.1 34.070 33.923 34.237 35.380 33.048 33.617 33.814 35.409 34.259 33.539 10 10.sup.0 36.585 36.109 36.813 37.786 36.030 37.271 37.633 37.500 36.098 36.604 11 10.sup.−1 Undet 38.289 38.254 39.541 38.572 39.894 Undet Undet 38.572 38.672

(38) When other primers and probes were used to detect *mycoplasma*, such as a primer MP03-F: 5′-GGTCGTCTACGTCAAAACTTGC-3′ (SEQ ID NO: 5), a primer MP03-R: 5′-GCCATTTGGTCCCCGTCAAAG-3′ (SEQ ID NO: 6), and a probe MP03-P: FAM-TACCTTGTTACGACTT-BHQ1 (SEQ ID NO: 7), there was a poor broad-spectrum activity, a typical S-type curve could not be provided for 2 of the 15 tested *mycoplasma* species, and a sensitivity was 102 copies/μL.

Example 3 Detection of *Mycoplasma* Contamination in a Cell Culture by the qPCR Method

(39) A total of 106 cell samples from various laboratories were detected by the qPCR method in Example 1, the cultivation method in the 2020 edition of the "*Veterinary Pharmacopoeia of the People's Republic of China*", and the commercial qPCR method for *mycoplasma* to investigate a coincidence rate of the qPCR method in Example 1 with the cultivation method in the 2020 edition of the "*Veterinary Pharmacopoeia of the People's Republic of China*".

1. The qPCR Method in Example 1

(40) A supernatant from each cell sample was taken to prepare a template through boiling. Specific steps were as follows: A supernatant was collected from a cell culture to be tested, added to a centrifuge tube, heated to 100° C. and boiled for 10 min, and cooled. A resulting supernatant was collected and centrifuged for 5 s to 6 s. A resulting supernatant was collected (or subjected to DNA extraction by a kit) as sample DNA for the qPCR detection method.

(41) 106 cell samples were detected by the qPCR method in Example 1. Results showed that 49 cell samples had a CT value of smaller than 38 (Table 9) and a typical amplification curve, and were positive for *mycoplasma*, as shown in FIGS. **4**A-**4**B. Thus, a positive detection rate was 46.23%.

(42) TABLE-US-00009 TABLE 9 CT values of the 106 cell samples detected by the qPCR method in Example 1 No. CT 1 19.488 2 Undet 3 16.9171 4 Undet 5 35.608 6 37.0916 7 12.060 8 Undet 9 Undet 10 Undet 11 15.107 12 Undet 13 32.115 14 17.542 15 Undet 16 Undet 17 Undet 18 32.582 19 15.626 20 Undet 21 35.800 22 21.760 23 22.583 24 35.458 25 Undet 26 26.516 27 Undet 28 37.493 29 Undet 30 16.7138 31 19.488 32 Undet 33 16.917 34 34.753 35 35.608 36 Undet 37 Undet 38 30.927 39 Undet 40 30.852 41 Undet 42 36.566 43 Undet 44 Undet 45 Undet 46 24.987 47 Undet 48 33.207 49 Undet 50 Undet 51 29.805 52 Undet 53 Undet 54 Undet 55 Undet 56 Undet 57 21.937 58 Undet 59 37.089 60 35.917 61 Undet 62 Undet 63 36.080 64 Undet 65 33.638 66 Undet 67 Undet 68 Undet 69 30.617 70 Undet 71 34.184 72 Undet 73 32.004 74 30.830 75 20.887 76 Undet 77 Undet 78 Undet 79 Undet 80 30.945 81 37.245 82 33.399 83 Undet 84 23.304 85 Undet 86 32.201 87 25.145 88 Undet 89 Undet 90 34.495 91 31.295 92 35.264 93 Undet 94 Undet

95 Undet 96 Undet 97 Undet 98 32.169 99 Undet 100 Undet 101 Undet 102 28.480 103 Undet 104 20.488 105 Undet 106 Undet Positive control 21.011 Negative control Undet

2. Detection of *Mycoplasma* in Cell Samples by the Isolation and Cultivation Method

(43) According to the cultivation method in the 2020 edition of the "*Veterinary Pharmacopoeia of the People's Republic of China*", each cell sample was subjected to liquid and solid cultivation. At the end of cultivation, if no *mycoplasma* grew in a medium into which a cell sample was inoculated, the cell sample was qualified, otherwise, the cell sample was unqualified.

(44) If *mycoplasma* grew, a color of a liquid medium would also change (pink or yellow). After aerobic cultivation in a solid medium at 37° C. for 30 d, if *mycoplasma* grew, pinpoint-like colonies could be observed by naked eyes in a medium and fried egg-like colonies could be observed under a microscope. In this experiment, known negative and positive samples were taken as negative and positive controls, respectively.

(45) After about 21 d of cultivation, results showed that liquid media of 24 cell samples turned yellow (a pH decreased), and media of 7 cell samples turned pink (a pH increased). As a result, it was determined that these 31 cell samples were contaminated with *mycoplasma*. Cultures undergoing a color change each were inoculated into a solid medium and cultivated for about 30 d, and then fried egg-like colonies were formed on the solid medium. Thus, a positive detection rate was 29.25%.

(46) 3. Detection of *Mycoplasma* in Cell Samples by the Commercial qPCR Kit

(47) Each cell sample was detected with the commercial qPCR kit, MycAway™ *Mycoplasma* Real-time qPCR Detection Kit (Yeasen BioTechnologies co., Ltd.). A specific method was implemented according to instructions.

(48) 106 cell samples were detected by the commercial qPCR kit. Results showed that 41 cell samples had a CT value of smaller than 40 (Table 10) and a typical amplification curve, and were positive for *mycoplasma*, as shown in FIGS. **5**A-**5**B. Thus, a positive detection rate was 38.68%.

(49) TABLE-US-00010 TABLE 10 CT values of 106 cell samples detected by the commercial qPCR kit No. CT 1 Undet 2 37.423 3 Undet 4 Undet 5 30.938 6 Undet 7 11.151 8 Undet 9 32.281 10 Undet 11 28.043 12 Undet 13 23.739 14 Undet 15 Undet 16 14.656 17 Undet 18 Undet 19 27.768 20 Undet 21 Undet 22 38.322 23 36.643 24 Undet 25 37.423 26 Undet 27 Undet 28 30.938 29 33.146 30 Undet 31 Undet 32 32.281 33 Undet 34 28.043 35 Undet 36 23.739 37 31.751 38 Undet 39 Undet 40 Undet 41 25.991 42 23.770 43 Undet 44 11.607 45 Undet 46 Undet 47 27.221 48 Undet 49 11.800 50 Undet 51 Undet 52 Undet 53 Undet 54 Undet 55 22.229 56 Undet 57 13.693 58 Undet 59 35.904 60 Undet 61 Undet 62 Undet 63 Undet 64 35.029 65 Undet 66 33.146 67 Undet 68 Undet 69 Undet 70 30.731 71 Undet 72 Undet 73 Undet 74 Undet 75 Undet 76 29.715 77 22.427 78 Undet 79 22.270 80 17.900 81 Undet 82 Undet 83 20.428 84 Undet 85 Undet 86 35.029 87 Undet 88 Undet 89 Undet 90 Undet 91 31.751 92 Undet 93 Undet 94 Undet 95 Undet 96 30.731 97 Undet 98 31.068 99 Undet 100 Undet 101 Undet 102 16.515 103 29.855 104 Undet 105 13.274 106 27.545 Pos 11.27 Neg Undet

4. Comparison of the Three Methods

(50) The qPCR method in Example 1 completed the detection within 1 h, the commercial qPCR kit completed the detection in about 3 h, and the isolation and cultivation method took 21 d to 29 d to complete the detection of all cell samples.

(51) TABLE-US-00011 TABLE 11 Comparison of detection performance of different methods Coincidence rate with the Time Detection gold standard (isolation Method consumption rate and cultivation method) qPCR in Example 1 1 h 46.23% 100% Isolation and 21-29 d 29.25% 100% cultivation method Commercial qPCR 3 h 38.68% 87.10%.sup.

(52) The collected 106 random cell samples were detected for *mycoplasma*, and results were shown in Table 11. 49 samples were detected as positive for *mycoplasma* contamination by the qPCR method in Example 1, which had a coincidence rate of 100% with the detection results of the classical cultivation method (a number of cell samples detected as positive by both methods/a

number of cell samples detected as positive by the classical cultivation method * 100%). 41 samples were detected as positive by the commercial qPCR kit, and 4 samples were missed compared with the classical cultivation method. A coincidence rate of the commercial qPCR kit with the cultivation method was only 87.10%.

(53) Therefore, when used in the detection of *mycoplasma*, the qPCR method in Example 1 is significantly superior to the prior art in terms of broad-spectrum activity, sensitivity, and accuracy.

(54) TABLE-US-00012 A sequence of the gene fragment Spiroplasma (SEQ ID NO: 4) was as follows:

AACATAACAACAAAAGATAATCATTTAATCAATGAATATCCGTCATTAAAGCTAGGAACAAA
AACGATATTTTTTAATGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCATGCCTAAT
ACATGCAAGTCGAACGGGGTGCTTGCACCCAGTGGCGAACGGGTGAGTAACACGTATCTAA
TCTACCCATTAGCGGGGGATAACAGTTGGAAACGACTGATAATACCGCATACGACATTTTCT
GGCATCAGAGAATGTTAAAAGGTCCGTTTGGATCACTAATGGATGAGGATGCGGCGTATTAG
TTAGTTGGTGGGGTAATGGCCTACCAAGACAATGATACGTAGCCGAACTGAGAGGTTGATC
GGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTAGGGAATTTT
TCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGACTGAAGACGGTCTTCGGATTGT
AAAAGTCTGTTGTAAGGGAAGAACAGTAAGTATAGGAAATGATACTTATTTGACGGTACCTT
ACCAGAAAGCCACGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGTGGCAAGCGTT
ATCCGGATTTATTGGGCGTAAAGCGTGCGCAGACGGTTTAACAAGTTTGGGGTCAAATCCT
GGAGCTCAACTCCAGTTCGCCTTGAAAACTGTTAAGCTAGAGTGTAGGAAAGGTCGATGG
AATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGT
CGACTGGCCTATCACTGACGTTTAGGCACGAAAGCGTAGGGAGCAAATAGGATTAGATACC
CTAGTAGTCTACGCCGTAAACGATGAGTACTAAGTGTCGGACTAAGTTCGGTGCTGCAGCT
AACGCATTAAGTACTCCGCCTGAGTAGTATGCTCGCAAGAGTGAAACTCAAAGGAATTGAC
GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAAGGCTTGACATCCAGTGCAAAGCTGTAGAAATACAGTGGAGGTTAACATTGAGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCAGTAACGAGCGCA
ACCCTTGCCGTTAGTTACTCCATTAAGTTGAGATACTCTAACAGGACTGCTAGTGTAAGCTA
GAGGAAGGTGGGGATGACGTCAAATCAGCATGCCCCTTATATCTTGGGCTACACACGTGCT
ACAATGGTCGGTACAAACAGTTGCGATCTCGTAAGAGGGAGCTAATCTGAAAAAGCCGATC
TCAGTTCGGATTGAGGGCTGCAACTCGCCCTCATGAAGCCGGAATCGCTAGTAATCGCGAA
TCAGCAATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCACACCATGAG
AGTTGATAATACCAGAAGTCGGTATTCTAACCGCAAGGAGGAAGCCGCCCAAGGTAGGATT
GATGATTAGGGTGAAGTCGTAACAAGGTATCCGTACGAGAACGTGCGGATGGATCACCTCC
TTTCTATGGAGTTAATACTTTATAGTAATTAACTAGTTTTAATGACCGTTATGTTTAGTTTTCA
GAGATTAGTTTCTCTGAAAATAACAAGTAAATGTTATTGGAATTGTTCTTTGAAAACTGGAT
AATAGACATCTAGTTATTTTAATCACATGATTAAAATAACAATAATTCAAAATTTCTGTTATTT
TTAAAAAATAACTAAAATTTCACAGTTATATTTGTAAATGATTCTCAAAAAACTGATTTAAAA
TCAGGTCAAATAATTTATAAAACTTTGAAGTTACAAAGGGCGTATGGTGAATGCCTTGG.

Claims

1. A composition for detecting mycoplasma, wherein the composition is an aqueous solution comprising a primer M-F consisting of SEQ ID NO: 1; a primer M-R consisting of SEQ ID NO: 2; and a probe M-P consisting of SEQ ID NO: 3, wherein the probe M-P further comprises a fluorophore carboxyfluorescein (FAM) linked at a 5′ terminus and a quencher black hole quencher 1 (BHQ1) linked at a 3′ terminus.

2. The composition according to claim 1, wherein the primer M-F, the primer M-R, and the probe M-P are in a molar concentration ratio of 1:(0.8-1.2):(0.8-1.2).

3. A kit for detecting mycoplasma, comprising the composition according to claim 1.

4. The kit according to claim 3, wherein the primer M-F, the primer M-R, and the probe M-P in the kit are in a molar concentration ratio of 1:(0.8-1.2):(0.8-1.2).
5. The kit according to claim 4, wherein the kit further comprises a positive plasmid, and the positive plasmid is obtained by inserting a fragment consisting of SEQ ID NO: 4 into a pUC57 plasmid vector.
6. A method for detecting mycoplasma using the composition according to claim 1 for a non-diagnostic purpose, comprising the following steps: (1) extracting DNA from a sample; (2) with the DNA of the sample as a template, conducting a quantitative polymerase chain reaction (qPCR) detection using the primer M-F, the primer M-R, and the probe M-P; and (3) when a cycle threshold (Ct) value of the qPCR detection for the DNA of the sample is smaller than 38 and there is a typical S-type amplification curve, determining the sample as positive, indicating that there is the mycoplasma in the sample; and when the Ct value of the qPCR detection for the DNA of the sample is larger than or equal to 38 or there is no Ct value or there is no typical S-type amplification curve, determining the sample as negative, indicating that there is no mycoplasma in the sample.
7. The method according to claim 6, wherein a reaction system for the qPCR detection comprises: 12.5 μL of a fluorescent polymerase chain reaction (PCR) solution, 1 μL of the DNA of the sample, 3 μL of the composition, and 8.5 μL of double distilled water (ddH.sub.2O).
8. The method according to claim 7, wherein a procedure for the qPCR detection is as follows: 95° C. for 3 min; 95° C. for 15 sec, and 60° C. for 30 sec, with 40 amplification cycles in total.
9. The method according to claim 6, wherein the sample is a biological product.
10. The method according to claim 9, wherein the biological product is a cell, a serum, or a vaccine.