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Establishment and Application of a Dual TaqMan Real-Time PCR Method for *Proteus Mirabilis* and *Proteus Vulgaris*

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Abstract

Proteus species are common opportunistic bacteria and foodborne pathogens. The proper detection of *Proteus* can effectively reduce the occurrence of food-borne public health events. *Proteus mirabilis* and *Proteus vulgaris* are the two most important pathogens in the *Proteus* genus. In this study, a dual TaqMan Real-Time PCR method was established to simultaneously detect and distinguish *P. mirabilis* and *P. vulgaris* in samples. The method exhibited good specificity, stability, and sensitivity. Specifically, the minimum detection concentrations of *P. mirabilis* and *P. vulgaris* in pure bacterial cultures were 6.08×10^2 colony forming units (CFU)/ml and 4.46×10^2 CFU/ml, respectively. Additionally, the minimum detectable number of *P. mirabilis* and *P. vulgaris* in meat and milk was 10^3 CFU/g. In addition, the method can be used to distinguish between strains of *P. mirabilis* and *P. vulgaris* within two hours. Overall, it is a sensitive, easy-to-use, and practical test for the identification and classification of *Proteus* in food.

K e y   w o r d s: *Proteus mirabilis*, *Proteus vulgaris*, TaqMan Real-Time PCR, food-borne pathogens, food poisoning

Introduction

Bacterial food-borne diseases are an increasing public health concern to the World Health Organization (Johnson 2011). The consumption of food and drinking water contaminated by pathogenic bacteria is the leading cause of food-borne disease outbreaks (Park et al. 2011). More than 250 types of foodborne diseases caused by the pathogen with various virulence genes have been identified (Mangal et al. 2016). In recent years, the number of food-borne diseases caused by *Proteus*, including vomiting, gastroenteritis, and acute diarrhea, has been increasing.

Proteus species are Gram-negative bacteria belonging to the *Morganellaceae* family that widely exist in nature (Adeolu et al. 2016; Drzewiecka 2016). The genus mainly includes *Proteus mirabilis*, *Proteus vulgaris*, *Proteus penneri*, *Proteus hauseri*, *Proteus terrae*, *Proteus cibarius,* unnamed geomospecies 4, 5, and 6 (Behrendt et al. 2015, Gu et al. 2020). *P. mirabilis* and *P. vulgaris* are the most well-known species in the genus *Proteus*, and they carry several virulence factors that contribute to gastrointestinal (GI) pathogenesis,

such as LPS, hemolysin, and O-antigen (Drzewiecka 2016; Hamilton et al. 2018). Because people living in developed countries generally have access to improved sanitation, food poisoning caused by *Proteus* is less common. However, researchers from developed countries also recently noted that *Proteus* strains are closely associated with GI diseases. Hamilton and coworkers (Hamilton et al. 2018) revealed that *P. mirabilis* and *P. vulgaris* were essential for the pathogenesis of infections in the GI tract and called on other researchers to pay closer attention to the role of these two species. In developing countries that are highly populated (e.g., China), *Proteus* species are among the leading bacterial causes of food poisoning. In 1996, the Ministry of Health of the People's Republic of China issued the WS/T 9-1996: Diagnostic Criteria and Principles of Management for Food Poisoning of *Proteus*, which remains in effect. The document explicitly states that Proteus can easily contaminate animal food, and therefore, inspections for animal food safety should include the identification and classification of *Proteus* strains. In 2008, Beijing reported a case of collective food poisoning caused by *P. mirabilis*, in which 13 individuals

displayed symptoms related to food poisoning, including fever, vomiting, and diarrhea (Wang et al. 2010). China recorded 2,795 foodborne illness outbreaks between 2003 and 2008; in particular, there were 110 (3.93%) public health crises caused by *Proteus* species as foodborne pathogens, resulting in 3,093 individuals to have symptoms of food poisoning (Wu et al. 2018).

In developed countries, researchers generally do not consider *Proteus* as a foodborne pathogen, and thus, they pay little attention to the identification of *Proteus* in food. Surprisingly, no study has reported the use of quantitative fluorescence PCR (Q-PCR) to identify *P. vulgaris* even though the technique has already been applied to the detection of *P. mirabilis* (Liu et al. 2019). At present, 16S ribosomal RNA (rRNA) sequencing, selective media, biochemical identification, and serological tests remain the mainstay modalities for distinguishing between strains of *P. mirabilis* and *P. vulgaris* (O'Hara et al. 2000). In this study, we used a dual TaqMan Real-Time PCR for the rapid and accurate identification and classification of *P. mirabilis* and *P. vulgaris* in food samples.

Experimental

Materials and Methods

Reagents, instruments and bacterial strains. Bacterial strains: thirty-six strains, including eight strains of *P. mirabilis* (four standard strains, and four clinical isolates), seven strains of *P. vulgaris* (four standard strains, and three clinical isolates) and 21 other strains (Table I), were used in this study. Bacteria were cultured according to Bergey's Manual of Systematic Bacteriology (9th edition) (Garrity et al. 2004).

Reagents: The Premix Ex TaqTM (Probe qPCR) was purchased from TaKaRa (Japan), Bacterial DNA and Gel Extraction Kits were purchased from OMEGA (USA), and the QIAamp PowerFecal QIAcube HT Kit was from QIAGEN (Germany).

Instruments: The LightCycler96 fluorescence quantitative polymerase chain reaction (PCR) instrument was purchased from Roche (Switzerland), the QIAcune HT automatic nucleic acid extraction system was purchased from QIAGEN (Germany), and the Nanodrop-2000 ultramicro nucleic acid protein analyzer was from Thermo Fisher (USA).

Target genes, primers, and probes. We analyzed the gene sequences of *Proteus* species published in Gene-Bank, compared the nucleotide sequences of the *16s rRNA*, *tuf*, *rpoB*, *atpD*, *ureR*, *blaA*, and *blaB* genes, and the *ureR* and *blaB* were selected as the target genes for the test. Primer Premier 6.0 (Premier Biosoft, CA, USA) was used to design two pairs of primers and two probes,

Table I
Bacterial strains used in the specificity assessment.

Strain	Origin	FAM	HEX
Proteus mirabilis	CVCC4106	+	–
Proteus mirabilis	CVCC1969	+	–
Proteus mirabilis	ATCC35659	+	–
Proteus mirabilis	CMCC49001	+	–
Proteus mirabilis	Isolated by lab	+	–
Proteus mirabilis	Isolated by lab	+	–
Proteus mirabilis	Isolated by lab	+	–
Proteus mirabilis	Isolated by lab	+	–
Proteus vulgaris	ATCC6896	–	+
Proteus vulgaris	CMCC 49008	–	+
Proteus vulgaris	CMCC49027	–	+
Proteus vulgaris	CMCC 49105	–	+
Proteus vulgaris	Isolated by lab	–	+
Proteus vulgaris	Isolated by lab	–	+
Proteus vulgaris	Isolated by lab	–	+
Proteus penneri	ATCC 33519	–	–
Proteus penneri	NTCC 35198	–	–
Providencia rettgeri	ATCC 31052	–	–
Providencia rettgeri	CVCC3947	–	–
Morganella morganii	ATCC9237	–	–
Morganella morganii	CICC71786	–	–
Salmonella	ATCC14028	–	–
Salmonella	CVCC2227	–	–
Salmonella	Isolated by lab	–	–
Salmonella	Isolated by lab	–	–
Escherichia coli	CICC52719	–	–
Escherichia coli	Isolated by lab	–	–
Escherichia coli	Isolated by lab	–	–
Staphylococcus aureus	ATCC25923	–	–
Staphylococcus aureus	Isolated by lab	–	–
Campylobacter jejuni	CICC 22936	–	–
Campylobacter jejuni	Isolated by lab	–	–
Mannheimia haemolytica	ATCC29695	–	–
Pseudomonas aeruginosa	Isolated by lab	–	–
Lactobacillus acidophilus	CICC6074	–	–
Bacillus subtilis	CICC20445	–	–

ATCC – American Type Culture Collection; CICC – China Center of Industrial Culture Collection; CMCC – China Medical Culture Collection; CVCC – China Veterinary Culture Collection Center, NTCC – National Type Culture Collection, China

and all primers were compared and analyzed by BLAST to ensure their specificity. The primers and probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and the nucleotide sequences are shown in Table II.

Genomic DNA extraction. The Bacterial DNA Kit was used to extract DNA from fresh bacterial culture medium (24 h). The QIAamp PowerFecal QIAcube HT Kit was used to extract bacterial DNA from meat

Table II
Primer and TaqMan probe sequences used in this study.

Pathogen	Target gene	Accession number	Primer (position)	Sequence (5' → 3')	Amplicon length (bp)
Proteus mirabilis	*ureR*	CP044134.1	64F	ACTACCCATCAGATTATGTCAT	101
			165R	CTGTTTGAGGAAAATGCAATTTA	
			136P	FAM-ATTCACACCCTACCCAACATTCAT-BHQ1	
Proteus vulgaris	*blaB*	D37831.1	503F	TCGTAAAGAGCCTGAATTAA	229
			732R	ATCACCACTACCGGTTTTATC	
			532P	HEX-TCATGGTGATCCTCGTGATACTA-BHQ1	

and milk according to the manufacturer's instructions. The purity and concentration of template DNA were detected using a Nanodrop-2000.

Preparation of the standard plasmid. The target fragment was amplified from *P. mirabilis* and *P. vulgaris* by PCR (PCR primers are shown in Table II), purified using the Gel Extraction Kit, and cloned into a PMD19-T vector (TaKaRa, Japan), which was then transformed into *Escherichia coli* DH5α. After transformation, *E. coli* DH5α were cultured for 18–24 h with shaking (37°C, 120 rpm), and the plasmid was extracted using a Plasmid Mini Kit. The purity and concentration of the extracted plasmid were examined using a Nanodrop-2000, and the copy number of the plasmid in a 1 μl of aliquots was converted, according to the Avogadro constant.

Specificity test. Thirty-six strains of bacteria were used to evaluate the specificity using the method. Components of the 25-μl-reaction system included 12.5 μl PCR Mix, four primers (0.5 μl for each, a total of 2 μl), two probes (0.5 μl for each, a total of 1 μl), 1 μl genome template and 8.5 μl aseptic deionized water. The reaction procedure was as follows: initially denatured at 95°C for 30 s, denatured at 95°C for 5 s, annealed at 56°C for 10 s, and extended at 72°C for 20 s. Fluorescence from both the FAM and HEX channels was measured for 40 cycles. The reagent kit used was Premix Ex TaqTM, and the equipment model was LightCycler96.

Standard curve. The two-plasmid samples were diluted ten times with ultra-pure water in a gradient manner. The fluorescence quantitative PCR reaction was performed for the optimized system. The standard curve of the relationship between the plasmid copy number and cycle threshold (*Ct*) was established. **Repeatability assessment.** The plasmid mixture containing the *ureR* and *blaB* genes was tested, and 15 repeated tests were conducted on the same sample in the same experiment to determine the coefficients of variation of *Ct* values and examine the reproducibility of the method.

Evaluation of detection limits for *P. mirabilis* and *P. vulgaris*. *P. mirabilis* and *P. vulgaris* in the logarithmic growth phase were diluted 10-fold continuously with aseptic saline (0.9%, NaCl) and inoculated on tryptic soy agar plates (three parallel controls were prepared). The number of colony-forming units (CFU)/ml was determined by plate counting. Then, the genomic DNA was extracted using a Bacterial DNA Kit, and absolute quantitative Real-Time PCR was performed.

Detection of *P. mirabilis* and *P. vulgaris* in contaminated meat and milk. Sterile saline (0.9%, NaCl) was used to prepare 10-fold gradient dilutions of *P. mirabilis* and *P. vulgaris* cultures, and the concentration of bacteria was determined by the plate counting method. Two bacterial suspensions were diluted to generate six gradient concentrations (10^2–10^7 CFU/ml), mixed in an isovolumetric manner, and added to a 1.5 ml sterile centrifuge tube for centrifugation at $10,000 \times g$ for 10 min. The supernatant was removed to collect the bacteria. Fresh pork (200 g, treated with a tissue homogenizer) with quarantine approval and a carton of germ-free milk (255 ml) were purchased from the market. Then, food samples were separately mixed with the bacteria in the different centrifuge tubes. The concentration gradients of *P. mirabilis* and *P. vulgaris* in food were in the range of 10^2–10^7 CFU/g. Then, the QIAamp PowerFecal DNA Kit was used to extract the total genome from the sample for quantitative Real-Time PCR.

Results

Genomic DNA extraction. The Bacterial DNA Kit was used to extract the genomic DNA from pure cultures of 36 strains of bacteria. The concentration of the genomic DNA was in the range of 78–205 ng/μl, and the OD 260/280 was in the range of 1.81–1.92. The QIAamp PowerFecal QIAcube HT Kit was used to extract the total genomic DNA from the pork and milk contaminated by the mixed bacterial solution. In pork and milk, the total genomic DNA concentrations were in the range of 280–325 ng/μl and 94–154 ng/μl, and the OD260/280 values were 1.92–1.99 and 1.83–1.95, respectively.

Test's specificity. The test's specificity results are listed in Table I. Eight *P. mirabilis* strains exhibited a typical 'S' amplification curve in the FAM channel,
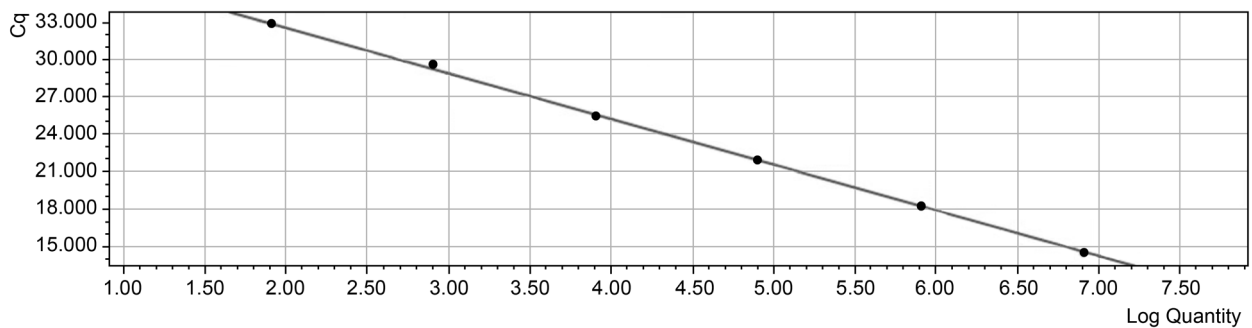
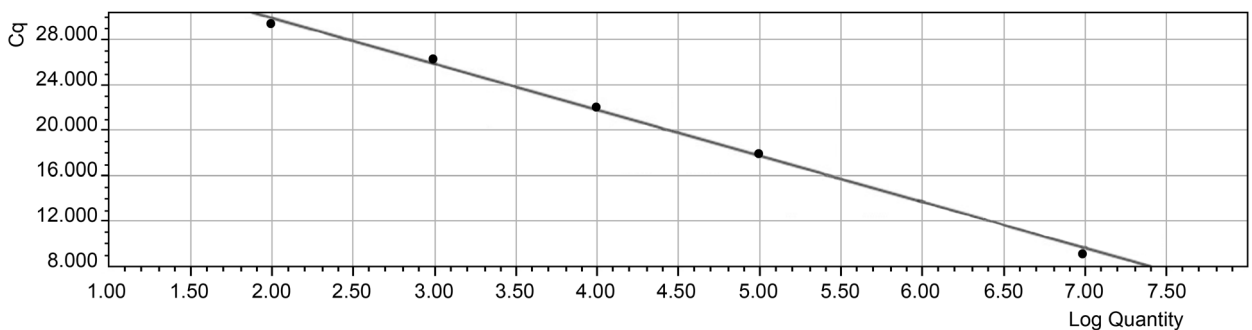Fig. 1. *P. mirabilis* standard curve (Note: log = $\log_{10}$).

Fig. 2. *P. vulgaris* standard curve (Note: log = $\log_{10}$).

while the other strains showed no typical amplification curve. In addition, seven *P. vulgaris* strains showed a typical 'S' amplification curve in the HEX channel, while the other strains exhibited no typical amplification curve. Together, these results show that the established method has high specificity.

Standard curve. The standard curve was established, and the plasmid copy number of different concentrations was the x-coordinate, and the corresponding *Ct* value was the y-coordinate. In the FAM channel, the standard curve for the *P. mirabilis ureR* gene ($8.17 \times 10 - 8.17 \times 10^6$ copies/µl) is shown in Fig. 1. The slope = 3.7136, intercept = 40.38, $R^2 = 1$, and the linear equation was as follows: $Ct = -3.7136 \lg X + 40.38$. In the HEX channel, the standard curve for the *P. vulgaris blaB* gene ($9.93 \times 10 - 9.93 \times 10^6$ copies/µl) is shown in Fig. 2. The slope = −4.0846, intercept = 38.09, $R^2 = 1$, and the linear equation was as follows: $Ct = -4.0846 \lg X + 38.09$.

Repeatability assessment. In the same experiment, 15 repeated tests were conducted using the same mixed plasmid sample ($ureR = 8.17 \times 104$ copies/µl, $blaB = 9.93 \times 10^4$ copies/µl). The results showed that when the *ureR* gene of *P. mirabilis* in the FAM channel was tested 15 times, the *Ct* value was 23.52 ± 0.091, and the coefficient of variation was 0.38%. When the *blaB* gene of *P. vulgaris* in the HEX channel was tested 15 times, the *Ct* value was 20.30 ± 0.116, and the coefficient of variation was 0.57%. These results showed that the method established in these experi-

ments exhibited good reproducibility for the detection of *P. mirabilis* and *P. vulgaris*.

Detection limits. Seven concentrations of *P. mirabilis* and *P. vulgaris* were tested (the genomes were extracted for each concentration after isovolumetric mixing). The corresponding concentrations of *P. mirabilis* were $6.08 \times 10 - 6.08 \times 107$ CFU/ml, and the concentration of *P. vulgaris* was $4.46 \times 10 - 4.46 \times 10^7$ CFU/ml. As shown in Fig. 3, when the concentration of *P. mirabilis* was $\geq 6.08 \times 10^2$ CFU/ml, the typical 'S' amplification curve was obtained. Additionally, when the concentration of *P. vulgaris* was $\geq 4.46 \times 10^2$ CFU/ml, the typical 'S' amplification curve was obtained. The results showed that the lowest detection limits of *P. mirabilis* and *P. vulgaris* were 6.08×10^2 CFU/ml and 4.46×10^2 CFU/ml, respectively.

Detection of the bacteria in contaminated meat and milk. The use of this method to detect food contamination was evaluated by testing pork and milk contaminated by *P. mirabilis* and *P. vulgaris*. The results showed that *P. mirabilis* and *P. vulgaris* in pork or milk could be detected when their content was ≥ 103 CFU/g (Fig. 4 and 5).

Discussion

An increasing number of diseases and public health events are caused by food-borne pathogens, to which society attaches great importance. As *Proteus* is not
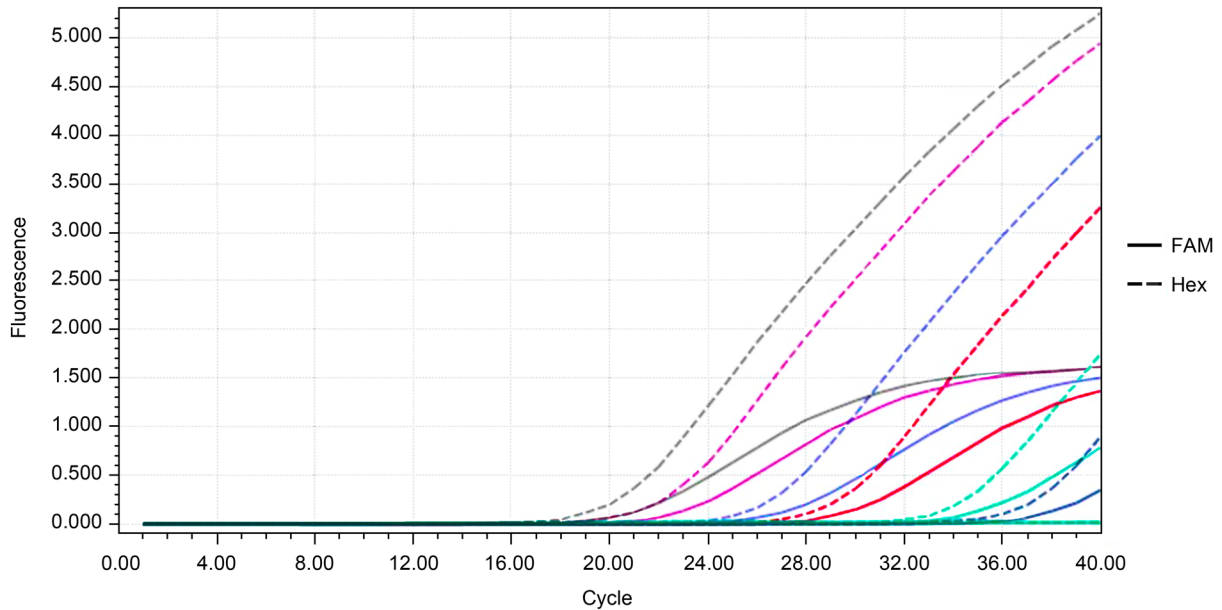
Fig. 3. Detection limits of *P. mirabilis* and *P. vulgaris* in a Dual TaqMan Real-Time PCR Method.

The FAM channel was used to detect *P. mirabilis*, and the concentration of the 'S' amplification curve from left to right was in the range of $6.08 \times 10^7 - 6.08 \times 10^2$ CFU/ml. When the concentration of *P. mirabilis* was 6.08×10 CFU/ml, no amplification curve was obtained. The HEX channel was used to detect *P. vulgaris*, and the concentration of the 'S' amplification curve from left to right was in the range of $4.46 \times 10^7 - 4.46 \times 10^2$ CFU/ml. When the concentration of *P. vulgaris* was 4.46×10 CFU/ml, no amplification curve was obtained.
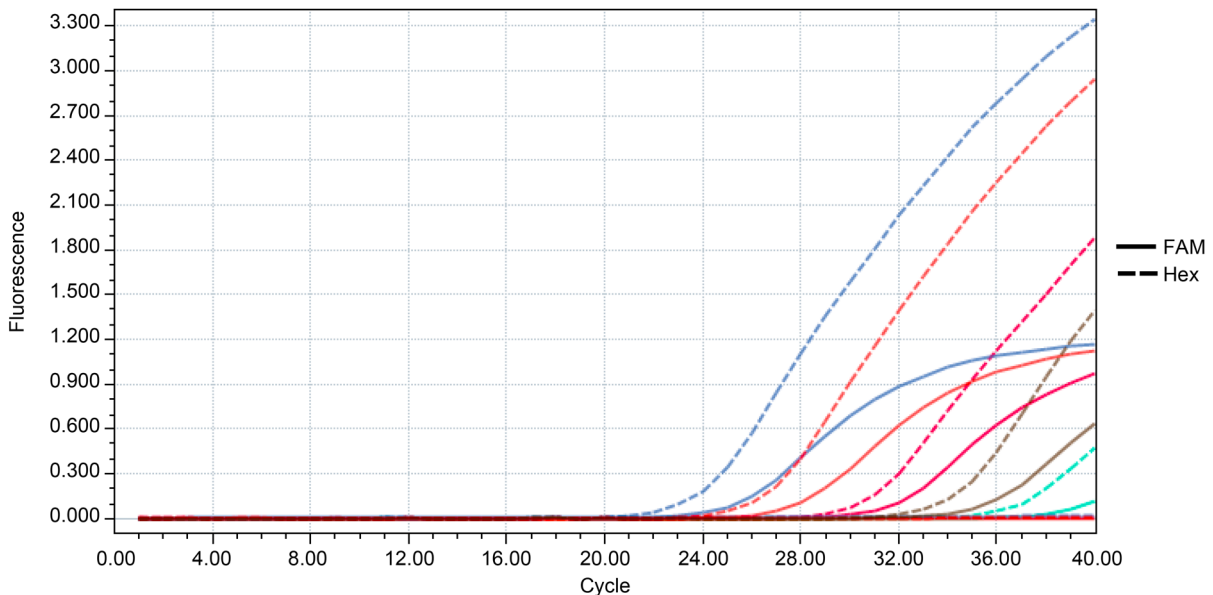

Fig. 4. Detection limits of *P. mirabilis* and *P. vulgaris* in contaminated pork.

The FAM channel was used to detect *P. mirabilis*, and the concentration of the 'S' amplification curve from left to right was in the range of $10^7 - 10^3$ CFU/g. The HEX channel was used to detect *P. vulgaris*, and the concentration of the 'S' amplification curve from left to right was in the range of $10^7 - 10^3$ CFU/g.

a common cause of food poisoning in developed countries, researchers in these countries may not believe that *Proteus* can cause foodborne illness. Conversely, *Proteus* is an important foodborne pathogen in countries with developing economies. *Proteus* species are most commonly found in the human intestinal tract. Because they comprise less than 0.05% of the human microbiome (Yatsunenko et al. 2012), their presence

can be easily overlooked. It has been demonstrated that unclean hands may have an important role in the hand-to-mouth spread of *Proteus* species. Smith et al. (2009) found strains of *P. vulgaris* on the handsets of telephone booths in Nigeria. Padaruth et al. (2014) reported that *Proteus* species were present on the hands of primary school students in Mauritius. Qadripur et al. (2001) identified *P. mirabilis* colonization on painters' hands.
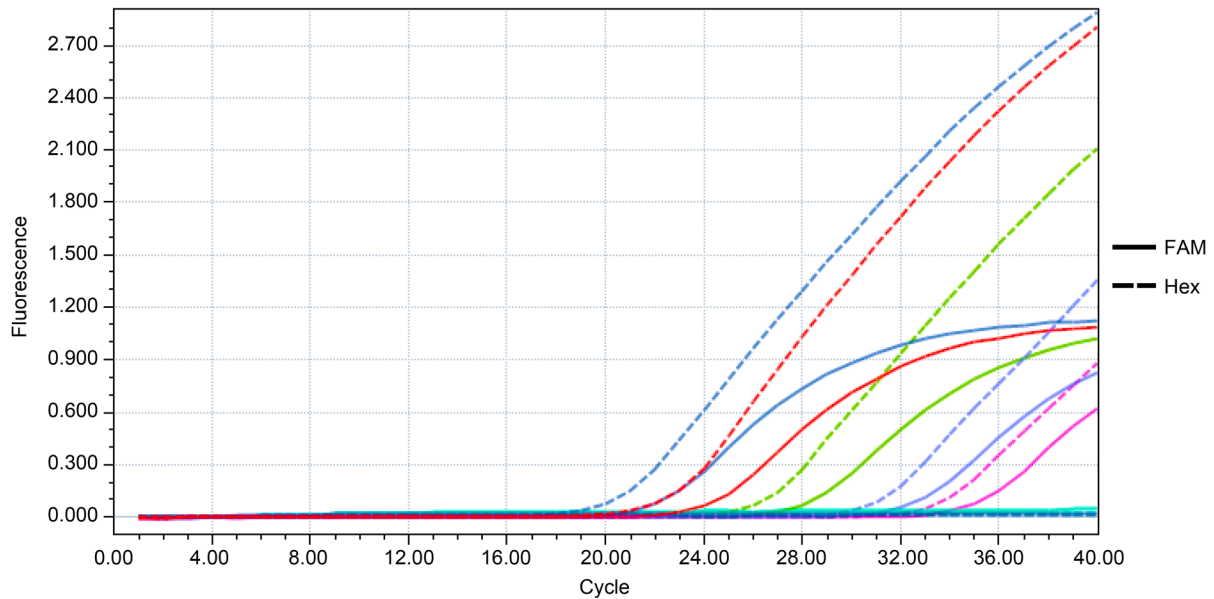
Fig. 5. Detection limits of *P. mirabilis* and *P. vulgaris* in contaminated milk.

The FAM channel was used to detect *P. mirabilis*, and the concentration of the 'S' amplification curve from left to right was in the range of $10^7 - 10^3$ CFU/g. The HEX channel was used to detect *P. vulgaris*, and the concentration of the 'S' amplification curve from left to right was in the range of $10^7 - 10^3$ CFU/g.

In developed countries, people usually maintain daily cleanliness by practicing good sanitary habits; contrarily, people living in developing countries are less aware of personal hygiene's importance. It is a major reason why *Proteus* is a much higher public health concern in underdeveloped countries.

TaqMan Real-Time PCR is a rapid, sensitive, specific, and efficient detection method that is widely used in food hygiene inspection, pathogen detection, and high throughput analysis (Kralik et al. 2017). In the detection of complex samples, obtaining high-quality DNA is essential to ensure the accuracy of detection (Cremonesi et al. 2014). During the analysis process, proven automated nucleic acid extraction technology and the supporting genome extraction kit are used to extract the genome in contaminated food samples to reduce the influence of human-related factors as much as possible and ensure the reliability and repeatability of genome extraction.

Target genes, primers, and probes are the decisive factors that ensure the detection method's specificity and sensitivity. The *ureR* gene is a urease gene regulator and an essential virulence factor for the genus *Proteus*. Researchers have already confirmed the activity of urease in *P. mirabilis*, *P. vulgaris*, and *P. penneri*, which allows these species to quickly adapt to the digestive tract and grow rapidly in an environment at pH of 5–10 (Mobley et al. 1987, 1991). It makes the *ureR* gene one of the most widely accepted target genes for detecting and identifying *P. mirabilis* (Liu et al. 2019; Wang et al. 2019). We believe that the β-lactamase gene *blaB* exists in the genome of *P. vulgaris*. Matsubara et al. (1981) reported

that *P. vulgaris* strains were mostly resistant to β-lactam antibiotics. Aspiotis and coworkers (Aspiotis et al. 1986) found that the resistance to β-lactam antibiotics was facilitated by the presence of β-lactamase in *P. vulgaris*, and even strains sensitive to β-lactam antibiotics could produce low levels of β-lactamase. Ishiguro and coworkers (Ishiguro et al. 1996) found that the production of β-lactamase in *P. vulgaris* was regulated by the *blaB* and *blaA* genes, making them the essential genes for β-lactamase production. The studies mentioned above provided ample evidence that *blaB* is extensively present in *P. vulgaris*. To verify this viewpoint, we analyzed 12 whole-genomes of *P. vulgaris* obtained from GenBank. As a result, the *blaB* gene was found in every genome. On this basis, we used *ureR* and *blaB* as the target genes for detection. Primers and probes were designed according to the conserved sequences of the two genes. The nucleotide sequences of the primers and probes were compared using NCBI BLAST. The primers and probes were able to identify target locations for PCR amplification in *P. mirabilis* and *P. vulgaris*.

This method can identify and distinguish between *P. mirabilis* and *P. vulgaris* efficiently and accurately. At present, 16S rRNA sequencing, selective media, biochemical identification, and serological tests are the major methods for identifying and classifying *Proteus* species (O'Hara et al. 2000). However, these methods are complex, and they require more than 24 h to complete all procedures. Against this backdrop, we developed a timesaving method that enables species identification and classification within two hours. As food poisoning is largely an emergency, quick identifying of

the pathogen can facilitate the development of a suitable treatment plan. Additionally, our method also displays a high degree of sensitivity. Wang et al. (2019) developed a multiplex PCR-based method to classify six different pathogenic bacteria, and the minimum detectable concentration of *P. mirabilis* was 8.6×10^3 CFU/ml. Compared with multiplex PCR, Q-PCR is substantially more sensitive. Liu et al. (2019) used Q-PCR to detect 12 foodborne pathogenic bacteria under the same reaction condition, and the minimum detectable concentration of *P. mirabilis* in the pork samples was 1×10^4 CFU/g. When using this method, researchers should assure that the reaction condition meets the requirements for PCR amplification of all bacteria. However, this does not necessarily mean that the reaction provides the best condition for the TaqMan Real-Time PCR amplification of *P. mirabilis*. Compared with the method introduced by Liu et al. (2019), our design has a higher degree of sensitivity. For contaminated pork and milk, the minimum detectable concentrations of *P. mirabilis* and *P. vulgaris* were both 1×10^3 CFU/g, indicating an advancement in the identification and classification of *Proteus* species.

To conclude, we have created a practical, easy-to-use, and highly efficient method based on TaqMan Real-Time PCR for the identification and classification of *P. mirabilis* and *P. vulgaris* in food that can be widely used in food safety and inspection service.

Authors' contributions

Yang R. and Xu G.Y. carried out most of the experiments and wrote the manuscript and should be considered as first authors. Yang R. and Fu L.Z. conceived and designed the experiments. Xu G.Y., Wang X.Y. and Qing Z.C. performed the experiments. Yang R. and Xu G.Y. designed the probes and primers, and analyzed the data. Yang R., Xu G.Y. and Fu L.Z. wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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