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Original article

Optimization of a multiplex PCR assay for simultaneous detection of key bovine respiratory infections

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Abstract

The control of respiratory infections is integral to a healthy livestock farm. The current study was conducted to optimize a multiplex PCR assay for simultaneous detection of *Pasteurella multocida* (*P. multocida*), *Staphylococcus aureus* (*S. aureus*) and *Mycobacterium bovis* (*M. bovis*) in nasal samples of cattle having suspicion of respiratory tract infections. The nasal samples were collected from 10 dairy farms affected with respiratory disease outbreaks in the recent past. These outbreaks were associated with respiratory tract infections caused by bacterial pathogens *P. multocida*, *S. aureus* and *M. bovis*. A multiplex PCR assay was therefore optimized to enable the simultaneous detection of these bacterial pathogens directly from nasal samples. The multiplex PCR assay was optimized using primers already validated for efficient amplification of specific DNA segments in reference strains of targeted bacterial pathogens. The standardized assay was specific and sensitive enough to detect ≥100 genome equivalents of target DNA segments in each of *P. multocida*, *S. aureus* and *M. bovis*. The assay was successfully applied for the detection of the three bacterial pathogens in 50 nasal samples. PCR amplicons were subjected to Sanger dideoxy sequencing followed by phylogenetic analysis to determine if species identification was specific. In short, the optimized multiplex PCR assay may prove as a reliable, economical, and simple tool for the management of bovine respiratory tract infections caused by some key bacterial pathogens.

Keywords: cattle, dairy farms, *Mycobacterium*, *Pasteurella*, *Staphylococcus*

Introduction

Livestock, a rapidly growing subsector of agriculture, is one of the productive modes on which most households rely to fulfill their food security demand (Weyori et al. 2019). In Pakistan, livestock contributes about 60% share to agriculture and 12% to the national gross domestic production (Mushtaq et al. 2021). More than 8 million rural families in the country are involved in livestock production and earn about 35-40 % of their livelihood from this occupation. There are around 52 million cattle and 43 million buffaloes in the country (Pakistan Economic Survey 2020-21).

The rural economy of the country has often suffered from natural disasters such as heavy floods and infectious disease outbreaks. The most common types of diseases reported from different livestock farms across the country include respiratory disorders, lumpy skin disease, foot and mouth disease, parasitic infestations, hemorrhagic septicemia, digestive diseases and infections to name a few (Naqvi and Fatima 2012, Ghafar et al. 2020, Nadeem et al. 2022). According to Naqvi and Fatima (2012), endo-parasites were found to be dominant health risk (25.3%) followed by respiratory diseases (24.74%) in livestock in Gilgit-Baltistan.

Bovine Respiratory Disease Complex (BRDC) is the most common manifestation among livestock animals and has a significant economic impact worldwide (Lubbers and Turnidge 2015, Wolfger et al. 2015). The annual disease burden from BRDC in US alone accounts for more than 1 billion dollars (Griffin et al. 2010, Loy et al. 2018). While clinical signs related with BRD show a fast onset, exact diagnosis of BRD is still a critical challenge due to involvement of multiple etiological agents (Wolfger et al. 2015). The respiratory pathogens include viruses such as IBR, BVD, PI3, BSRV and bacterial species such as *P. haemolitica*, *P. multocida*, *Haemophilus somnus*, *S. aureus*, *Mycoplasma bovis* and *Clostridium perfringens* (Uzal et al. 2002, Fernandez-Miyakawa et al. 2007). Among these bacterial species, *Pasteurella multocida* causes haemorrhagic septicaemia (Kuranasree 2016) and results in high mortality in cattle and buffalo in Asia (Benkirane and De Alwis 2002). Likewise, *S. aureus* colonizes the respiratory tract and can cause pneumonia, a serious infection that can be life-threatening, especially in vulnerable populations such as the elderly, immunocompromised, and those with underlying lung disease (He and Wunderink 2020). *S. aureus* may be a key concern because of its frequent presence in various indoor and outdoor environments (Kozajda et al. 2019). Aside from *P. multocida* and *S. aureus*, there are many more bacterial pathogens whose presence in the air can lead to a variety of diseases such as bovine pneumonia and bovine tuberculosis in livestock farms (Radostits et al. 2000, Eames et al. 2009).

While these respiratory diseases are a remarkable burden on economy and require the use of antimicrobials in livestock, their early and rapid detection is an important aspect that needs to be improvised. Culture based techniques are tedious, requiring a few days to acquire bacterial separation, and a few types such as *M. bovis* are slow-culturing. Moreover, these techniques are designed to identify one species at a time (Cusack et al. 2003, Loy 2020). Consequently, new advances need to be assessed to improve the determination and early identification of respiratory pathogens (Kardos et al. 2007, Hu et al. 2011, Wolfger et al. 2015, Loy 2020). For example, multiplex PCR is faster than culture and can detect several targets in a single reaction (Dalmasso et al. 2004, Safdar et al. 2014, Chen et al. 2015, Elbrissi et al. 2017, Moriconi et al. 2017)

In the current study, three key bacterial respiratory pathogens *P. multocida*, *S. aureus* and *M. bovis* were chosen for the development of multiplex PCR so that simultaneous detection of these bacteria could be achieved in nasal swabs from cattle and buffaloes. The assay proved to be robust, reliable, rapid, specific, and sensitive for amplification of target DNA segments in *P. multocida*, *S. aureus* and *M. bovis*.

Materials and Methods

Sample collection

Ten livestock farms were selected from two districts of Lahore Division i.e. Lahore and Kasur (Fig. 1). The selection was made on the basis of information obtained from outdoor clinic of University of Veterinary and Animal Sciences Lahore regarding recent respiratory disease outbreaks (such as BRD, haemorrhagic septicemia). Further details regarding the selection criteria, location and general information of the selected farms can be obtained from Jamil et al. (2023). Nasal samples were collected by expert veterinarian from these farms by swabbing nasal cavity of all those 50 animals that were suspicious of having respiratory tract infections. Recent respiratory disease outbreaks in selected livestock farms have been associated with infections by *M. bovis, P. multocida* and *S. aureus*. It was therefore assumed that these bacterial respiratory pathogens might be involved in causing symptoms such as nasal discharge in sampled cattle and buffaloes. Consequently, a multiplex PCR assay was optimized for simultaneous detection of these pathogens.

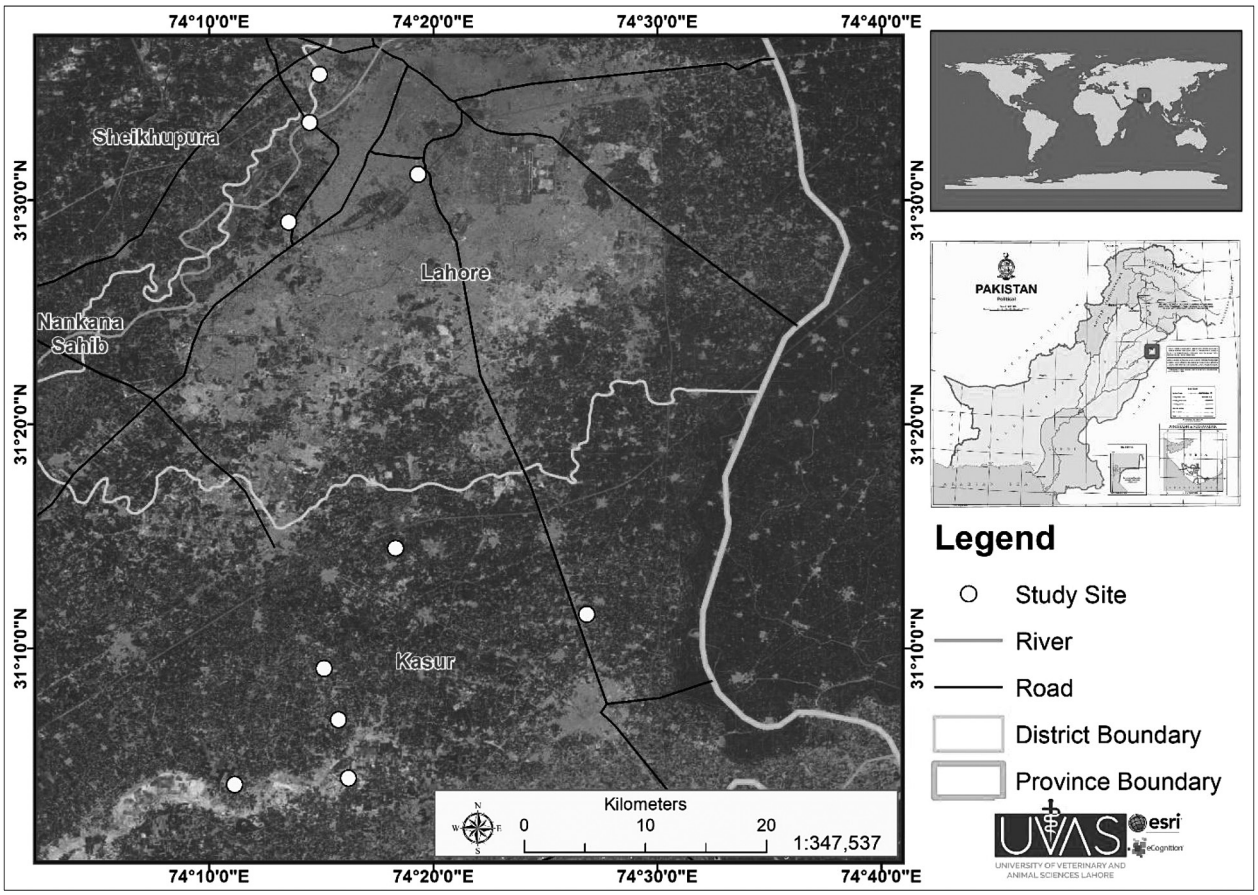

Fig. 1. Map of the study sites.

Bacterial strains

The reference bacterial strains of *M. bovis, P. multocida* and *S. aureus* were obtained from Veterinary Research Institute (Government of the Punjab, Pakistan) and University Diagnostic Lab (University of Veterinary and Animal Sciences Lahore, Punjab, Pakistan).

Bacterial DNA extraction and quantification

DNA extraction from reference strains was performed using GeneJet Genomic DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) and from nasal swabs using PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The DNA bands were separated by agarose gel electrophoresis and visualized under UV light in a Gel Documentation System (BioRad, Hercules, CA, USA). The quality and quantity of DNA was estimated using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The details of NanoDrop values for reference strains are given in Table 1.

Multiplex PCR assay

Multiplex PCR assay was performed in 25 µl of reaction mixture. PCR thermal profile was optimized using 3 different temperatures with initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C/58°C/60°°C for 30 s and extension at 72°C for 30 s, followed by final extension at 72°C for 10 min. The PCR product were separated by agarose gel electrophoresis and analyzed under UV light. The specificity of multiplex PCR assay was tested on DNA samples isolated from some non-target bacterial species. The presence of inhibitors was checked by spiking the PCR-negative nasal samples with standard bacterial species. DNA was isolated from the spiked samples and amplified using standardized multiplex PCR. Nasal swabs from healthy animals served as negative control. These nasal samples were collected from Friesian cattle farm of the university.

Bacterial detection in nasal swabs by multiplex PCR assay

The target bacterial respiratory pathogens were detected from nasal swab samples using optimized recipe and thermal profile of multiplex PCR. The identity of all positive samples was confirmed by PCR

Table 1. NanoDrop values of extracted DNA samples used as standard for assay optimization.

Sample	ng/ul	A 260	A 280	Ratio 260/280	Ratio 260/230
SAM01	32.8	0.630	0.370	1.99	0.64
SAM02 *Staphylococcus aureus*	29	0.585	0.344	1.56	0.76
Sample 03	43	0.734	0.457	1.74	1.03
Sample 04 *Mycobacterium bovis*	28	1.323	0.678	1.98	1.66
Sample 05	7.7	0.155	0.087	1.93	0.34
Sample 06 *Pasteurella multocida*	46.7	0.992	0.324	1.98	0.65

Table 2. Primer sequences for multiplex PCR assay.

Target Organism	Target Gene	Primer name	Primer Sequences	Amplicon size	References
S. aureus	16SrRNA	Sa-F Sa-R	5′- GAACCGCATGGTTCAAAAGT -3′ 5′- CATTTCACCGCTACACATGG -3′	518bp	(Ashraf et al. 2017)
P. multocida	Kmt1	F- KMT1T7 R- KMT1SP6	5′-ATCCGCTATTTACCCAGTGG-3′ 5′- GCTGTAAACGAACTCGCCAC -3′	460bp	(Mahrous et al. 2022)
P. multocida	Omp87	Forward Reverse	5’- AGGTGAAAGAGGTTATG -3’ 5’- TACCTAACTCAACCAAC -3’	219bp	(Rodriguez-Castillo et al. 2017)
M. bovis	16SrRNA	Mb-F Mb-R	5′- GATGTTTAGCGGGGTTGAGA -3′ 5′- TTGAGCCCCAAAATTTAACG -3′	343bp	(Ashraf et al. 2017)

amplification of 16S rRNA gene in these samples followed by Sanger dideoxy sequencing. The sequencing results were analyzed using NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular Evolutionary Genetics Analysis (MEGA) software was used to conduct sequence alignments and phylogenetic analysis (Tamura et al. 2011).

Results

Optimization and application of multiplex PCR assay

For optimization of multiplex PCR assay, 25 µl reactions were prepared using following PCR components: 4 µl 5× PCR buffer with 1.5 mM final $MgCl_2$, 1 µl each of *M. bovis* Primer-F (10 ρmoles/µl) and Primer-R (10 ρmoles/µl), 1 µl each of *P. multocida* Primer-F (10 ρmoles/µl) and Primer-R (10 ρmoles/µl), 1 µl each of *S. aureus* Primer-F (10 ρmoles/µl) and Primer-R (10 ρmoles/µl), 2 µl dNTPs (2.5 mM), 2 µl DNA sample (25 ηg/µl), 0.25 µl Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific) and 10.75 µl ddH_2O. DNA from reference strains of *M. bovis, P. multocida* and *S. aureus* were used as positive control. The detail of primers targeting reference strains is given in Table 2.

The thermal profile of multiplex PCR assay was optimized using 3 different annealing temperatures viz: 53°C, 58°C, 60°C. All the subsequent reactions were performed by following the thermal profile: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. A final step of extension at 72°C for 10 min was followed by 4°C hold for infinity.

The optimized multiplex PCR assay was capable of efficiently detecting and differentiating *P. multocida, M. bovis* and *S. aureus* (Fig. 2). The assay was applied to screen nasal swabs from clinically suspected cases of BRDC and some of them were found to contain DNA from the targeted bacterial respiratory pathogens (Fig. 3). No sample turned to contain coinfection by more than one pathogen. All samples were tested in triplicates along with positive control, negative control, no template control and negative controls spiked with DNA of known identity. The positive samples were reconfirmed by performing the assay on different days with freshly extracted DNA. There was no error found in repeated testing of positive nasal samples.

The positive samples were also PCR-amplified using universal 16S rRNA gene primers and amplicons obtained thus were Sanger-sequenced for verifying the identity of *M. bovis, P. multocida* and *S. aureus*. The phylogenetic clustering of these entire bacterial 16S rRNA gene sequences was achieved accurately (Figs. 3, 4, 5 and 6).

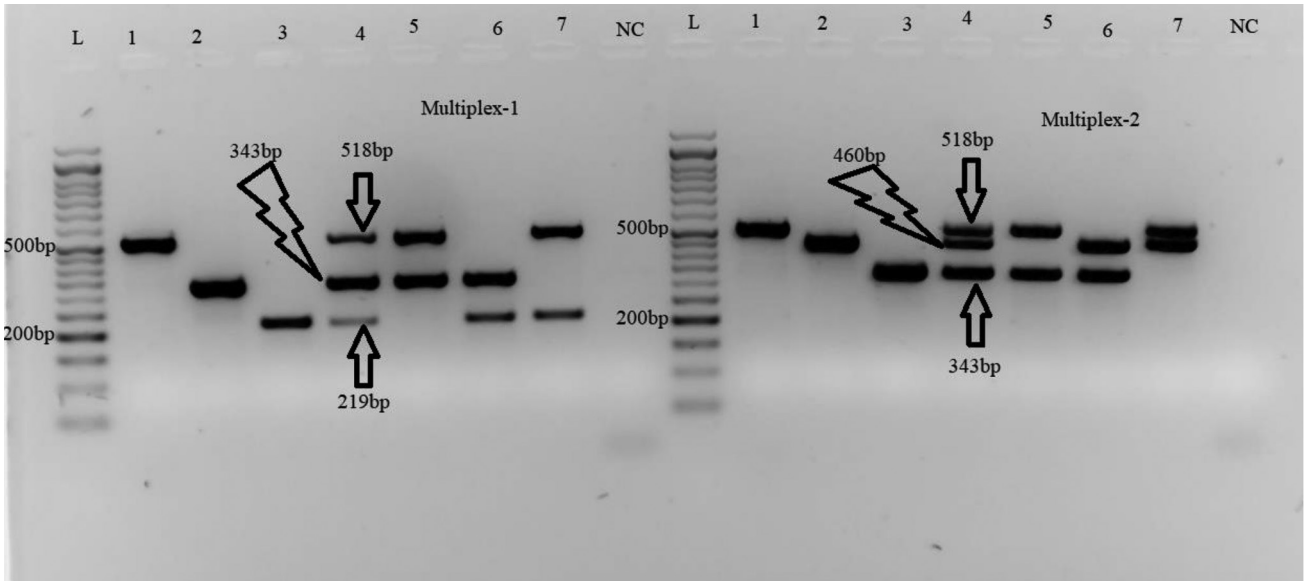

Fig. 2. Multiplex PCR-1 assay for respiratory bacterial pathogens. Lane L – 100 bp DNA ladder, Lane 4 – *S. aureus* (518 bp), *M. bovis* (343 bp) & *P. multocida* (219 bp). Multiplex PCR-2 assay for respiratory bacterial pathogens. Lane L – 100 bp DNA ladder, Lane 4 – *S. aureus* (518 bp), *M. bovis* (343 bp) & *P. multocida* (460 bp).

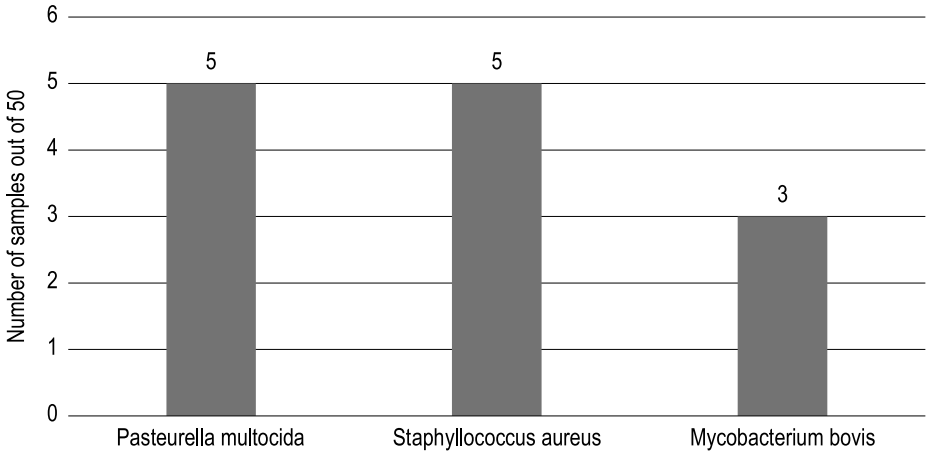

Fig. 3. The detection of respiratory bacterial pathogens in collected samples.

Determination of specificity of multiplex PCR assay

The PCR products were sequenced to confirm the specificity of the multiplex PCR assay. The genomic DNAs were adjusted to a 50 ηg/μL concentration, and 1 μL of each DNA sample was used as a template for multiplex PCR amplification. The purpose of using genomic DNAs from different bacterial species was to ensure that the optimized multiplex PCR is specific to the target organisms and does not amplify DNA from other organisms. The specificity of multiplex PCR assay was tested on DNA samples from non-target bacterial species (*Escherichia coli, Salmonella* and *Clostridium perfringens*).

Determination of sensitivity of multiplex PCR assay

The primers chosen for standardization of multiplex PCR assay have shown robust amplification of target DNA molecules with substantial sensitivity in previous studies (Ashraf et al. 2017, Rodriguez-Castillo et al. 2017, Mahrous et al. 2022). The sensitivity of these primers in multiplex PCR format was determined by serially diluting DNA representing the target bacterial pathogens. Serial dilutions contained 10^1 to 10^7 genome equivalent of bacteria in background bovine DNA (25-50 ηg). The optimized multiplex PCR assay was found sensitive enough to detect ≥ 100 genome equivalents of target DNA segments in each of *P. multocida*, *S. aureus* and *M. bovis*. There was seen no interference by bacterial and/or background bovine DNA in detection of at least 100 genome equivalents of any of three

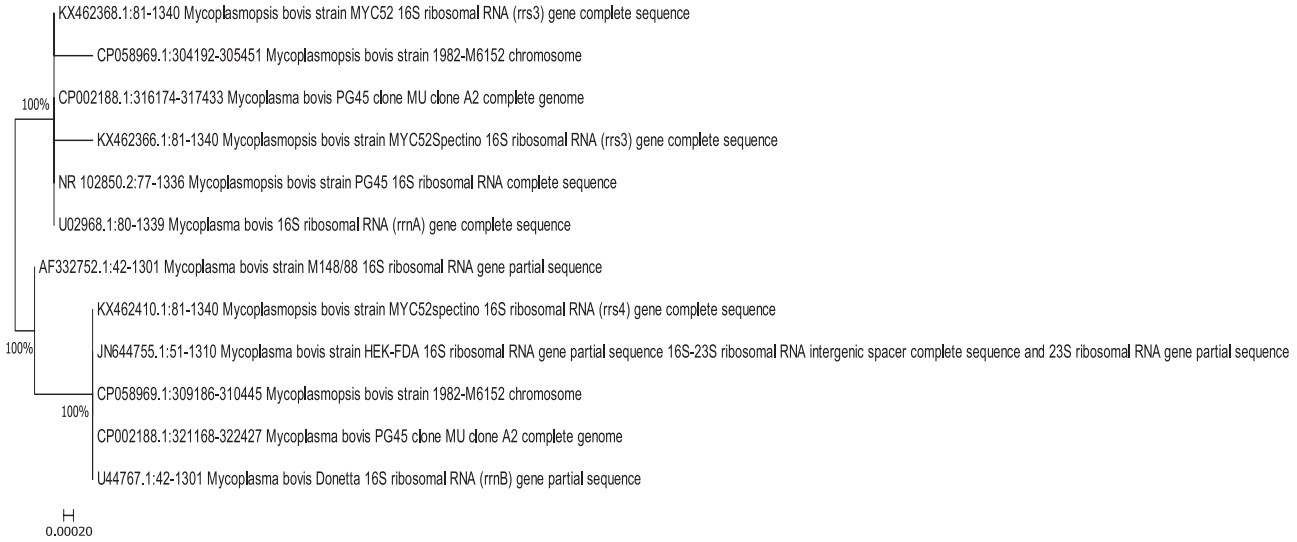

Fig. 4. Phylogenetic tree of partial 16S rRNA gene sequence of *M. bovis*.

Fig. 5. Phylogenetic tree of partial 16S rRNA gene sequence of *P. multocida*.

target pathogens. It is important to note that the sensitivity of the multiplex PCR assay may be influenced by factors such as quality of the DNA template, and concentration and annealing temperature of the primers. These factors were therefore optimized to ensure the best possible sensitivity of the assay.

Discussion

Both viruses and bacteria can cause BRD by single or dual infections in bacterial-bacterial, viral-bacterial and viral-viral combinations (Lachowicz-Wolak et al. 2022, Uprety et al. 2023). They may interact synergistically or antagonistically, or their coinfection may be unrelated (Babawale and Guerrero-Plata 2024). Viruses are the primary pathogens infecting the susceptible animals and developing a scenario for secondary bacterial

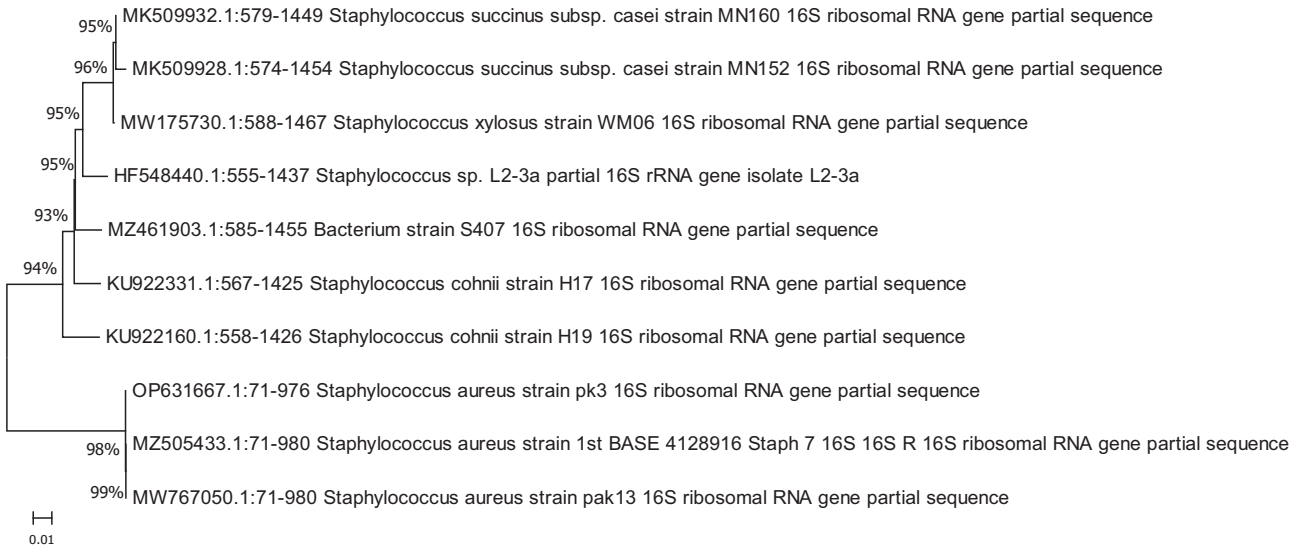

Fig. 6. Phylogenetic tree of partial 16S rRNA gene sequence of *S. aureus*.

superinfections. The viral priming may increase bacterial adherence and colonization of the respiratory tract (Gaudino et al. 2022). Of note, viral infections can be easily diagnosed by visual symptoms (Grissett et al. 2015), while bacterial infections are complicated and can lead to fatality (Panciera and Confer 2010). The most of viral and bacterial respiratory tract infections have a moderate individual pathogenicity. On the contrary, their coinfections are associated with increased severity of the respiratory disease (Lion et al. 2021) and mortality rates (Sid et al. 2015). In vivo studies have demonstrated that a primary viral and/or bacterial infection can alter the pathogenicity of a secondary viral and/or bacterial infection, and results in severe disease outcomes (Gaudino et al. 2022).

The interactions between respiratory pathogens are specific to different combinations, suggesting that differential interactions underlie distinct mechanisms. There is therefore a need for novel diagnostic methods to accurately identify multiple pathogens causing BRD to avoid misdiagnosis (Babawale and Guerrero-Plata 2024). Traditional culture methods are insufficiently sensitive for detecting secondary bacterial respiratory tract pathogens, hindering the formulation of prevention strategies against the spread of the disease (Lubbers and Turnidge 2015). The focus of the current was therefore to establish a rapid and sensitive multiplex PCR assay for the early detection and control of bacterial pathogens closely related to BRDC: *P. multocida, M. bovis* and *S. aureus*. The use of multiplex PCR has gained massive popularity in recent years in the field of molecular biology because it facilitates with low-cost detection of multiple pathogens in a single reaction (Rajalakshmi 2017, Khalil 2021). A multiplex PCR thus saves time by avoiding the performance of monoplex reactions for multiple targets (Moriconi et al. 2017).

It is important to consider factors that may affect the specificity, sensitivity, and rapidity of the assay when designing a multiplex PCR. The choices of target gene and primer design are crucial factors in this process. In the current study, the Omp87, 16S rRNA and Kmt1 genes were chosen as markers for the detection of *P. multocida, M. bovis* and *S. aureus* (Table 2), due to their high intraspecies conservation and previous use in detection methods (Ashraf et al. 2017, Rodriguez-Castillo et al. 2017, Mahrous et al. 2022). The primers targeting the selected genes appeared to be highly effective in multiplex PCR amplification (Fig. 2), indicating their potential for use in diagnostic applications.

The primers used in multiplex PCR can interfere with each other, making optimization of their concentration and annealing temperature necessary to obtain the highest possible efficacy for each primer pair. The concentration and annealing temperature of the primers were therefore optimized for finalizing the multiplex PCR assay. The final multiplex PCR assay showed equal efficacy in obtaining all of three target products, indicating that the concentration and annealing temperature of the primers had been successfully optimized. It appears that the concentration and annealing temperature of the primers are the most critical parameters in determining the efficacy of the multiplex PCR while the concentration of dNTPs and Mg^{2+} in the reaction mixture, as well as the annealing time and cycle number, have a relatively smaller impact. Overall, these findings highlight the importance of careful optimization of the PCR reaction parameters to achieve optimal results in multiplex PCR experiments.

It is an established fact that validating the method using real clinical specimens would be the best, but

it is difficult to collect such specimens that meet the requirements of the study. Therefore, in the current study, the bacteria were first enriched in a culture medium and then detected through the multiplex PCR. Although the enrichment process was time-consuming, the total time for completing the detection was not longer than eight hours, which is much shorter than the time required for the conventional bacterial isolation method. The final multiplex PCR was optimized to directly detect the three bacteria in nasal samples, further reducing the time and money required for the detection process. There was found no disparity in results obtained from enriched specimens and those originated directly from nasal swabs.

The management of BRD is quite challenging because it results from complex interactions between pathogens, environment and host (Gaudino et al. 2022). The disease can be prevented by adopting biosecurity measures, nutritional interventions, regular health checkups, and active surveillance which involves the use of diagnostic methods capable of rapid and accurate identification of pathogens in laboratory setups at livestock farms. The multiplex PCR assay standardized in this study may be implemented for active surveillance and diagnosis of single as well as dual infections by *P. multocida, M. bovis* and *S. aureus*.

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