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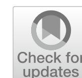

Developing multiplex PCR for the rapid and simultaneous detection of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* associated with sheep respiratory tract infections

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Abstract
Respiratory tract infections in sheep are among the important health problems that affect all sheep ages around the world. Nine bacterial isolates obtained from sheep with respiratory tract infections were selected to be used in the current study. The isolates included 3 *Staphylococcus aureus,* 4 *Klebsiella pneumoniae*, and 2 *Pseudomonas aeruginosa*. Following the primers design by the Primer3Plus software tool and optimization of the conventional polymerase chain reaction (PCR), the primers were validated for their use in the multiplex PCR experiments. The MFEprimer program was used to check the suitability of the primer set combinations for multiplex PCR. The MFEprimer software was successful in designing the multiplex-PCR experiments and determining the optimal primer set combinations. Multiplex PCR was able to amplify specific DNA sequences of one, two or three target genes of these mixed microorganisms in the same PCR reaction tube. This technique efficiently detected combinations of two organisms, either *S. aureus* with *K. pneumoniae, S. aureus* with *P. aeruginosa* or *K. pneumoniae* with *P. aeruginosa*. Moreover, multiplex PCR was also able to detect the presence of the three organisms together in the same reaction tube. To conclude, this study confirmed multiplex-PCR as a specific, sensitive, rapid, accurate, and cost-effective molecular diagnostic method for identification and differentiation of three clinically important bacteria associated with sheep respiratory tract infections, including *S. aureus, P. aeruginosa,* and *K. pneumoniae*. This can efficiently support control and treatment of such diseases and would increase the economy of the animals' owners and wellbeing of the animals.

Keywords Multiplex PCR · Sheep · Respiratory infections · *S. aureus* · *P. aeruginosa* · *Klebsiella pneumoniae*

Abbreviations

PCR	Polymerase Chain Reaction
DNA	Deoxyribo-Nucleic Acid
API	Analytical Profile Index
BLAST	Basic Local Alignment Search Tool
NCBI	the National Center for Biotechnology Information
Tm	melting temperature
SNPs	Single Nucleotide Polymorphisms

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Introduction

Respiratory infections are among the important health problems of sheep of all ages throughout the world. Concerning Iraq, a study performed in Mosul city, northern part of the country, found that respiratory infections were more common in sheep following gastrointestinal disturbances (Dahl et al. 2021). These infections, either acute or chronic, have economic impacts on the performance of flocks (Thompson 2019). Respiratory diseases may affect individual animals or groups, leading to weak gain of live weight with high mortality rate (Kumar et al. 2000). This causes large economic losses to owners due to reduced production of milk, meat, and wool as well as decreased offspring numbers (Chakraborty et al. 2014). Small ruminants are extremely vulnerable to pulmonary infections, which cause death in roughly 50% of them (Kumar et al. 2014).

Various bacteria can infect the upper and lower respiratory tract and frequently lead to the development of bronchitis, pneumonia or other respiratory disorders. Many bacterial species either single or mixed with other types have been isolated from sheep respiratory infections (Chakraborty et al. 2014). Using conventional diagnostic methods based on phenotypic tests often need longer time to generate and analyze the results, and in most cases, extra tests are essential (Gandra et al. 2016). Furthermore, phenotypic tests can be associated with false-negative results, indicating the impact of environmental factors on gene expression (Downes and Ito 2001), adding to the relatively lower specificity and sensitivity of these tests than molecular assays (Chakraborty et al. 2014). Thus, a reliable diagnostic tool can reduce the problems of diagnosis and the possible economic losses resulting from respiratory diseases (Scott 2011).

Therefore, early, fast, and precise detection of such infections is of significant value for the small ruminants (Chakraborty et al. 2014; Kumar et al. 2014). Molecular techniques based on PCR have been proved to be valuable for the diagnostic and epidemiological studies (Yang et al. 2007; Kumar et al. 2014). However, molecular detection of the respiratory infections in these animals is considered as a challenge in Southeast Asian and African countries because of their inadequate laboratory resources. This situation makes large numbers of these animals to be slaughtered due to respiratory infections outbreaks (Daniel et al. 2006; Scott 2011).

Mixed infections have been observed for many times by researchers. Isolation and identification of these infections could be boring and time consuming (Kumar et al. 2012; Saleh and Allam 2014). Therefore, improvement of molecular diagnostic tools, such as multiplex PCR, has been validated to be useful for the detection and characterization of the causative agents of such complex infections (Dhama et al. 2014). Multiplex PCR has helped diagnosticians to decrease confusion in cases of mixed infection because such techniques are able to discriminate between different species in the same genus (Chakraborty et al. 2014). Moreover, this technique has been used successfully for detecting multiple pathogens simultaneously in the same reaction tube with high sensitivity, specificity, throughput, with low cost (Datukishvili et al. 2015; Hu et al. 2020). The main advantages of using multiplex PCR over traditional PCR is its cost-effectiveness. Multiplexing decreases the amounts of reagents, e.g., DNA polymerase, applied for each reaction. In addition, it does not require more time for preparation and analysis of results than using multiple tubes for simplex PCR (Phuektes et al. 2001). Therefore, this study was designed in an attempt to develop multiplex-PCR for the cost-effective, rapid, and accurate identification of three bacteria causing respiratory tract infections in sheep, including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *K. pneumoniae.*

Materials and methods

Bacterial isolates

Nine bacterial isolates were used in this study, including: 4 *K. pneumoniae*, 3 *S. aureus,* and 2 *P. aeruginosa.* These bacteria were obtained from the study of Aziz and Lafta (2021) performed on sheep with respiratory tract infections and reared in different farms in Baghdad, Iraq. The bacteria were already identified by the corresponding analytical profile index (API staph, API 20NE or API 20E, as well as the automated Vitek2 system (data not shown).

Molecular diagnosis

Primers design and preparation

Molecular identification of *S. aureus, P. aeruginosa,* and *K. pneumoniae* was done by partial amplification of their *nuc, rpoS* and *gapA* genes, respectively, by using specific primers designed in this study through the use of Primer3Plus software tool. The primers were checked by in silico PCR amplification as well as the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) before running the conventional PCR.

To validate the primers before being used in the multiplex PCR experiments, the MFEprimer program was used to check the specificity of the primers against the background DNA that can act as a competitor to the target DNA. Here, the background DNA was genomic DNA extracted from the bacteria under study. Furthermore, the major purpose of using this program was to evaluate the candidate primers, to examine the formation of primer dimers to discard the inappropriate primers, and finally to determine the optimal primer set combination for multiplex PCR by providing a virtual electrophoresis to assist users choose the best combination of primer sets (Qu and Zhang 2015).

Prior to their use, the lyophilized primers (Macrogen, Korea) were dissolved in a suitable volume of nuclease free water to give a final concentration of 100 pmol/μL as a stock solution, yet their working concentration was 10 pmol/μL. The accession numbers of the genes used to design the primers as well as the amplicon sizes are mentioned in the Table 1 below.

DNA extraction and quantification

Genomic DNA was isolated from the bacterial growth according to the protocol of ABIOpure Extraction. Then,

Table 1 The genes and their accession numbers used to design the specific primers and sizes of the PCR amplicons

Genes' Name	Accession No.	Primers' Sequence 5′---------3'	Product Size (bp)
nuc	DQ399678.1	F- TAGCTCAGCAAATGCATCAC R- CGTTGTCTTCGCTCCAAATA	506
gapA	NC_016845.1	F- GTGATGGGCGTTAATGAGAG R- AAGCATTGTTCACCTCTTCG	391
rpoS	NC_002516.2	F- AGCCACCACTTCCTTCTCTT R- ACAGTCCGCGATTGACATAG	236

Quantus Fluorometer was used to detect the DNA concentration of the samples.

Uniplex and multiplex PCR optimization

To examine the optimum annealing temperature of the primers, uniplex PCR was optimized initially by amplifying one DNA template with the corresponding primer pair at different annealing temperatures of 50, 52, 55, and 58 °C. The PCR cycling conditions involved the use of the following program: denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s; annealing at one of the four temperatures mentioned above for 30 s; and extension at 72 °C for 30 s. A final extension step was done for 7 min at 72 °C, followed by 10 min incubation at 4 °C to stop the reactions. The PCR reaction mixture composed of 20 µL total volume containing 10 µL GoTaq Green Master Mix (2×); 1 µL of each primer (10 pmol/µL); 6 µl nuclease free water, and 2 µL of template DNA.

Following the optimization of the uniplex PCR conditions, multiplex PCR was run where the reaction mixture composed of the same components and volumes mentioned above for the uniplex PCR, except that the template DNA amount was increased to 3 µL and the nuclease free water volume was decreased. Concerning the PCR program, an annealing temperature of 58 °C was used for all the primers, and temperatures of the other steps were the same.

Results

Validation of the primers

The MFEprimer software was successful in designing the multiplex-PCR experiments and determining the optimal primer set combinations. Briefly, it stated melting temperatures (Tm) of each primer, primer binding numbers, and the amount of energy required (delta G measured by Kcal/mol). Importantly, the report showed absence of hairpins and primer dimers. Amplicon details were also described in the report for each primer set combination, such as the expected size of the amplicon, GC contents, and delta G of each primer (data not shown).

Optimum conditions for PCR

Fig. 1 shows amplification of partial regions of the *nuc, gapA,* and *rpoS* genes of *S. aureus, Klebsiella pneumoniae,* and *P. aeruginosa,* respectively using conventional PCR. It is clear from the image that each of the temperatures 50, 52, 55 and 58 °C was able to efficiently amplify the aforementioned genes, despite the presence of non-specific bands that appeared at 50 and 52 °C in case of the *gapA* gene. Therefore, the highest annealing temperature of 58 °C was used for the next experiments.

Multiplex PCR

Multiplex PCR was successful in targeting one of the three microorganisms, i.e. either *S. aureus, K. pneumoniae* or *P. aeruginosa* when their primers were put together in the same PCR reaction tube (Fig. 2). Similarly, this technique efficiently detected combinations of two organisms, either *S. aureus* with *K. pneumoniae, S. aureus* with *P. aeruginosa* or *K. pneumoniae* with *P. aeruginosa* (Fig. 3). Importantly, multiplex PCR was also able to detect the presence of the three organisms together in the same reaction tube (Fig. 3).

Discussion

Mixed bacterial infections are often seen in various animal disorders; thus, isolation and identification of such causative agents is always boring and wearing to carry out (Kumar et al. 2012). Due to evolution in the PCR technique, massive progress in the detection of animal respiratory infections has occurred (Munir et al. 2013). Advances in molecular biology and biotechnology have resulted in progression of different diagnostic procedures, including multiplex PCR among others (Deb and Chakraborty 2012; Dhama et al. 2013a, b).

This study focused on three different bacterial species associated with sheep respiratory tract infections, including:

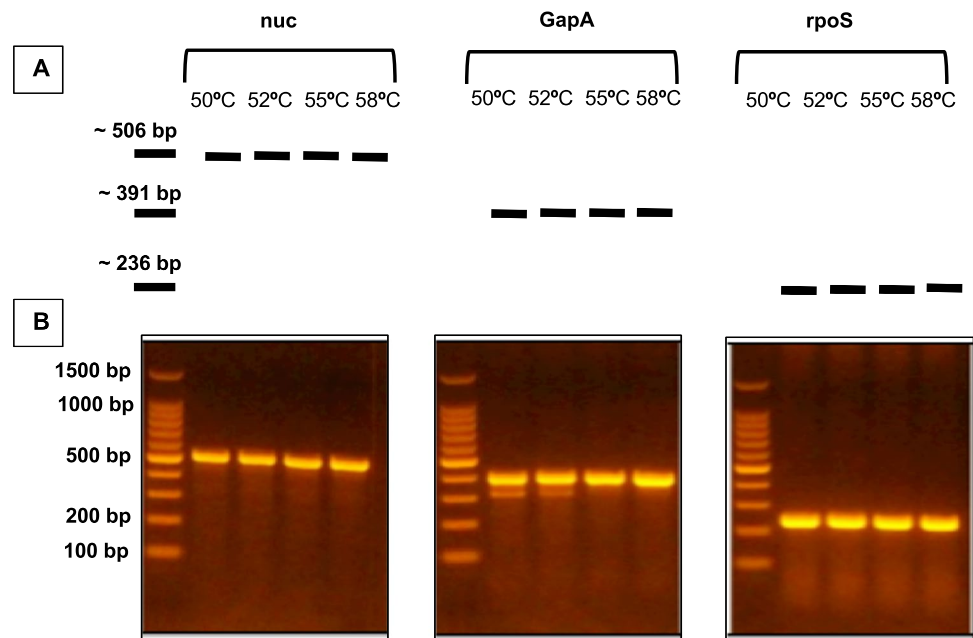

Fig. 1 Optimization of the PCR reaction conditions. **A-** Diagram showing the virtual electropherogram to choose the optimum PCR annealing temperatures for three genes. **B-** Agarose gel electrophoresis for the PCR products amplified under different annealing temperatures of 50, 52, 55 and 58 °C shows bands of ~506 bp representing the *nuc* gene of *S. aureus* (left panel), bands of ~391 bp representing the *gapA* gene of *Klebsiella pneumoniae* (middle panel), and ~ 236 bp for the *rpoS* gene of *P. aeruginosa* (right panel)

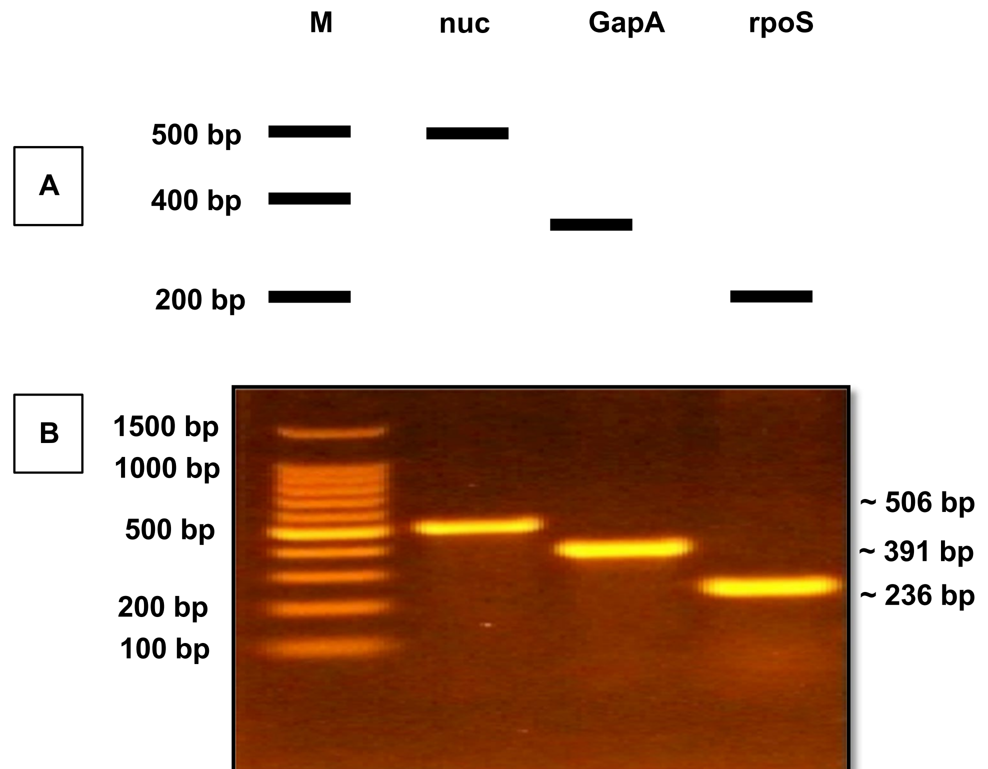

Fig. 2 Gel electrophoresis for the multiplex-PCR targeting one of three isolates in the same reaction tube. **A-** Diagram showing the virtual electropherogram of the multiplex PCR targeting one bacterium. **B-** The experimentally validated electrophoretogram for the multiplex-PCR targeting DNA of one bacterial isolate. Bands of ~506, ~391, and ~ 236 bp represent partial amplification of the *nuc, gapA,* and *rpoS* genes of *S. aureus*, *Klebsiella pneumoniae*, and *P. aeruginosa,* respectively

K. pneumoniae, S. aureus, and *P. aeruginosa.* These bacteria are the primary pathogens associated with morbidity and mortality in less developed countries due to respiratory infections (Hu et al. 2020). Other studies stated that both *S. aureus* and *K. pneumoniae* are among the ovine pathogens that have been isolated from respiratory infections (Azizi et al. 2013; Saleh and Allam 2014). In addition, *P. aeruginosa,* which is also opportunistic pathogen that exists in different ecosystems, can be implemented with dangerous animal diseases (Yang et al. 2015; Bird et al. 2017; Dapgh et al. 2019). It can cause many diseases in sheep, such as respiratory illness and mainly pneumonia that is linked with physiological and physical stress resulting in high economic losses and considerable mortality rates (Bangar et al. 2016).

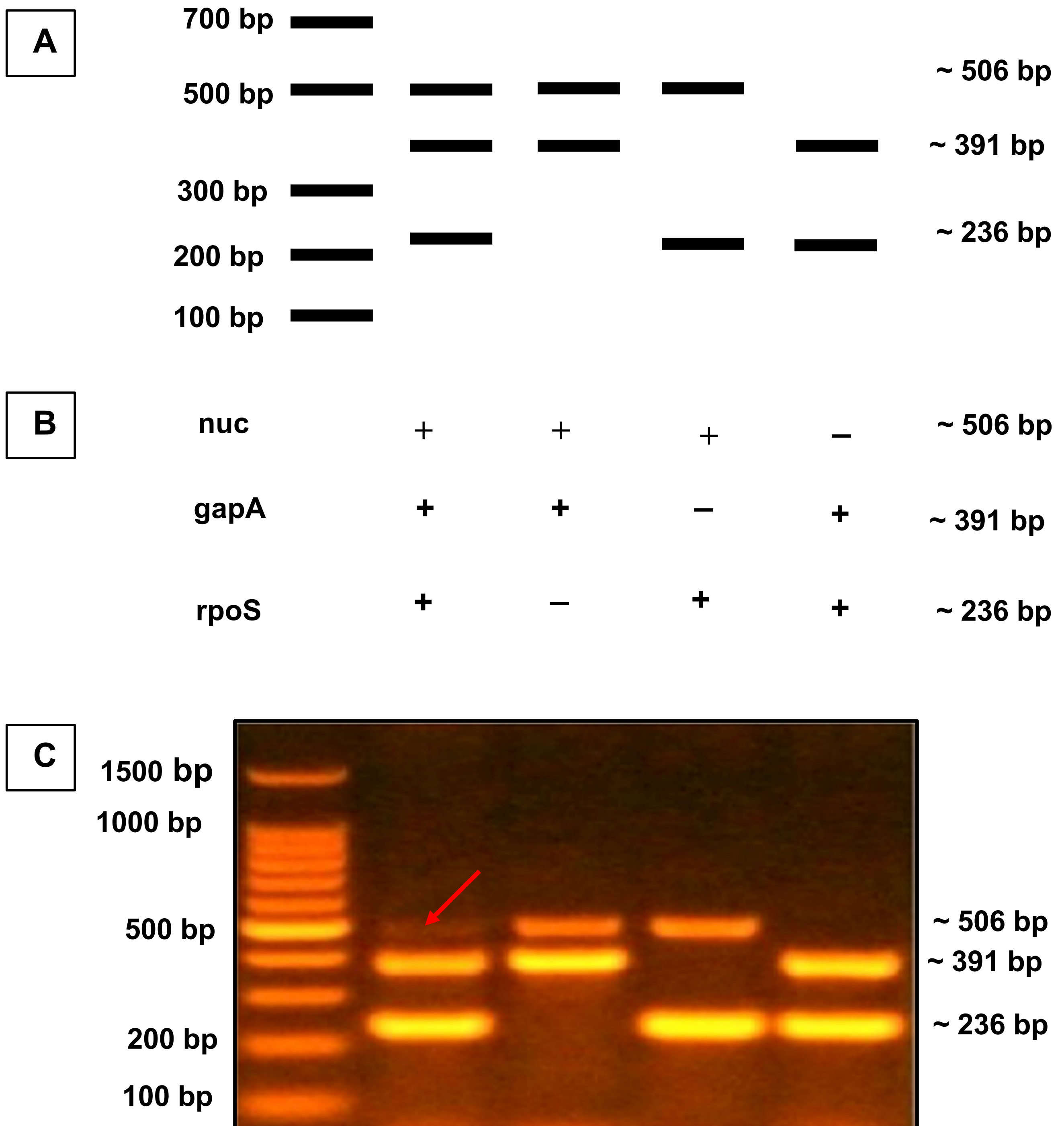

Fig. 3 Gel electrophoresis for the multiplex-PCR targeting two or three isolates in one reaction tube. **A-** Diagram showing the virtual electropherogram of the multiplex PCR targeting two or three bacterial combinations. **B-** Diagram showing the presence (+) or absence (−) of a certain bacterium as represented by the corresponding gene (*nuc, gapA, rpoS*). **C-** The experimentally validated electropherogram for the multiplex-PCR targeting two or three bacterial isolates. Bands of ~506, ~391, and ~236 bp represent partial amplification of the *nuc, gapA,* and *rpoS* genes of *S. aureus*, *Klebsiella pneumoniae*, and *P. aeruginosa,* respectively

In the current study, the designed primer pairs were efficient in amplifying their targets in the above microorganisms when tested separately by uniplex PCR, where one amplicon only was obtained from each target. Similarly, the same amplification efficiency was obtained when a single bacterium was identified using multiplex PCR. Although Hu et al. (2020) observed that the sensitivity of detecting a single organism in multiplex PCR was 5–100 times lower than

that of uniplex PCR, and the sensitivity of identifying four bacteria using multiplex PCR was 5–50 lower than that of one bacterium using the multiplex PCR. This was illustrated by the fact that using combination of primers in the same reaction tube could make specific primers less available than necessary. Likewise, competition between nucleotides and enzymes might occur if numerous targets are present in one reaction.

Here, two targets were also detected successfully and efficiently by multiplex PCR. Combinations of *K. pneumoniae* with *S. aureus*, *K. pneumoniae* with *P. aeruginosa,* or *S. aureus* with *P. aeruginosa* were detected effectively and concurrently in a single reaction by this technique. However, this assay has been commonly used by many researchers to amplify DNA targets belonging to the same species or same genus by using several pairs of primers in the same reaction (Okolie et al. 2015a, b; Gandra et al. 2016). It has been demonstrated by Datukishvili et al. (2015) that concentrations of the templates and primers are crucial factors in some multiplex PCRs reactions.

Nevertheless, a weak band of ~506 bp, which represents partial amplification of the *nuc* gene of *S. aureus,* observed in this study. This can be attributed to many factors, for instance, low concentration of DNA of *S. aureus,* or due to inadequate nucleotides available for amplifying a sharp band of such large size due to competition between the primers, especially the volume of the PCR reaction mixture applied in this study was 20 µL, which is less than 50 µL used by many investigators. Weak and not clear bands were suggested by Gandra et al. (2016) to occur when there is low concentration of the template DNA (~10^2 CFU/mL). Other authors proposed different detection limits in experiments involving multiplex PCR. These variations in the detection limit of this technique and the lack of amplification in the majority of cases when DNA concentrations less than 10^3 CFU/mL are used, can be linked to DNA purity and/or presence of inhibitors within the samples or microorganism culture media, which might cause false negative results (Gandra et al. 2016).

A fact already confirmed by Tamarapu et al. (2001) indicates that when the same primers are used in multiplex and uniplex PCR, in the latest case the detection limit was found to move from 10^2 to 10^1. The study of Gandra et al. (2016) showed that the detection limit was 10^3 CFU/mL when individual target (either *S. aureus* or *Yersinia enterocolitica*) was used. However, the detection limit became 10^4 CFU/mL when both bacteria were used together in multiplex PCR. The authors stated that the difference in the detection limit can be related to the reaction itself (Gandra et al. 2016). Increasing the amount of transgenic material in the samples leads to consistent increase in the DNA bands intensity (Datukishvili et al. 2015). Furthermore, Ramesh et al. (2002) confirmed the effect of the primer numbers on the detection limit. In the current study, the primers targeting the *nuc* gene of *S. aureus* were successful in amplifying this gene when only one or two bacteria were present in the same tube, yet the multiplex PCR efficiency decreased in amplifying that gene when three bacteria existed together.

Overall, the primers designed in this study worked successfully in amplifying and detecting three different targets related to three different bacteria. This might be due to using the software program MFEprimer-3.0 (Wang et al. 2019) for primers quality control. This software has been developed and used for checking the presence of primers dimers, hairpins, non-specific amplicons, single nucleotide polymorphisms (SNPs) within the binding sites, as well as other important parameters (Shen et al. 2010; Qu and Zhang 2015).

Conclusions

To conclude, the present study highlighted the importance of using the in silico software programs, such as MFEprimer-3.0, for primers quality control. Here, multiplex PCR was proved to be specific, sensitive, fast, accurate, and cost-effective molecular diagnostic method for identification and differentiation of three clinically prevalent bacteria, including *K. pneumoniae, S. aureus*, and *P. aeruginosa* associated with ovine respiratory infections. This can efficiently support control and treatment of sheep respiratory diseases. This in turn, would increase the economy of the animals' owners and wellbeing of the animals.

Data availability Available upon request.

Declarations

Consent to participate All authors have agreed to participate.

Consent for publication All authors have agreed to publish.

Conflicts of interest/Competing interests None.

References

Aziz TA, Lafta IJ (2021) Isolation and antimicrobial resistance of *Staphylococcus* spp., enteric bacteria and *Pseudomonas* spp. associated with respiratory tract infections of sheep. Iraqi J Vet Sci. 35 (Supplement III): 53–58. Proceedings of the 13th (2nd International) Scientific Conference, College of Veterinary Medicine, University of Baghdad. https://doi.org/10.33899/ijvs.2021.131098.1917

Azizi SH, Korani FS, Oryan A (2013) Pneumonia in slaughtered sheep in South-Western Iran: pathological characteristics and aerobic bacterial aetiology. Vet Ital 49(1):109–118. https://doi.org/10.1155/2019/5169040

Bangar YC, Pachpute ST, Nimase RG (2016) The survival analysis of the potential risk factors affecting lamb mortality in Deccani sheep. J Dairy Vet Anim Res 4(2):266–270. https://doi.org/10.15406/jdvar.2016.04.00114

Bird VY, Chastain-Gross R, Sutkowski R, Bird VG, Vyas P, Joseph R (2017) *Pseudomonas aeruginosa* as an etiologic agent of nephrolithiasis in deep water divers. J Endourol Case Rep 3(1):4–6. https://doi.org/10.1089/cren.2016.0117

Chakraborty S, Kumar A, Tiwari R, Rahal A, Malik Y (2014) Advances in diagnosis of respiratory diseases of small ruminants. Vet Med Int 508304. https://doi.org/10.1155/2014/508304

Dahl MO, Hamdoon OK, Abdulmonem ON (2021) Epidemiological analysis for medical records of veterinary teaching hospital, University of Mosul during 2017 to 2019. Iraqi J Vet Sci 35(3):541–548. https://doi.org/10.33899/ijvs.2020.127141.1468

Daniel JA, Held JE, Brake DG, Wulf WB (2006) Epperson, evaluation of the prevalence and onset of lung lesions and their impact on growth of lambs. Amer J Vet Res 67(5):890–894. https://doi.org/10.2460/ajvr.67.5.890

Dapgh AN, Hakim AS, Abouelhag HA, Abdou AM, Elgabry EA (2019) Detection of virulence and multidrug resistance operons in *Pseudomonas aeruginosa* isolated from Egyptian Baladi sheep and goat. Vet. World. 12(10):1524–1528. https://doi.org/10.14202/vetworld.2019.1524-1528

Datukishvili N, Kutateladze T, Gabriadze I, Bitskinashvili K, Vishnepolsky B (2015) New multiplex PCR methods for rapid screening of genetically modified organisms in foods. Frontiers in Microbiol 6:757. https://doi.org/10.3389/fmicb.2015.00757

Deb R, Chakraborty S (2012) Trends in veterinary diagnostics. J Vet Sci Technol 3:e103. https://doi.org/10.4172/2157-7579.1000e103

Dhama K, Chakraborty S, Kapoor S, Tiwari R, Kumar A, Deb R, Rajagunalan S, Singh R, Vora K, Natesan S (2013a) One world, one health-veterinary perspectives. Adv Ani Vet Sci 1(1):5–13 http://www.nexusacademicpublishers.com/uploads/files/20130420082020.pdf

Dhama K, Verma AK, Tiwari R, Chakraborty S, Vora K, Kapoor S, Deb R, Karthik K, Singh R, Munir M, Natesan S (2013b) A perspective on applications of geographical information system (GIS), an advanced tracking tool for disease surveillance and monitoring in veterinary epidemiology. Adv Ani Vet Sci 1(1):14–24 http://www.nexusacademicpublishers.com/uploads/files/Nexus_33.pdf

Dhama K, Karthik K, Chakraborty S, Tiwari R, Kapoor S, Kumar A, Thomas P (2014) Loop-mediated isothermal amplification of DNA, (LAMP)-a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. Pakis J Biol Sci 17(2):51–166. https://doi.org/10.3923/pjbs.2014.151.166

Downes FP, Ito H (2001) Compendium of methods for the microbiological examination of foods. 4.ed. Washington: American Public Health Association- APHA, 676 p

Gandra EA, Silva JA, Fernandez MA, da Silva WP (2016) Detection by multiplex PCR of *Staphylococcus aureus*, *S. intermedius* and *S. hyicus* in artificially contaminated milk. Ciencia Rural Microbiol 46(8):1418–1423. https://doi.org/10.1590/0103-8478cr20151391

Hu L, Han B, Tong Q, Xiao H, Cao D (2020) Detection of eight respiratory bacterial pathogens based on multiplex real-time PCR with fluorescence melting curve analysis. Canad J Infect Dis Med Microbiol 2697230. https://doi.org/10.1155/2020/2697230

Kumar R, Katoch RC, Dhar P (2000) Bacteriological studies on pneumonic gaddi sheep of Himachal Pradesh. Indian Vet J 77(10):846–848

Kumar A, Verma AK, Gangwar NK, Rahal A (2012) Isolation, characterization and antibiogram of *mycoplasma bovis* in sheep pneumonia. Asian J Animal and Vet Adv 7(2):149–157. https://doi.org/10.3923/ajava.2012.149.157

Kumar A, Tikoo SK, Malik P, Kumar AT (2014) Respiratory diseases of small ruminants. Vet Med Int 373642. https://doi.org/10.1155/2014/373642

Munir M, Zohari S, Berg M (2013) Current advances in molecular diagnosis and vaccines for Peste des Petits ruminants. Springer Briefs Animal Science 105–133. https://doi.org/10.1007/978-3-642-31451-3_6

Okolie CE, Wooldridge GK, Turner DP, Cockayne A, James R (2015a) Development of a heptaplex PCR assay for identification of *Staphylococcus aureus* and CoNS with simultaneous detection of virulence and antibiotic resistance genes. BMC Microbiol 15:1471–2180. https://doi.org/10.1186/s12866-015-0490-9

Okolie CE, Wooldridge KG, Turner DP, Cockayne A, James R (2015b) Development of a new pentaplex real-time PCR assay for the identification of poly-microbial specimens containing *Staphylococcus aureus* and other staphylococci, with simultaneous detection of staphylococcal virulence and methicillin resistance markers. Mol Cell Probes 29:315–322. https://doi.org/10.1016/j.mcp.2015.03.002

Phuektes P, Mansell PD, Browning GF (2001) Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. J Dairy Sci 84:1140–1148

Qu W, Zhang C (2015) Selecting specific PCR primers with MFEprimer. In: Basu C (ed) PCR Prmier Design, Methods in Molecular Biology, vol 1275. Springer Science, Business Media, New York

Ramesh A, Padmapriya BP, Chrashekar A, Varadaraj MC (2002) Application of a convenient DNA extraction method and multiplex PCR for the direct detection of *Staphylococcus aureus* and *Yersinia enterocolitica* in milk samples. Mol Cell Probes 16(4):307–314. https://doi.org/10.1006/mcpr.2002.0428

Saleh NS, Allam TS (2014) Pneumonia in sheep: bacteriological and clinicopathological studies. Amer J Res Commun 2(11):73–88

Scott PR (2011) Treatment and control of respiratory disease in sheep. Vet Clin North America-Food Animal Practice 27(1):175–186. https://doi.org/10.1016/j.cvfa.2010.10.016

Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, Hang X, Wang X, Zhao D, Zhang C (2010) MPprimer: a program for reliable multiplex PCR primer design. BMC Bioinform 11:143. https://doi.org/10.1186/1471-2105-11-143

Tamarapu S, McKillip JL, Drake M (2001) Development of a multiplex polymerase chain reaction assay for detection and differentiation of *Staphylococcus aureus* in dairy products. J Food Protec 64(5):664–668. https://doi.org/10.4315/0362-028X-64.5.664

Thompson M (2019) Respiratory diseases in sheep. Vet Practice Today 7(4)

Wang K, Li H, Xu Y, Shao Q, Yi J, Wang R, Cai W, Hang X, Zhang C, Cai H, Qu W (2019) MFEprimer-3.0: quality control for PCR primers. Nucleic Acids Res 47:W610–W613. https://doi.org/10.1093/nar/gkz351

Yang Y, Su X, Yung Y, Kang C, Li Y, Zhang W, Zhong X (2007) Detection of *Staphylococcus aureus* in dairy products by polymerase chain reaction assay. Agric Sci China 6:857–862. https://doi.org/10.1016/S1671-2927(07)60122-9

Yang J, Zhao HL, Ran LY, Li CY, Zhang XY, Su HN, Shi M, Zhou BC, Chen XL, Zhang YZ (2015) Mechanistic insights into elastin degradation by pseudolysin, the major virulence factor of the opportunistic pathogen *Pseudomonas aeruginosa*. Sci Rep 23(5):9936. https://doi.org/10.1038/srep09936

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