Journal of the American Association for Laboratory Animal Science
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Vol 61, No 5
September 2022
Pages 441–447

Single-tube Multiplex Nested PCR System for Efficient Detection of Pathogenic Microorganisms in SPF Rodents

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PCR testing is increasingly important for microbial control in SPF facilities. However, most current PCR methods are time-consuming and require compromise between high sensitivity and high multiplexing. We developed a one-tube multiplex nested PCR strategy (MN-PCR) for simultaneous direct (that is, without culturing) detection of multiple pathogens. We first aligned sequences for the 16S rDNA genes of selected target bacteria and a panel of closely related organisms. From these data, we designed a pair of universal primers and multiple sets of species-specific PCR primers to amplify the target sequences; the universal primers were modified to include various degenerate bases and locked nucleic acids. In a single tube, 16S rDNA sequences were amplified by using the nested PCR primers under high temperature (that is, above 65 °C) during the first stage of the MN-PCR procedure, when the target-species–specific PCR primers do not support amplification due to their short length. In addition, the concentration of the nested PCR primers during the first stage was adjusted to ensure that they were consumed and did not yield visible bands themselves. During the second stage, the enriched 16S rDNA sequences then served as templates for amplification of the species-specific fragments by using the multiple PCR primers at low annealing temperatures (that is, below 60 °C). The results showed that our MN-PCR method detected as little as 1 fg of target bacterial DNA in a 20-µL reaction volume, whereas conventional multiplex PCR detected a minimum of 1 pg only. Compared with traditional multiplex PCR assays, our MN-PCR system is an effective and efficient culture-free process.

Abbreviations: MN-PCR, multiplex nested PCR system

This article contains supplemental materials online.

DOI: 10.30802/AALAS-JAALAS-21-000117

Introduction

Rodent research colonies harbor numerous adventitious agents that can cause clinical disease and interfere with research results. To manage these microbes, barrier housing practices and health monitoring programs have been developed to exclude these pathogens and efficiently detect them when they are inadvertently introduced into the colony. Therefore, reliable detection of microbial agents is essential for meaningful health monitoring in research animal facilities. Accurate and rapid detection methods are crucial for monitoring and identifying microorganisms in samples.[5,24] Traditional methods of pathogenic microbial detection include culture and separation; biochemical and serologic detection; and immunology and nucleic acid detection.[25] Most of these methods require bacterial culturing to detect pathogens through the analysis of biochemical indicators and are often time-consuming and labor-intensive.[4,7] In addition, in vitro culture supports the quantification of only the dominant growing microorganisms; however, many microorganisms cannot be cultured routinely. Detection of these nonculturable microbes has become a driving force for the development of culture-free methods.[10,26,27]

Pathogenic diagnostic technology is rapidly developing, especially molecular diagnostic techniques such as PCR amplification. Due to their precision, rapidity, and sensitivity, PCR methods are widely used to detect pathogenic microorganisms. Because PCR-based analyses are much less time-consuming than other conventional techniques, numerous derivative PCR methods have arisen recently,[19] including quantitative PCR, multiplex PCR, RT-PCR, extreme PCR,[3] co-amplification at lower denaturation temperature (COLD) PCR,[15] heat pulse–extension (HPE) PCR,[16] nanoparticle PCR,[11] loop-mediated isothermal amplification (LAMP),[9] droplet digital (dd) PCR,[23] and high-throughput next-generation sequencing (NGS) systems.[6] However, for most of these strategies, effectively balancing high throughput and accuracy is difficult.

To mitigate the shortcomings associated with current PCR-based methods, we developed a single-tube nested multiplex PCR technique (MN-PCR method) for target pathogen detection. Our MN-PCR method offers the advantages of both the sensitivity of nested PCR testing and the high throughput of multiplex PCR assays. As an example, we used our MN-PCR method to detect 4 target bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Rodentibacter pneumotropicus*) in samples from research mice.[1] We found that MN-PCR analysis is more sensitive and convenient than multiplex PCR assays for the detection of bacteria in clinical samples from mice.

Submitted: 19 Oct 2021. Revision requested: 18 Jan 2022. Accepted: 21 Apr 2022.
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Materials and Methods

Animals. Sixty male C57BL/6 J mice (25 to 30 g, 8 to 10 wk of age) were obtained from Shanghai SLAC Laboratory Animal Company (Shanghai, China). All mice were kept for a 12:12-h light/dark cycle and free to obtain water and food (XieTong, Jiangsu, China). The microbial infection status of the mice was determined by a third-party testing organization (Xishan Biotechnology, Suzhou, China). Animal use conformed to the *Guideline for the Care and Use of Laboratory Animals* established by the Chinese Council on Animal Care and were approved by the Shanghai Jiao Tong University Ethics Committee.

Bacterial strains and clinical samples. Strains of *P. aeruginosa* (ATCC9027), *R. pneumotropicus* (NCTC8141), *S. aureus* (ATCC6538) and *K. pneumoniae* (ATCC46117) were obtained from Guangdong Microbial Culture Collection Center (Guangdong, China). Strains of *Streptococcus pneumoniae* (ATCC49619), *Staphylococcus epidermidis* (CMCC50115), *Pasteurella multocida* (ATCC12945), *Salmonella typhimurium* (SL1344), and *Escherichia coli* (CMCC44102) were donated by the Shanghai Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Beijing, China).

Clinical samples were collected from mice infected with *K. pneumoniae*, *S. aureus*, *R. pneumotropicus*, or *P. aeruginosa*. For all samples, sterile cotton swabs were used to collect secretions from the mouths and throats of mice. Swabs were soaked in 1.5 mL of sterile water, 750 µL of the resulting solution was centrifuged at 7,100 × *g* for 2 min, and the supernatant discarded. The bacterial pellet was suspended in 15 µL of TE buffer; 1.5 µL of this suspension was used as a reaction template.

Bacterial culture and DNA isolation. A single colony of the Bacteria from a blood agar plate was inoculated into 5 mL of LB medium. For clinical samples, throat swabs from mice were immersed in 1.5 mL of sterile water for 10 min, which was then divided equally into two 750-µL portions. One was prepared as described below in the *Preparation of clinical samples* section, and the other was added to 5 mL of LB medium. The cultures were grown at 37 °C for 18 to 24 h with shaking. Template DNA was isolated from bacterial cultures by using a Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions.

MN-PCR primers for the detection of laboratory animal pathogens. We designed a pair of universal primers (UP-F/UP-R) that bound to conserved regions of the 16S rDNA sequence and that yielded a 1500-bp PCR product. Using the variable regions of the 16S rDNA sequence, we designed 4 pairs of primers specific to our selected target bacteria (*K. pneumoniae*, KP-F/KP-R; *S. aureus*, SA-F/SA-R; *R. pneumotropicus*, RP-F/RP-R; and *P. aeruginosa*, PA-F/PA-R; Figure 1). Each universal primer was required to be at least 27 bp, with an annealing temperature above 65 °C; species-specific primers had to be 20 bp or shorter, with an annealing temperature below 56 °C.

Primer	Sequence (5′ to 3′, as synthesized)	Expected amplicon
UP-F	acc ttg tta cga ctt cAc ccc art Cat	1500 bp
UP-R	gag ttt gat cmt ggc tcA grw tga aCg c	
KP-F	tca tcg att gac gtt acc ct	139 bp
KP-R	ttt cac atc cga ctt gac ag	
SA-F	aac tct aga gat aga gcc	342 bp
SA-R	tag cga ttc cag ctt cat g	
RP-F	gtt tgg tta ata gcc aag	428 bp
RP-R	cca gac ggt cga ttt atc a	
PA-F	cta ctg agc tag agt acg	662 bp
PA-R	tcc gga cta cga tcg gt	

Figure 1. Sequences of primers used in the current study. Uppercase letters indicate linked nucleic acids.

Multiplex PCR detection for four target pathogens. The common multiplex PCR reaction system is 20 µ, including 10 µL of 2× Taq Master Mix (Vazyme,Nanjing, China) and 0.15 µM of the specific primer (KP-F, KP-R, SA-F, SA-R, RP-F, RP-R, PA-F and PA-R). Reaction conditions were denaturation at 95 °C for 5 min followed by 15 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 10 min.

Optimization of reaction conditions for MN-PCR analysis. We then optimized the reaction conditions for MN-PCR analysis (MyCycler, Bio-Rad, Hercules, CA) in terms of annealing temperature, primer concentration, and number of cycles. For the current study, the optimal annealing temperature range for the universal primers (UP-F/UP-R) was 63 to 66 °C, whereas the species-specific primers were productive between 50 and 55 °C, with no product amplified above 60 °C. Thus, using temperatures above 60 °C during the first stage ensured that only the universal primers supported amplification; the specific primers yielded product only during the second (lower temperature) stage of the MN-PCR process.

The optimal concentration of the universal primers was 0.005 to 0.01 µM. At 0.01 µM, the universal primers generated ample reaction products but did not yield visible primer-specific bands in the electrophoresis gel. Thus, residual universal primers did not interfere with amplification during the second stage of the MN-PCR process.

To optimize the number of cycles for each of the 2 stages of the MN-PCR procedure, we compared 3 configurations of 40 total cycles: 1) enrichment phase, 5 cycles; detection phase, 35 cycles; 2) enrichment phase, 10 cycles; detection phase, 30 cycles; and 3) enrichment phase, 15 cycles; detection phase, 25 cycles. The greatest amplification efficiency was obtained by using 15 cycles for the enrichment phase and 25 cycles for the detection phase.

The optimized reaction conditions included a reaction volume of 20 µL that contained 2× Taq Master Mix (Vazyme, Nanjing, China); 0.01 µM each of UP-F and UP-R primers, and 0.15 µM of each species-specific primer (KP-F, KP-R, SA-F, SA-R, RP-F, RP-R, PA-F and PA-R). Reactions were run at 95 °C for 5 min; followed by 15 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; and then for 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

Simultaneous detection of multiple organisms by MN-PCR. To detect multiple target species by using MN-PCR analysis, the reaction volume was 20 µL, containing 2× Taq Master Mix (1 U of Taq DNA polymerase, 3 mM $MgCl_2$, and 400 µM of each dNTP), 0.15 µM of each specific primer (KP-F, KP-R, SA-F, SA-R, RP-F, RP-R, PA-F and PA-R; Figure 1), and 1 ng template DNA. Amplification conditions were 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

Sequence analysis. To verify the accuracy of the MN-PCR method for the analysis of clinical samples, we isolated the amplified PCR products from agarose gels by using a QIAquick Gel Extraction Kit (Qiagen, Germantown, MD) and performed bidirectional sequencing of the products. Sequences were submitted to the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned by using the BLAST program.

Results

Developmental strategy for MN-PCR assay. When developing our MN-PCR method, we implemented several strategies

that addressed primer design sites, primer modification and concentration, and annealing temperatures to combine the benefits of both nested PCR assays and multiplex PCR analysis into a single-tube process. First, we aligned the 16S rDNA genes from the 4 target bacteria we selected and a panel of closely related bacteria. From the obtained characteristic sequences, we designed a set of universal PCR primers according to 16s rDNA regions conserved among the target bacteria. We used degenerate bases and locked nucleic acids to avoid amplification at 16s rDNA sites corresponding to closely related bacteria and species-differential sites and to increase the primer annealing temperature. Then, within the product amplified by using the universal primers, we designed species-specific PCR primers according to variable regions in the 16s rDNA sequence of each selected target bacterial species (Figure 2).

Due to the different design strategies for the 2 types of primers, the first stage of MN-PCR analysis used a higher annealing temperature to enrich amplification of the full-length product from the universal primers and to ensure that the species-specific primers did not bind. In addition, we reduced the concentration of the universal primers to minimize their interference during the second stage of the MN-PCR process. Furthermore, we optimized the number of amplification cycles according to the 2 stages of our MN-PCR method.

We tested 4 bacteria common in research animals—*S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *R. pneumotropicus*—as an example to verify the utility of the MN-PCR method. These 4 bacteria are the most prevalent opportunistic pathogens in experimental facilities worldwide,[20] and are associated with various clinical manifestations, including eye, genital tract, and respiratory infections. Furthermore, these bacterial species must be excluded from SPF mouse facilities according to Chinese national standards (GB14922.2–2011).

Assessment of specificity and sensitivity of MN-PCR method for pathogen detection. We used several bacteria closely related to the target species to verify the specificity of our MN-PCR method. The results showed that MN-PCR amplification specifically generated bands from each target bacterial template but not from related control bacteria (Figure 3). Subsequent results from clinical samples likewise showed that the MN-PCR method detected positive samples with high specificity (Supplementary Figure 4). Next, we compared the sensitivity of MN-PCR analysis with that of a multiplex PCR method. With regard to detection of the 4 target bacterial species, the MN-PCR

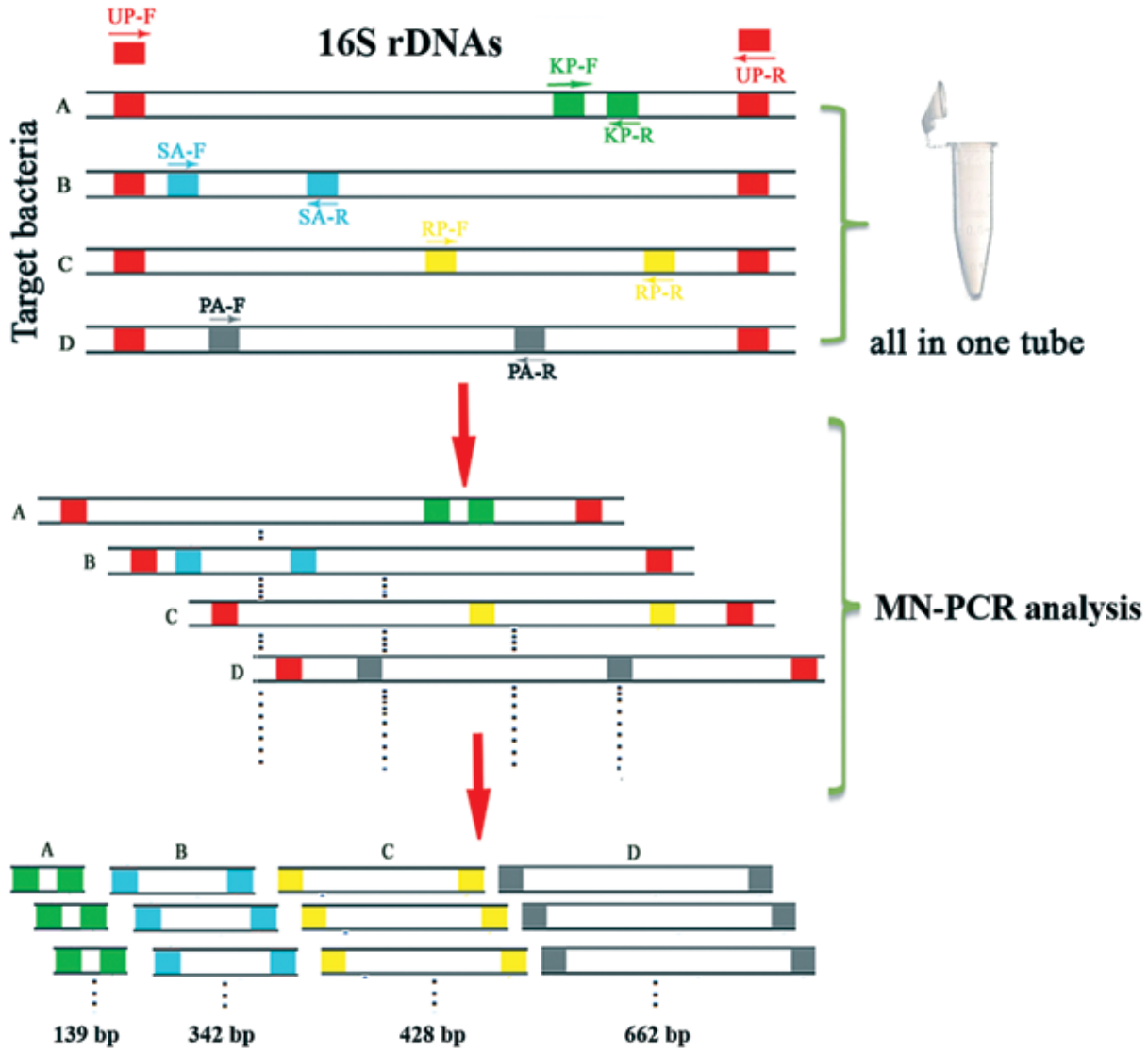

Figure 2. Schematic overview of MN-PCR analysis. Universal primers UP-F/UP-R (red) are designed to 16s rDNA regions conserved among the target bacteria; the specific primers KP-F/KP-R, SA-F/SA-R, RP-F/RP-R, PA-F/PA-R are from variable regions of the 16S rDNA sequence.

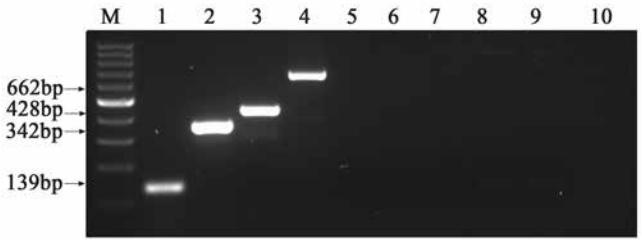

Figure 3. Verification of specificity MN-PCR assay . Lanes: 1, *Klebsiella pneumoniae* (139 bp); 2, *Staphylococcus aureus* (342 bp); 3, *Rodentibacter pneumotropicus* (428 bp); 4, *Psueudomonas aeruginosa* (662 bp); 5, *Streptococcus pneumoniae*; 6, *Staphylococcus epidermidis*; 7, *Pasteurella multocida*; 8, *Salmonella typhimurium*; 9, *Escherichia coli*; 10, blank.

procedure was 10 to 100 times more sensitive than the multiplex PCR method (Figure 5).

Simultaneous detection of 4 pathogens by MN-PCR analysis. We then evaluated whether MN-PCR analysis correctly detected target bacteria in mixed samples. MN-PCR templates were prepared by mixing the DNA of targeted bacteria in 2, 3, or 4 different combinations. In all cases, the bands of PCR products from both the single bacteria and the combinations were clear and could be distinguished from each other (Figure 6).

Next, we used template DNA representing various combinations of the 4 target pathogens to compare the efficiencies of the MN-PCR and multiplex PCR assays. Even at template concentrations below 1 fg per 20 µL, the MN-PCR method reliably detected the components of mixed samples, whereas multiplex PCR did not, even at concentrations of 1 pg per 20 µL (Figure 7).

Application of MN-PCR analysis to clinical samples. MN-PCR analysis showed high diagnostic sensitivity and specificity for the 2 types of clinical samples. In the first case, MN-PCR amplified 4 different bacteria that had been obtained from infected mouse samples and identified by a third party company. After sequencing, the amplified bands were aligned by using BLAST on the NCBI website (Data S1).

We also collected throat swab samples from 36 mice of unknown infection status and processed them by using MN-PCR analysis. The results showed that 10 of the 36 samples were positive for target bacterial species (*R. pneumotropicus*, $n = 9$; *P. aeruginosa*, $n = 1$; Figure 4). We then cultured these 10 positive samples and extracted DNA for sequencing and detection by other PCR methods (Table 1).

Discussion

Reliable detection of unwanted organisms is essential for meaningful health monitoring in research animal facilities. Currently, rodents are often housed in IVC systems that prevent airborne transmission of pathogens between cages. Traditionally, sentinel animals have been used as key method for health surveillance of research rodent colonies. However, this method requires using numerous live animals. Important drawbacks to sentinel monitoring include the poor transmission (and thus poor detection) of some pathogens through soiled bedding.[18] Recent advances in PCR-based surveillance methods,[2,21] including exhaust air dust (EAD) PCR analysis, present alternatives to the use of sentinel monitoring.

Samples collected from the exhaust plenums of IVC racks can be used as environmental samples for molecular detection

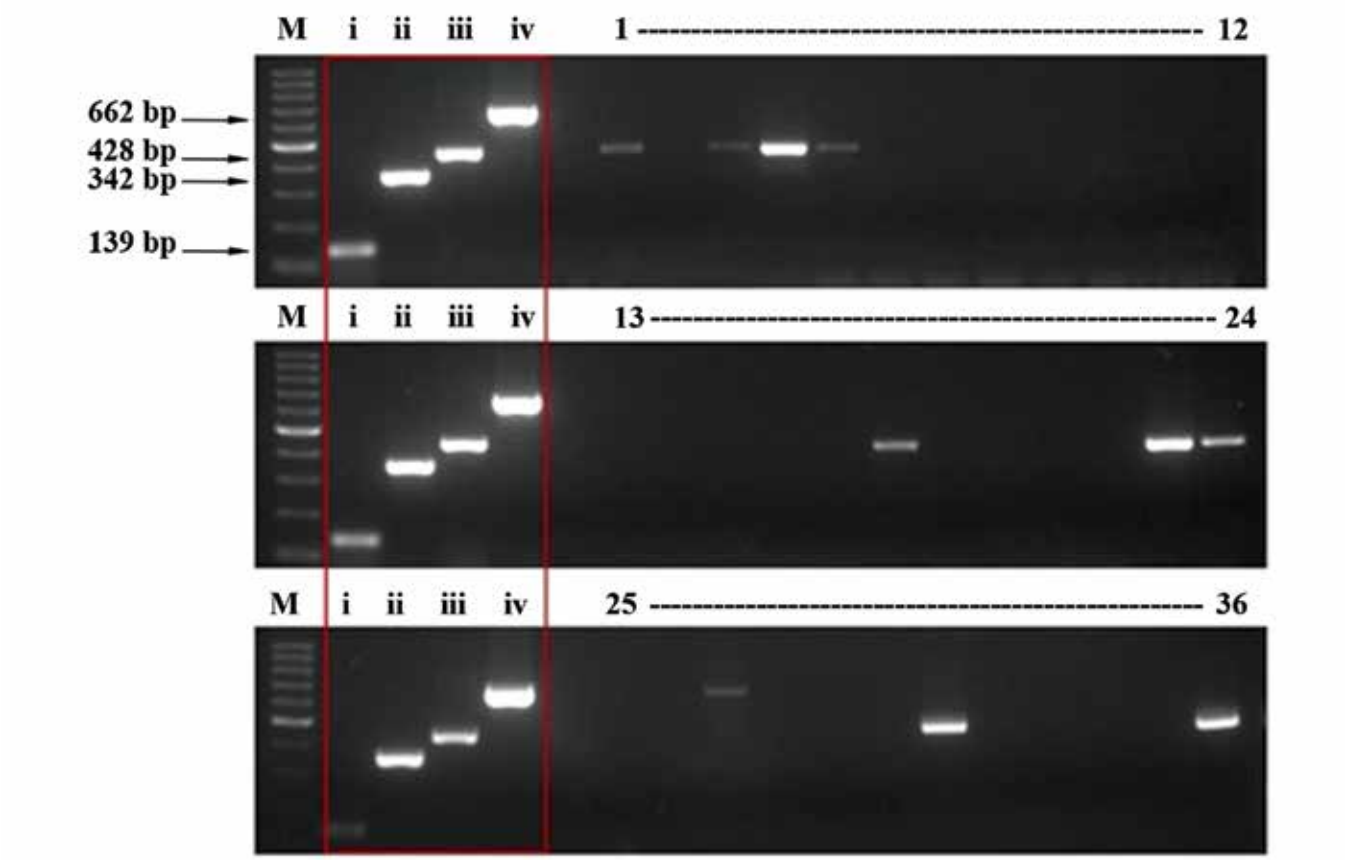

Figure 4. Detection of 4 target pathogens in 36 clinical samples from mice (throat swabs). Lanes: i-iv: Positive controls of *K. pneumonia; S. aureus; R. pneumotropicus*; *P. aeruginosa*, respectively. Lanes 1-36: the 36 clinical samples.

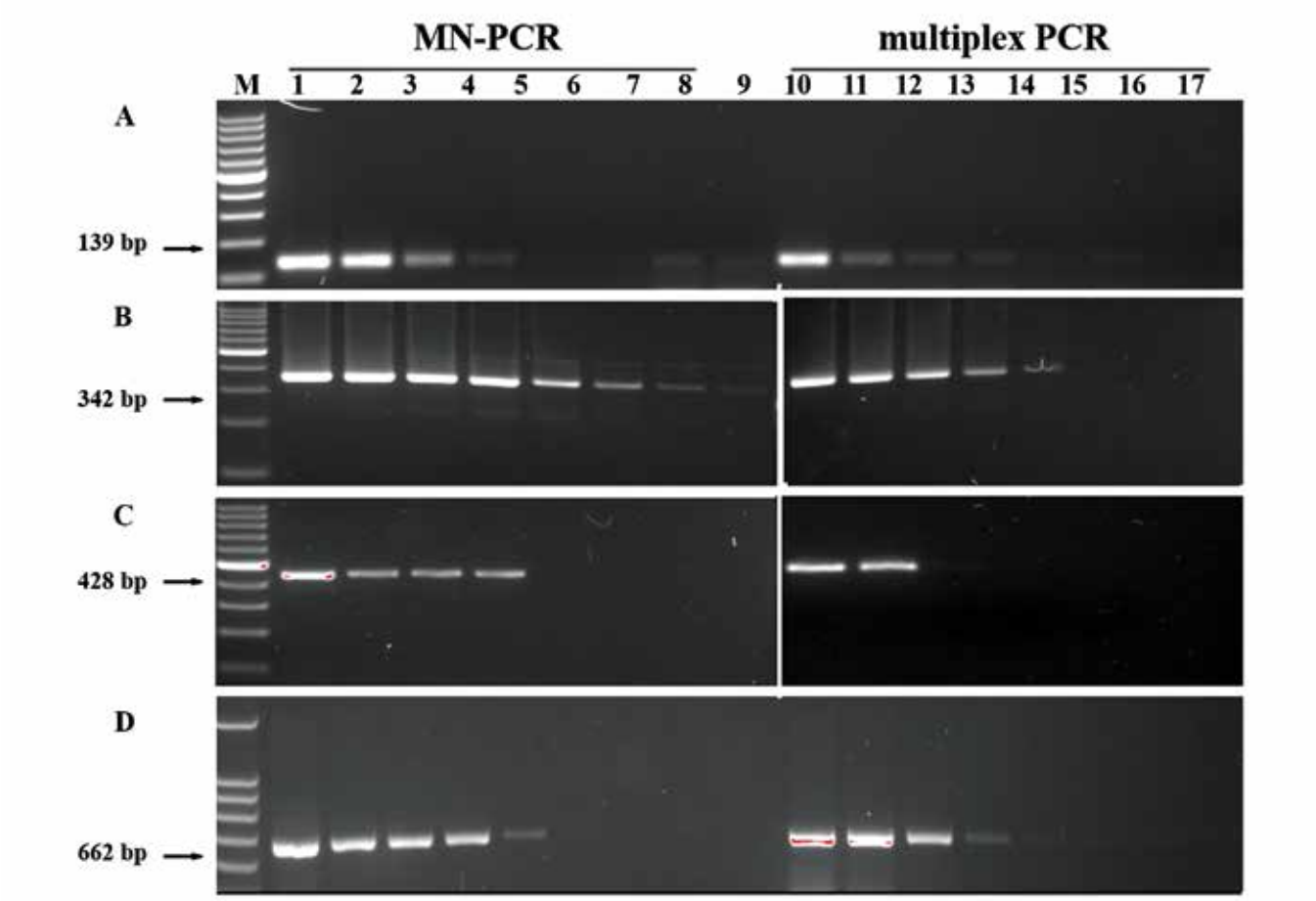

Figure 5. Sensitivity of MN-PCR assay compared with multiplex PCR assay in detecting a single template. (A) *Klebsiella pneumoniae.* (B) *Staphylococcus aureus.* (C) *Rodentibacter pneumotropicus.* (D) *Pseudomonas aeruginosa.* Lanes 1-8 and 10-17: bacterial genomic DNA Conc. in 20 µL reaction system with 1 pg, 100 fg, 10 fg, 1 fg, 0.10 fg, 0.01 fg, 0.001 fg, and 0.0001 fg, respectively; M, 100-bp DNA ladder.

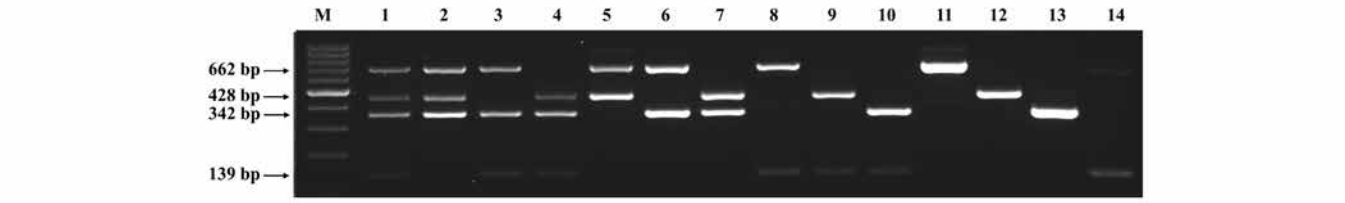

Figure 6. Simultaneous detection of 4 targeted bacteria by MN-PCR analysis. Lanes: 1, mixed template representing all 4 target bacterial species; lanes 2 through 4: mixed template representing various combinations of 3 target bacteria; lanes 5 through 10, mixed templates representing various combinations of 2 target bacteria; lanes 11 through 14, single-bacteria templates; M, 100-bp DNA ladder.

methods such as conventional PCR, real-time quantitative PCR, and multiplex PCR assays and sequencing approaches. However, most environmental methods are not particularly efficient: most of them detect only a single pathogen—not multiple pathogens concurrently—in a sample.[12-14] In contrast, multiplex PCR assays amplify multiple DNA fragments simultaneously, and this method has been successfully applied in many areas of DNA testing. However, multiplex PCR assays have several disadvantages. Due to the presence of multiple pathogens and differing pathogen abundance in samples, differences in amplification preference between products and interference between multiple primers may lead to the accumulation of nonspecific fragments. Balancing the level of multiplexing with detection accuracy is challenging with multiplex PCR assays and often leads to unknown specificity regarding clinical samples.[8,17,22]

To overcome disadvantages of multiplex PCR methods, researchers often use nested PCR strategies to enrich the desired template and then use multiple subsequent reactions to amplify specific bands. However, in addition to being time-consuming and laborious, this multistep process is prone to sample contamination. With all of these considerations in mind, we developed the MN-PCR technique to increase the sensitivity and specificity of pathogen detection in samples from mice. Our MN-PCR method takes advantage of both homologous and variant regions of the 16S rDNA genes in bacteria. To this end, we first generated a pair of universal primers to enrich a region of the 16S rDNA gene that was conserved among the 4 target bacteria we selected (that is, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *R. pneumotropicus*) and then we designed sets of species-specific primers for variable regions in the 16S rDNA sequences of the respective target species. In addition, by

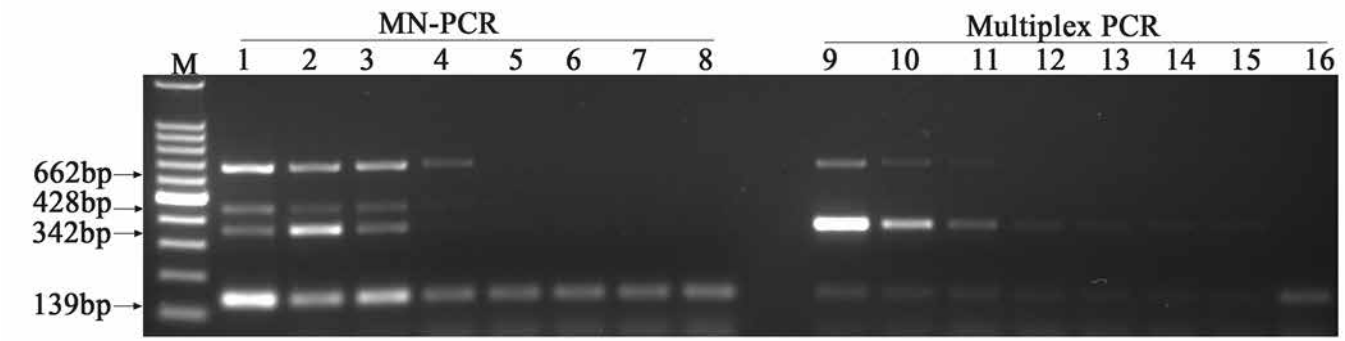

Figure 7. Sensitivity comparison of MN-PCR and multiplex PCR using 4 mixed templates. Lanes 1 through 8, template DNA mass (in 20 µL) of 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg, 0.01 fg, 0.001 fg, and 0.0001 fg, respectively; lanes 9 through 16, same as lanes 1 through 8. M, DNA molecular size marker (100 bp DNA ladder; Takara)

Table 1. BLAST analysis of MN-PCR products from clinical samples

Description	Maximum score	Total score	Query cover	E value	% Identity	Accession no.
Klebsiella pneumoniae strain GXNN3 16S ribosomal RNA gene, partial sequence	255	255	100%	2E-64	100	KU936064.1
Staphylococcus aureus strain S1 16S ribosomal RNA gene, partial sequence	656	656	100%	0	100	MK881023.1
Pseudomonas aeruginosa gene for 16S ribosomal RNA, partial sequence, strain: VR5	1223	1223	100%	0	100	AB862140.1
Rodentibacter pneumotropicus strain NCTC 8141 16S ribosomal RNA, partial sequence	817	817	100%	0	100	NR042887.1

incorporating multiple strategies into primer design, annealing temperatures, and the number of amplification cycles, we were able to incorporate both the universal and species-specific components into a single-tube tube method. Our resulting MN-PCR method is much more sensitive than multiplex PCR analysis and minimizes the trade-off between sensitivity and multiplicity in the detection of microorganisms in samples.

In addition to providing improved sensitivity, the MN-PCR system uses a nested PCR strategy, which enables direct detection of target pathogens from clinical samples without culturing. This feature is especially helpful in detecting microorganisms that are difficult to culture. Because they contain animal somatic cells, bacteria, viruses, and other substances, fecal samples and specimens collected by swabbing are complex. When this complexity is coupled with low abundance of the target bacteria, multiplex PCR methods typically cannot detect these pathogens without enrichment through culturing. Thus, multiplex PCR analysis is a time-consuming and laborious process, with associated risks of contamination and potential false-positive results. In comparison, the MN-PCR method can directly detect target bacteria from sample swabs or fecal specimens without the need for microbial culture and thus has high sensitivity and accuracy compared with multiplex PCR.

Rodent health-monitoring programs based on sampling exhaust air dust from IVC systems have enhanced and even replaced traditional sentinels for some pathogens. We believe that MN-PCR analysis is fast and convenient, saves time and labor, and likely can be used with samples of exhaust air dust. In addition, MN-PCR techniques have significant potential beyond exhaust air dust PCR analysis, with diverse possible applications in monitoring environmental water sources, establishing clinical diagnoses, and maintaining food safety, among others.

Supplementary Materials

Data S1. Sequence blast results of clinical samples. The bands amplified by MN-PCR were 16SrDNA sequences of 4 kinds of targeted bacteria.

Acknowledgments

This work was supported by the interdisciplinary program of Shanghai Jiao Tong University (YG2017MS07), the Natural Science Foundation of Shanghai (20ZR142700), and the Shanghai Jiao Tong University Decision-making Consultation Project (JCZXSJA-02). The authors declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

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