International Journal of Infectious Diseases 81 (2019) 73–80

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

A multiplex PCR assay for detection of *Vibrio vulnificus*, *Aeromonas hydrophila*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* from the isolates of patients with necrotizing fasciitis

Yao-Hung Tsai[a,b,*], Po-Han Chen[a,b], Pei-An Yu[a], Chi-Lung Chen[a,b], Liang Tseng Kuo[a,b], Kuo-Chin Huang[a,b]

[a] Department of Orthopedic Surgery, Chang Gung Memorial Hospital at Chia-Yi, Taiwan, Republic of China
[b] College of Medicine, Chang Gung University at Taoyuan, Taiwan, Republic of China

ARTICLE INFO

Article history:
Received 16 October 2018
Received in revised form 11 January 2019
Accepted 19 January 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Necrotizing fasciitis
Multiplex PCR
Diagnostics
Assay

ABSTRACT

Background: *Vibrio vulnificus*, *Aeromonas hydrophila*, methicillin-resistant *Staphylococcus aureus* (MRSA), group A *Streptococcus*, and group B *Streptococcus* are commonly detected causative agents of necrotizing fasciitis (NF) in Chia-Yi Chang Gung Memorial Hospital. The aim of this study was to develop and evaluate a multiplex PCR method for the simultaneous detection of five of the most important human pathogens involved in NF by using a novel combination of species-specific genes.
Methods: The samples used were collected from 99 patients with surgically confirmed NF of the extremities who were hospitalized consecutively between June 2015 and November 2017. Two sets of blood and tissue samples were collected from all patients; one set was sent to a microbiology laboratory for bacterial identification and the other set was sent to an immunohistochemistry laboratory for PCR amplification.
Results: The multiplex PCR results for the blood samples showed negative findings. The multiplex PCR results for the tissue specimens showed 28 positive findings. Fourteen (87.5%) of the 16 *V. vulnificus* culture-positive tissue specimens, six (75%) of the eight *A. hydrophila* culture-positive tissue specimens, and four (100%) of the four MRSA culture-positive tissue specimens were positive by PCR. Similarly, two (100%) of the group A *Streptococcus* and two (100%) of the group B *Streptococcus* were PCR-positive.
Conclusions: The accuracy rate of the multiplex PCR presenting positive results in these culture-positive tissue samples was 87.5% (28/32). This suggests that multiplex PCR of tissue specimens may be a useful and rapid diagnostic tool for the detection of these lethal microorganisms in patients with NF.

Introduction

Necrotizing fasciitis (NF) is a life-threatening deep soft tissue infection characterized by rapidly progressive necrosis of the fascia and the adjacent soft tissues. NF of the extremities is associated with high mortality rates, and immediate surgical intervention is required upon detection of NF infections (Sarani et al., 2009; Angoules et al., 2007; Anaya et al., 2005; Bellapianta et al., 2009).

Type I NF is a polymicrobial infection, a mixed infection with aerobic and anaerobic bacteria, and has been reported in some studies to cause nearly 80% of NF. However, in the past decade, the incidence of monomicrobial NF has been reported to be between 60% and 70.6% (Morgan, 2010; Anaya et al., 2009; Bair et al., 2009; Frazee et al., 2008; Oncul et al., 2008; Tsai et al., 2012).

In Europe and the USA, Gram-positive aerobic bacteria are the major causative organisms of NF. NF caused by β-hemolytic *Streptococcus* spp. and *Staphylococcus aureus* is often associated with a high mortality rate in Western countries (Frazee et al., 2008; Oncul et al., 2008; Tsai et al., 2012; Lee et al., 2007; Miller et al., 2005). Previous studies revealed that the Gram-negative aerobic pathogens, such as *Vibrio vulnificus*, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, and *Escherichia coli*, were the most frequently

* Corresponding author at: Department of Orthopedic Surgery, Chang Gung Memorial Hospital at Chia-Yi, No. 6, West Sec, Chia-Pu Road, Putz City, Cha-I, 613, Taiwan, Republic of China.
E-mail address: orma2244@adm.cgmh.org.tw (Y.-H. Tsai).

https://doi.org/10.1016/j.ijid.2019.01.037

isolated microorganisms in southwest Taiwan, and these were found to cause more rapid, fulminant, and deadly NF than Gram-positive pathogens (Tsai et al., 2012; Tsai et al., 2015). Early diagnosis and bacterial identification are essential for adequate surgical treatment and appropriate antibiotic administration in patients with NF. The clinical signs and symptoms of NF caused by Gram-positive and Gram-negative microorganisms are characteristically indistinguishable at the time of presentation. Moreover, it takes 3–4 days to obtain the results of microbiological analysis and antimicrobial sensitivity testing of the specimens. The improved accuracy and reliability of diagnosis for NF through the identification of group A *Streptococcus* or methicillin-resistant *S. aureus* (MRSA) by PCR has been described (Bruun et al., 2013; Zhu et al., 2016; Louie et al., 1998; Changchien et al., 2011; Miao et al., 2017).

In recent years, multiplex PCR assays have been developed, which contain more than one pair of primers to simultaneously detect different microorganisms in a single reaction. These assays may prove to be a relatively easy, inexpensive, and more rapid means of identifying specific pathogens. Multiplex PCR methods for the detection of *Vibrio* spp., such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, have been reported previously (Garrido-Maestu et al., 2014; Wei et al., 2014; Kim et al., 2015). The identification of *Aeromonas* spp., such as *A. hydrophila*, *A. veronii*, and *A. caviae*, is also possible using multiplex PCR methods (Rather et al., 2014; Chen et al., 2014). However, the isolates for these tests have usually been obtained from seafood samples or laboratory reference strains, and only seldom directly from human blood or tissue samples.

We designed a novel multiplex PCR method to simultaneously detect the following six pathogens: *V. vulnificus*, *V. cholerae* non-O1, *A. hydrophila*, MRSA, group A *Streptococcus* (*S. pyogenes*), and group B *Streptococcus* (*S. agalactiae*). These pathogens have been identified as the common causative agents of NF at Chia-Yi NF at Chang Gung Memorial Hospital. The PCR protocols that have been developed use several combinations of unique primers for the detection of molecular biomarkers found in these five pathogens.

The aim of this study was to develop and evaluate an open and straightforward multiplex PCR method for the simultaneous detection of the five most important human pathogens in NF. This would enable reliable and comprehensive diagnostics by using a novel combination of species-specific genes.

Materials and methods

Study design and setting

A total of 99 patients with surgically confirmed NF of the extremities were hospitalized consecutively at Chang Gung Memorial Hospital between June 2015 and November 2017. Urgent operations for fasciotomy and excisional debridement were performed in cases where NF was diagnosed at the time of admission to the emergency room or at the time of consultation on the ward. The diagnosis of NF was based on clinical, surgical, or histopathological findings.

The study was approved by the Ethics Committee of Chang Gung Memorial Hospital. Blood specimens were obtained in the emergency room, whereas the tissue specimens were obtained upon initial surgical debridement. Two sets of blood and tissue samples were collected from all patients; one set was sent to a microbiology laboratory for bacterial identification, while the other was sent to the immunohistochemistry laboratory of the hospital for PCR amplification.

Microbiology laboratory procedures

The culture specimens were obtained from the infected wounds or the blood, and were identified by microbiological evaluation. Microorganism identification was conducted using standard phenotypic methods employed in clinical microbiology laboratories. Antimicrobial susceptibility testing was performed in the hospital microbiology laboratory using the standard disk diffusion test. These antimicrobial susceptibility tests were performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) and the results were interpreted according to the CLSI criteria for these microorganisms. The five microorganisms to be used as positive controls in the multiplex PCR assay were isolated in the microbiology laboratory.

Primer design for the six pathogenic genes

The six genes that were selected were specifically chosen to effectively differentiate the six microorganisms. Primers for the *rpoS* gene (RPOS-F and RPOS-R), a gene responsible for hemolytic and cytolytic activity, were used to target *V. vulnificus*. The *toxR* gene, which has been shown to be a transcriptional activator of the cholera toxin operon, was used to target *V. cholerae* (toxR-F and toxR-R). Primers for the *mecA* gene (mecA-F and mecA-R), were used to target MRSA. The *gcat* gene (primers gcat-F and gcat-R), which encodes collagenase, was used as the target for *A. hydrophila*. Primers for the *dnaseB* gene (dnaseB-F and dnaseB-R), which is responsible for pyrogenicity, were used to target group A *Streptococcus*. Primers GB-F and GB-R for the *GB* gene, which is responsible for pyrogenicity, were used to target group B *Streptococcus*.

The specificity of the designed primer sets was evaluated and determined by conventional single PCR using various pathogenic bacteria. Then according to this identification approach, six pairs of oligonucleotide primers were designed to simultaneously detect six different microorganisms by multiplex PCR. Table 1 lists the primers used for the amplification of these genes and the predicted sizes of the amplification products. These genes have previously been confirmed as marker genes for the identification of the six pathogens in the literature (Changchien et al., 2011; Miao et al., 2017; Garrido-Maestu et al., 2014; Wei et al., 2014; Kim et al., 2015; Rather et al., 2014; Chen et al., 2014; Shirvani et al., 2014; Slinger et al., 2011; Kim et al., 2008a; Chakraborty et al., 2000).

Table 1
Nucleotide sequences of primers used in this study.

Pathogen	Target gene	Primer and probe sequences	bp
Vibrio vulnificus	RPOS-F RPOS-R	CAGACTCGAACCATCCGTTT CCTTCTCTCCATCTCCACCA	217
Vibrio cholerae	toxR-F toxR-R	TCGAATTCTTTGGCTGCTGG CAGAGTCGAAATGGCTTGGG	130
MRSA	mecA-F mecA-R	AGGTACTGCTATCCACCCTC CACCTTGTCCGTAACCTGAA	530
Aeromonas hydrophila	gcat-F gcat-R	CTCCTggAATCCCAAgTATCAg ggCAggTTgAACAgCAgTATCT	237
Group A *Streptococcus*	dnaseB-F dnaseB-R	TGATTCCAAGAGCTGTCGTG TGGTGTAGCCATTAGCTGTGTT	140
Group B *Streptococcus*	GB-F GB-R	TTTCACCAgCTgTATTAgAAgTA gTTCCCTgAACATTATCTTTgAT	200
16S rDNA	16S1 16S2	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1200

MRSA, methicillin-resistant *Staphylococcus aureus*.

DNA extraction and multiplex PCR analysis

Genomic bacterial DNA extraction was performed and 16S rDNA universal PCR (5′-AGAGTTTGATCCTGGCTCAG-3′) was performed for each sample to validate the results of the multiplex PCR assay. Total genomic DNA was purified using a Geneaid Bacteria Genomic DNA Kit (GBB300/301) and the amplification process is described as follows. PCR was performed in a reaction mixture (25 μl total volume) containing Mg++, dNTPs, Taq DNA (Invitrogen), and sterile distilled water. The following thermal cycling conditions were applied: 30 cycles of denaturation at 94 °C for 1 min, annealing at 52–58 °C for 1 min, extension at 72 °C for 1 min, and final elongation at 72 °C for 5 min. PCR amplicons were analyzed by electrophoresis in 2% agarose gel. The products were stained with ethidium bromide and visualized with a UV transilluminator. Positive controls with all components of the reaction mixture were included in each experiment.

All experiments were performed at least twice. In the first stage, the primers were evaluated by uniplex PCR and multiplex PCR. Once the correct functioning of each set had been verified, one isolate of reference pathogen was added as the PCR positive control and was tested retrospectively to confirm the specificity of this study primer in the second stage.

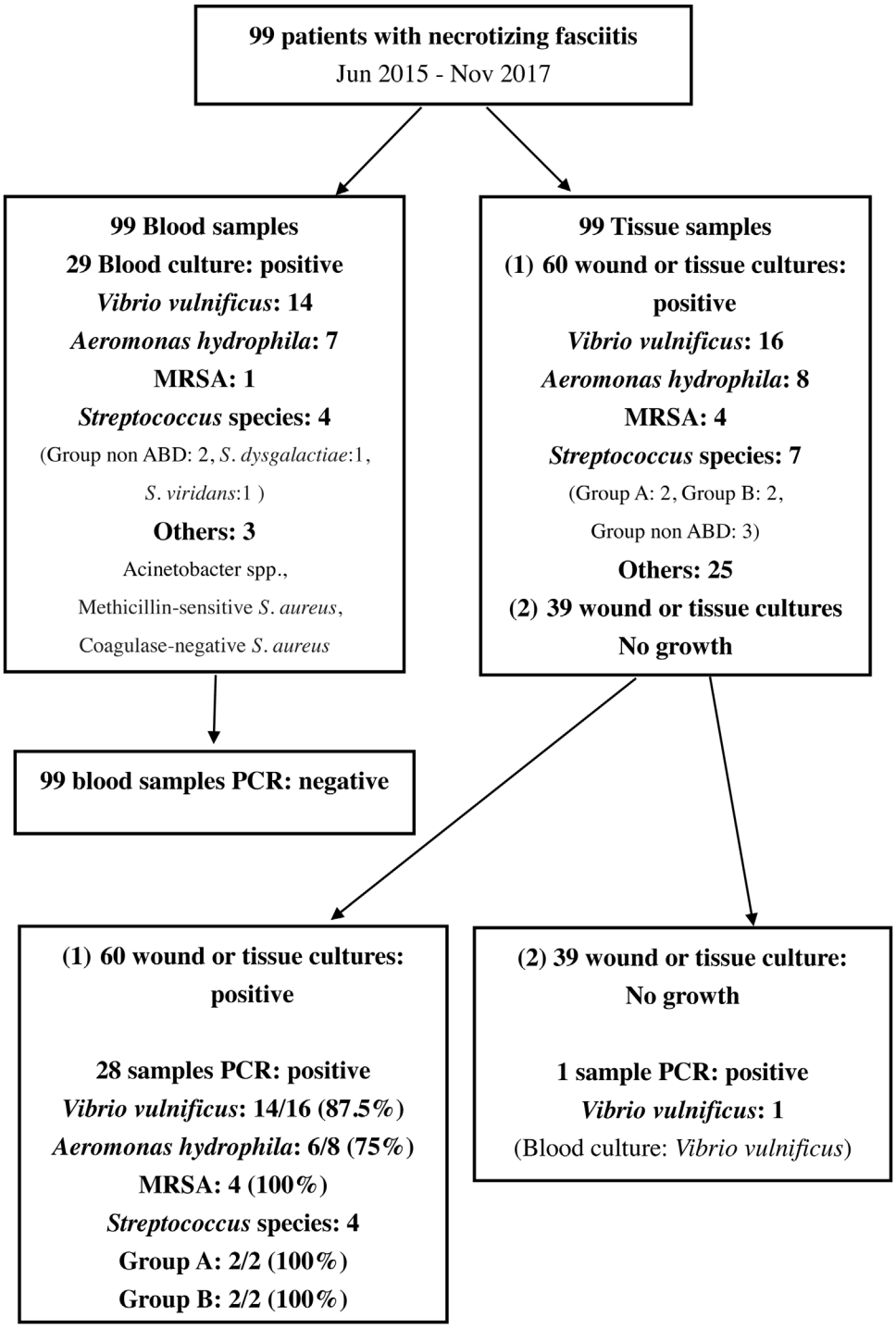

Figure 1. Flow chart of the 198 samples from the 99 patients with necrotizing fasciitis.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy rate of the multiplex PCR method were calculated.

Results

Culture specimens of 99 patients with NF were obtained from the blood or surgical wounds, and culture findings confirmed the bacterial species responsible for the infection after surgery. In total, 198 samples (99 blood samples and 99 tissue samples) were analyzed in order to evaluate the quality and relative accuracy of the multiplex PCR method. The PCR results were compared to those of bacteriological culture (Figure 1). Figure 2 shows the results of the single and multiplex PCR amplification of *V. vulnificus* by agarose gel electrophoresis: lanes 1–14 are the single PCRs; lanes 15 and 16 are the multiplex PCR amplification of all targeted gene segments for the detection of the reference pathogen and tested sample. The appearance of a white line in lane 16 which was displayed as the same as lane 15, was used to confirm that the clinical sample had a positive result.

Blood samples

In total, there were 29 positive blood cultures from the 99 samples analyzed, including 14 *V. vulnificus*, seven *A. hydrophila*, four *Streptococcus* species (two group non-ABD, one *S. dysgalactiae*, one *S. viridans*), one MRSA, and three other species (one *Acinetobacter spp*, one methicillin-sensitive *S. aureus*, one coagulase-negative *S. aureus*). The multiplex PCR failed to detect any of the target DNA sequences in the 99 blood samples.

Tissue samples

In total, 60 pus or wound bacterial cultures were positive, including 16 *V. vulnificus*, eight *A. hydrophila*, seven *Streptococcus* species, four MRSA, and 25 other species. No bacterial growth was detected in specimens obtained from the wounds of the remaining 39 patients.

The multiplex PCR reaction identified 28 positive results from the 60 viable tissue specimens. Fourteen (87.5%) of the 16 *V. vulnificus* culture-positive tissue specimens were positive by PCR. Similarly, six (75%) of the eight *A. hydrophila* culture-positive tissue specimens were PCR-positive. Additionally, the PCR results showed that all four (100%) of the MRSA culture-positive tissue specimens were positive. Finally, two (100%) of the group A *Streptococcus* and two (100%) of the group B *Streptococcus* culture-positive tissue specimens were PCR-positive (Figures 2–6).

One sample from the 39 culture-negative tissue specimens presented a positive PCR result for *V. vulnificus*. This finding was validated as a true-positive by tracing the blood culture result from this particular patient, which showed positive *V. vulnificus* (Table 2).

The overall sensitivity and specificity of the multiplex PCR according to the diagnosis of tissue specimens were 46.6% (28/60) and 97.4% (38/39), respectively. The PPV and NPV were 96.5% (28/29) and 54.3% (38/70), respectively. The accuracy rate of the multiplex PCR for the detection of culture-positive tissue samples was 87.5% (28/32).

Discussion

The PCR technique has been used widely to detect *Vibrio* spp., *Aeromonas* spp., and MRSA by cloning target genes using specific primer sequences in patients with skin and soft tissue infections. Changchien et al. identified the virulence factors and multilocus

Vibrio vulnificus

	Tissue Sample							*Vibrio vulnificus* (reference strain)							TS	RS
Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16S rDNA	+							+							+	+
RPOS		+							+						+	+
tox			+							+					+	+
mecA				+							+				+	+
gcat					+							+			+	+
*dnase*B						+							+		+	+
GB							+							+	+	+

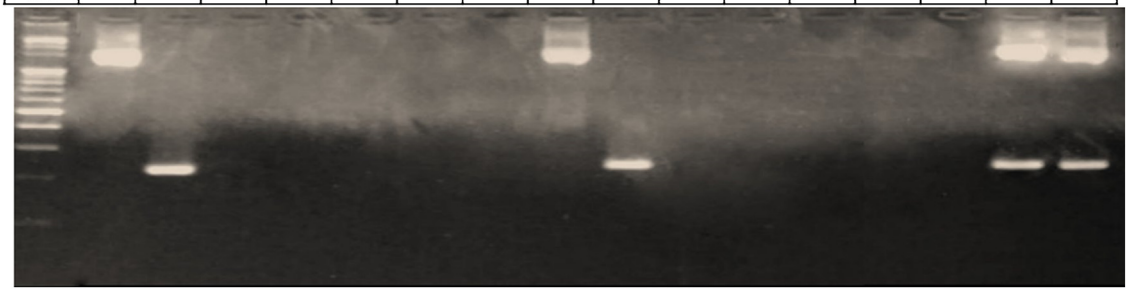

Figure 2. Agarose gel electrophoresis showing the results of single and multiplex PCR amplification of *Vibrio vulnificus*. Lane 1 and lane 8, 16S rDNA for positive control; lane 2 and lane 9, single PCR for the *rpoS* gene specific to *V. vulnificus*; lane 3 and lane 10, single PCR for the *tox* gene specific to *Vibrio cholerae*; lane 4 and lane 11, single PCR for the *mecA* gene specific to MRSA; lane 5 and lane 12, single PCR for the *gcat* gene specific to *Aeromonas hydrophila*; lane 6 and lane 13, single PCR for the *dnaseB* gene specific to group A *Streptococcus*; lane 7 and lane 14, single PCR for the *GB* gene specific to group B *Streptococcus*; lane 15 and lane 16, multiplex PCR amplification of all targeted gene segments. RS: reference pathogen strain isolated from the microbiology laboratory; TS: tissue sample from a necrotizing fasciitis patient. The *rpoS* gene was identical to that seen in lane 2 and lane 9 with specificity of multiplex PCR for identifying *V. vulnificus* in lane 15 and lane 16.

MRSA

	Tissue Sample							MRSA (reference strain)							TS	RS
Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16S rDNA	+							+							+	+
RPOS		+							+						+	+
tox			+							+					+	+
mecA				+							+				+	+
gcat					+							+			+	+
*dnase*B						+							+		+	+
GB							+							+	+	+

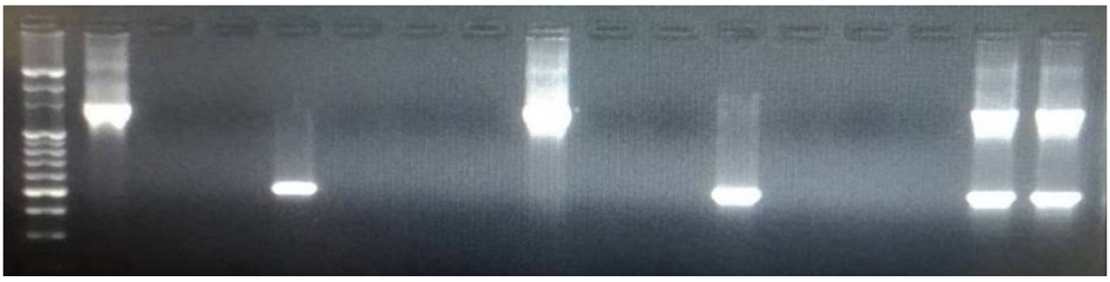

Figure 3. Agarose gel electrophoresis showing the results of uniplex and multiplex PCR amplification of MRSA. The *mecA* gene was identical to those seen in lanes 4, 11, 15, and 16.

Aeromonas hydrophila

	Tissue Sample							*Aeromonas hydrophila* (reference strain)							TS	RS
Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16S rDNA	+							+							+	+
RPOS		+							+						+	+
tox			+							+					+	+
mecA				+							+				+	+
gcat					+							+			+	+
*dnase*B						+							+		+	+
GB							+							+	+	+

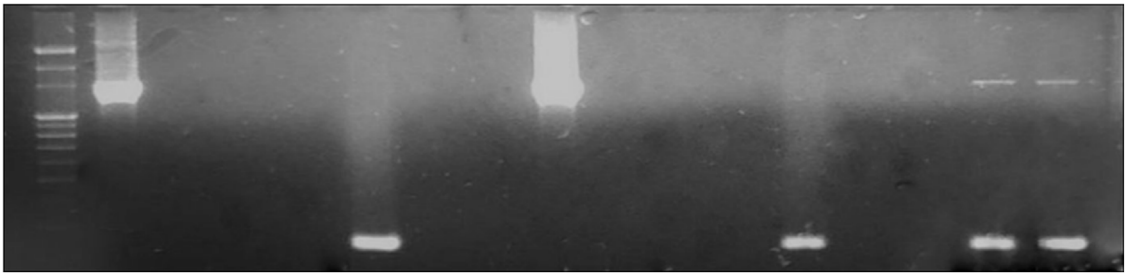

Figure 4. Agarose gel electrophoresis showing the results of uniplex and multiplex PCR amplification of *Aeromonas hydrophila*. The *gcat* fragments were identical to those seen in lanes 4, 11, 15, and 16. The *mecA* gene fragments were identical to those seen in lanes 5, 12, 15, and 16.

sequence types of MRSA strains that were isolated from wound or blood specimens of 16 NF patients by PCR methods (Changchien et al., 2011). Kim et al. used PCR to identify *toxR* in the blood samples of a *V. vulnificus* patient with a necrotizing skin and soft tissue infection (Kim et al., 2008b).

This appears to be the first report describing a comprehensive DNA array-based analysis of multiplex PCR-amplified gene segments for five different lethal microorganisms that are present in tissue samples obtained from patients with NF, and not from seafood samples or environmental strains (Garrido-Maestu et al., 2014; Wei et al., 2014; Kim et al., 2015; Rather et al., 2014; Chen et al., 2014). In this study, the PCR assay presented negative results for all 99 blood samples; however, positive PCR results were obtained for 29 tissue specimens sampled from the wounds. By comparing these results to those of the microbiology laboratory identification, the accuracy rates of the PCR results for these tissue samples were high (75–100%).

Although blood culture was positive for 14 *V. vulnificus*, seven *A. hydrophila*, and one MRSA, no pathogen DNA was detected in these culture-positive blood samples by either single or multiplex PCR. By

Group A *Streptococcus*

	Tissue Sample							Group A *Streptococcus* (reference strain)							TS	RS
Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16S rDNA	+							+							+	+
RPOS		+							+						+	+
tox			+							+					+	+
mecA				+							+				+	+
gcat					+							+			+	+
*dnase*B						+							+		+	+
GB							+							+	+	+

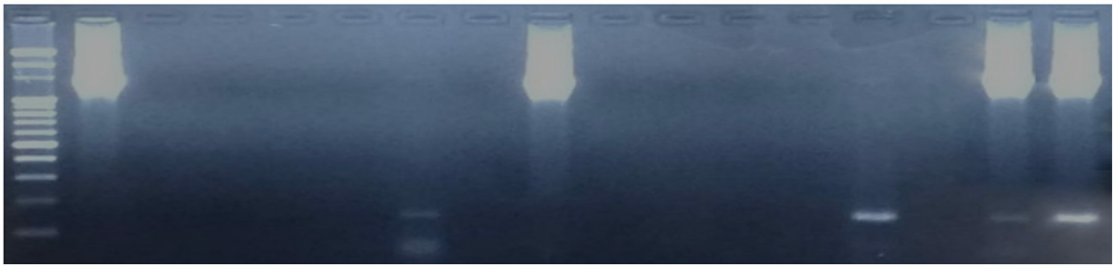

Figure 5. Agarose gel electrophoresis showing the results of uniplex and multiplex PCR amplification of group A *Streptococcus*. The *dnaseB* gene fragments were identical to those seen in lanes 6, 13, 15, and 16.

Group B *Streptococcus*

	Tissue Sample							Group B *Streptococcus* (reference strain)							TS	RS
Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16S rDNA	+							+							+	+
RPOS		+							+						+	+
tox			+							+					+	+
mecA				+							+				+	+
gcat					+							+			+	+
*dnase*B						+							+		+	+
GB							+							+	+	+

Figure 6. Agarose gel electrophoresis showing the results of uniplex and multiplex PCR amplification of group B *Streptococcus*. The GB gene fragments were identical to those seen in lanes 7, 14, 15, and 16.

reviewing the NF cases with both culture-positive blood and tissue specimens in Table 2, positive PCR results were only detected in the tissue samples of eight *V. vulnificus*, four *A. hydrophila*, and one MRSA. With regard to the main entry routes of *Vibrio* and *Aeromonas*, NF symptoms have often occurred after exposure of wounds to seawater, seafood, fresh or brackish water, and contaminated soil; some bacteria then enter the bloodstream to cause bacteremia (Tsai et al., 2012; Tsai et al., 2015). It was thought that the infected skin might contain higher levels of pathogens to cause extensive necrosis. Lee et al. studied patients with skin and soft tissue infections and reported that DNA numbers of *V. vulnificus* were higher in the tissues than in blood samples (Lee et al., 2017). They therefore concluded that necrotic skin tissues were more useful than blood samples as specimens for PCR analysis in *V. vulnificus* infection. Considering the fact that the infected tissues in patients with NF contained greater numbers of bacterial DNA as compared to blood samples, the multiplex PCR assay may be a clinically useful diagnostic tool for the rapid and early detection of these virulent microorganisms when specifically using tissue specimens.

Rapid diagnostic tests for these lethal microorganisms are really important for appropriate antibiotic treatment in patients with NF. This could reduce the hospitalization and medical costs.

Table 2
Culture results and PCR positive tissue samples.[a]

	Number of cases	PCR positive
Vibrio vulnificus		
Blood (P) Wound (P)	8	8
Blood (P) Wound (N)	6	1
Blood (N) Wound (P)	8	6
MRSA		
Blood (P) Wound (P)	1	1
Blood (P) Wound (N)	0	0
Blood (N) Wound (P)	3	3
Aeromonas hydrophila		
Blood (P) Wound (P)	4	4
Blood (P) Wound (N)	3	0
Blood (N) Wound (P)	4	2
Group A *Streptococcus*		
Blood (P) Wound (P)	0	0
Blood (P) Wound (N)	0	0
Blood (N) Wound (P)	2	2
Group B *Streptococcus*		
Blood (P) Wound (P)	0	0
Blood (P) Wound (N)	0	0
Blood (N) Wound (P)	2	2
Total	41	29

MRSA, methicillin-resistant *Staphylococcus aureus*.
[a] Blood (P), culture-positive blood sample; Blood (N), culture-negative blood sample; Wound (P), culture-positive surgical wound sample; Wound (N), culture-negative surgical wound sample.

Conventional microbiology laboratory culture tests take at least 3–4 days for confirmation of positive cultures. Of note, 39 tissue samples revealed no bacterial growth even though some of the patients were suspected to have *Vibrio* NF as a result of a history of contact with seawater or raw seafood. The cost of aerobic and anaerobic microbiology culture tests for two blood samples and two surgical tissue specimens at Chang Gung Memorial Hospital is approximately US$ 40. The multiplex PCR cost for one blood sample and one surgical tissue specimen is approximately US$ 30, and the results may be available as soon as 1–2 days after submission. The multiplex PCR technique could be a rapid and more cost-effective approach to detect and differentiate five types of lethal bacterial pathogen simultaneously in NF patients.

This study has two limitations. First, the blood and tissue samples used were frozen and tested retrospectively, rather than being examined immediately after collection. The possibility that freezing and thawing may have affected the viability of the samples cannot be excluded. Second, the detection rates of culture-positive blood samples by multiplex PCR assay were zero. However, one tissue sample that was tissue culture-negative with a positive *V. vulnificus* blood culture revealed a positive PCR result. Thus, improvement of the DNA extraction techniques may lead to an increase in the PCR products of each target gene and may provide more accurate and rapid identification.

In conclusion, the accuracy rate of the multiplex PCR that revealed positive results in these culture-positive tissue samples was 87.5% (28/32). This suggests that the multiplex PCR method designed here for the detection of these lethal microorganisms in tissue samples may be a useful and rapid diagnostic tool for patients with NF.

Funding

This work was supported in part by grants from the Chang Gung Memorial Hospital in Taiwan (grant number CORPG6E0033 to Yao-Hung Tsai).

Ethical approval and consent to participate

The study protocol was approved by the Institutional Review Board of Chang Gung Medical Foundation (103-2081B).

Conflict of interest

All contributing authors declare no conflicts of interest.

References

Anaya DA, McMahon K, Nathens AB, Sullivan SR, Foy H, Bulger E. Predictors of mortality and limb loss in necrotizing soft tissue infections. Arch Surg 2005;140:151–7.
Anaya DA, Bulger EM, Kwon YS, Kao LS, Evans H, Nathens AB. Predicting death in necrotizing soft tissue infections: a clinical score. Surg infect 2009;10:517–22.
Angoules AG, Kontakis G, Drakoulakis E, Vrentzos G, Granick MS, Giannoudis PV. Necrotizing fasciitis of upper and lower limb: a systematic review. Injury 2007;38S:18–25.
Bair MJ, Chi H, Wang WS, Hsiao YC, Chiang RA, Chang KY. Necrotizing fasciitis in southeast Taiwan: clinical features, microbiology, and prognosis. Intern J Infect Dis 2009;13:255–60.
Bellapianta JM, Ljungquist K, Tobin E, Uhl R. Necrotizing fasciitis. J Am Acad Orthop Surg 2009;17:174–82.
Bruun T, Kittang BR, de Hoog BJ, Aardal S, Flaatten HK, Langeland N, et al. Necrotizing soft tissue infections caused by *Streptococcus pyogenes* and *Streptococcus dysgalactiae* subsp. equisimilis of groups C and G in Western Norway. Clin Microbiol Infect 2013;19:545–50.
Chakraborty S, Mukhopadhyay AK, Bhadra RK, et al. Virulence genes in environmental strains of *Vibrio cholerae*. Appl Environ Microbiol 2000;66:4022–8.
Changchien CH, Chen YY, Chen SW, Chen WL, Tsay JG, Chu C. Retrospective study of necrotizing fasciitis and characterization of its associated Methicillin-resistant *Staphylococcus aureus* in Taiwan. BMC Infect Dis 2011;11:297.
Chen PL, Wu CJ, Tsai PJ, Tang HJ, Chuang YC, et al. Virulence diversity among bacteremic Aeromonas isolates: ex vivo, animal, and clinical evidences. PLoS One 2014;9:e111213.
Frazee BW, Fee C, Lynn J, Wang R, Bostrom A, Hargis C, et al. Community-acquired necrotizing soft tissue infections: a review of 122 cases presenting to a single emergency department over 12 years. J Emerg Med 2008;34:139–46.
Garrido-Maestu A, Chapela MJ, Penaranda E, Vieites JM, Cabado AG. In-house validation of novel multiplex Real-Time PCR gene combination for the simultaneous detection of the main human pathogenic vibrios (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*). Food Control 2014;37:371–9.
Kim DG, Ahn SH, Kim LH, Park KJ, Hong YK, Kong IS. Application of the rpoS gene for species-specific detection of *Vibrio vulnificus* by real-time PCR. J Microbiol Biotechnol 2008a;18:1841–7.
Kim DM, Cho HS, Kang JI, et al. Deferasirox plus ciprofloxacin combination therapy after rapid diagnosis of Vibrio vulnificus sepsis using real-time polymerase chain reaction. J Infection 2008b;57:489–92.
Kim HJ, Ryu JO, Lee SY, Kim ES, Kim HY. Multiplex PCR for detection of the Vibrio genus and five pathogenic Vibrio species with primer sets designed using comparative genomics. BMC Microbiol 2015;15:239.
Lee TC, Carrick MM, Scott BG, Hodges JC, Pham HQ. Incidence and clinical characteristics of methicillin-resistant *Staphylococcus aureus* necrotizing fasciitis in a large urban hospital. Am J Surg 2007;194:809–13.
Lee JY, Kim SW, Km DM, Yun NR, Kim CM, Lee SH. Clinical usefulness of real-time polymerase chain reaction for the diagnosis of *Vibrio vulnificus* infection using skin and soft tissues. Am J Trop Med Hyg 2017;97:443–6.
Louie L, Simor AE, Louie M, Mcgeer A, Low DE. Diagnosis of group A Streptococcal necrotizing fasciitis by using PCR to amplify the Streptococcal pyrogenic exotoxin B gene. J Clin Microbiol 1998;36:1769–71.
Miao J, Chen L, Wang J, Wang W, Chen D, Li L, et al. Current methodologies on genotyping for nosocomial pathogen methicillin-resistant *Staphylococcus aureus* (MRSA). Microb Pathogen 2017;107:17–28.
Miller LG, Perdreau-Remington F, Rieg G, et al. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N Engl J Med 2005;352:1445–53.
Morgan MS. Diagnosis and management of necrotizing fasciitis: a multiparametric approach. J Hosp Infect 2010;75:249–57.
Oncul O, Erenoglu C, Top C, Kucukardali Y, Karabudak O, Kurt Y, et al. Necrotizing fasciitis: a life-threating clinical disorder in uncontrolled type 2 diabetic patients. Diabetes Res Clin Pract 2008;80:218–23.
Rather MA, Willayat MM, Wani SA, Munshi ZH, Hussain SA. A multiplex PCR for detection of enterotoxin genes in *Aeromonas* species isolated from foods of animal origin and human diarrhoeal samples. J Appl Microbiol 2014;117:1721–9.
Sarani B, Strong M, Pascual J, Schwab CW. Necrotizing fasciitis: current concepts and review of the literature. J Am Coll Surg 2009;208:279–88.
Shirvani F, Shahrochi N, Radfar M, Lakestani D. Estimation of group B Streptococcus colonization in high risk neonates by PCR and standard culture. Arch Pediatr Infect Dis 2014;2:e17345.

Slinger R, Goldfarb D, Rajakumar D, Moldovan I, Barrowman N, Tam R, et al. Rapid PCR detection of group A streptococcus from flocked throat swabs: a retrospective clinical study. Ann Clin Microbiol Antimicrob 2011;10:33.

Tsai YH, Huang KC, Shen SH, et al. Microbiology and surgical indicators of necrotizing fasciitis in a tertiary hospital of southwest Taiwan. Int J Infect Dis 2012;16: e159–165.

Tsai Yao-Hung, Shen Shih-Hsun, Yang Tien-Yu, Chen Po-Han, Huang Kuo-Chin, Lee Mel S. Monomicrobial necrotizing fasciitis caused by *Aeromonas hydrophila* and *Klebsiella pneumoniae*. Med Princ Pract 2015;24:416–23.

Wei S, Zhao H, Xian Y, Hussain MA, Wu X. Multiplex PCR assays for the detection of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* with an internal amplification control. Diagn Microbiol Infect Dis 2014;79:115–8.

Zhu L, Olsen RJ, Horstmann N, Shelburne SA, Fan J, Hu Y, et al. Intergenic variable-number tandem-repeat polymorphism upstream of rocA alters toxin production and enhances virulence in *Streptococcus pyogenes*. Infect Immun 2016;84:2086–93.