Establishment of multiplex PCR for detection of genes related to Quinolone resistance

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Abstract:

Background/aims: It has been shown that quinolone resistance arises due to mutations in the quinolone resistance-determining regions of the drug targets. This study aimed to optimise a multiplex PCR assay to track plasmid-mediated low-level quinolone resistance profiles.

Subjects and methods: A multiplex PCR-based-method in which the primers were already established by our team. About 44 samples were collected from 44 patients who enrolled in this study by using surgical site infection (SSI).

Results: By targeting the conserved domains of *qnrA, qnrB, qnrS and the qnrVC* gene families, the primer number was reduced significantly to only four pairs in one multiplex PCR. Using multiplex PCR, 3/44 SSI samples were found to be carrying fluoroquinolone-resistance genes (*qnrA, qnrB, qnrS, qnrVC*).

Conclusion: A multiplex PCR for detecting pathogens as well as identifying quinolone resistance genes all in one reaction was successfully established.

Keywords:* *plasmid-mediated quinolone resistance genes, plasmids, SSI, qnr.

Classification numbers:* *3.3, 3.5

Introduction

Hospital-acquired bacterial infections and drugs resistance of bacteria are big healthcare problems not only in Vietnam but also worldwide. It not only causes severe consequences for patients but also causes economic losses and global diseases. Surgical site infection (SSI) is one of the most common nosocomial infection that occurrs in operated patients. The diagnosis of SSI seems to be easy but, sometimes, the identified pathogens and their profile of antibiotics resistance are inconcludable by culture. Bacterial culture is the classical method for identifying pathogens. However, there are many disadvantages such as, i) long waiting for the result and ii) false negative rate is still high.

Recent advances in nucleic acid testing have opened up doors for molecular detection of microbes. One of the latest methodologies employs multiplexing assays, thus, offering a chance to not only investigate various microbial pathogens causing SSI but also to quickly detect the antibiotic resistance genes.

In bacteria, the type II topoisomerases DNA gyrase and topoisomerase IV change the topology of DNA. These enzymes cleave both strands of DNA and allow one double-stranded DNA molecule to pass through another and then reseal the break. DNA gyrase wraps DNA around itself, which is responsible for introducing negative superhelical twists to plasmid DNA molecules that topoisomerase IV does not, and DNA becomes relaxed. Quinolones inhibit

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these enzymes by stabilising the DNA-DNA gyrase complex or topoisomerase IV and thus, block the progression of polymerase and DNA replication. The widespread use of these agents has contributed to the rise of bacterial quinolone resistance [1]. Previous studies have shown that the mechanisms of quinolone resistance arise by mutations in chromosomal genes [2] and plasmid-mediated low-level resistance mechanisms (*qnrA, qnrB, qnrS, qnrVC*) [3]. These genes have a large geographic distribution (mainly in *Enterobacteriaceae*) [4-6]. Since these genes appear in various bacteria species, the prevalence of detecting this type of resistance phenotypically is only partially known. Although these mechanisms cause low-level resistance, but they favour and complement the selection of other resistance mechanisms [4].

In this study, we aim to develop and optimise a multiplex PCR approach in order to track the plasmid-mediated low-level quinolone resistance mechanisms. By applying our assays to a collection of SSI samples, the first prevalence of fluoroquinolone resistance genes (*qnrA, qnrB, qnrS, qnrVC*) in Vietnam can be described.

Subjects and methods

Subjects

For this study, we included patients who were hospitalised at 108 Military Central Hospital, Hanoi, Vietnam from February 2012 to December 2012 with at least one of these conditions: (1) purulent incisional drainage from deep layers of soft tissue within 30 days after the operation or within 1 year after the operation if a prosthesis was inserted and (2) local signs and symptoms of pain or tenderness, swelling and erythema with the incision opened by the surgeon or confirmed by the attending surgeon or physician. Patients provided their written informed consent prior to participation, and the study was approved by the local ethics committee of the 108 Military Central Hospital.

Exudates from deep incisional surgical infection sites (aspirate beneath the incision area) were collected using sterile syringes and, in parallel, subjected to bacterial culture or stored at minus 80 degrees Celsius for molecular diagnostic.

In total, ninety-one patient samples (n=91) were enrolled in this study procedure. About 44/91 SSI samples were proved to be bacterial culture positive.

Primer design

In order to screen genes encoding important quinolone resistance plasmids *(qnrA, qnrB, qnrS and qnrVC),* the gene bank accession numbers of the *qnrA, qnrB, qnrS* and *qnrVC* gene families were acquired from http://blast.ncbi.nlm.nih.gov/Blast.cgi and imputed into Vector NTI software 11.5 (Invitrogen, California).

DNA extraction and multiplex PCR

Aliquots of exudates from SSIs were allocated for total bacterial culture and the remnants were immersed into a universal lysis solution (200 mM NaOH, 1% SDS) and heated for 5 minutes at 95°C. An equal volume of 1 M Tris-HCl was also added to neutralise the pH down to about 7.5. The resulting solution was subjected to standard phenol/chloroform/isoamyl alcohol extraction. The precipitated DNA was reconstituted in 150 µl TE (25 mM Tris-base, pH 8.0, 1 mM EDTA). About 5 µl of the reconstituted DNA was used as a template for multiplex PCR in 25 µl reactions containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM Mg^{2+} and 250 µM of each deoxynucleotide triphosphate and optimised amount of individual primers. Thermal amplification cycles were carried out as follows: initial denaturation at 95°C for 4 minutes, 35 cycles of 94°C for 25 seconds, 58°C for 45 seconds and 72°C for one minute.

Confirmatory sequencing of amplicons

To avoid false positive results due to cross-reactivity of the selected primer pairs with human DNA, amplified products were cleaned up and subjected to bidirectional PCR sequencing. The outcome was compared to an annotated gene-bank database (Blast search http://blast.

ncbi.nlm.nih.gov/Blast.cgi) in order to identify human genomic information.

Results

Obtained primers

By using the described assay for designing primers, which ones were selected by vector NTI software and targeted for PCR primer binding. The multiplex PCR assay SHPT@QNR (*qnrA, qnrB, qnrS, qnrVC*) was developed and optimised in a way that neighbouring PCR amplicons differed from each other by 60 to 100 bp, which helps to resolve the PCR fragments into visible bands in agarose gel electrophoresis. The primer details can be found in Table 1.

Table 1. Primers used for amplifying conserved domains of quinolone resistance gene families (*qnrA, qnrB, qnrS, qnrVC*).

Primer name	Primer sequencing (5' to 3')	Amplicon size (bp)
Tr-qnrB-F	TGARTTTATYGGCTGYCARTTYTATGATCG	216
Tr-qnrB-R	CAGGTGCGMGTRGTGATCATATTCAT	
Tr-qnrS-F	AACTGCAAGTTCATTGAACAGGGTGATATC	357
Tr-qnrS-R	AACACCTCGACTTAAGTCTGACTCTTTCA	
Tr-qnrVC-F	GGGTGYGATTTTTCTTAYKCKGATCTT	447
Tr-qnrVC-R	CTGYTGCCACGARCAKATTTTTACACC	
Tr-qnrA-F	CAAGAGGATTTCTCACGCCAGGATTT	562
Tr-qnrA-R	CCCCMTCGAGGTTGACCCG	

SHPT@QNR multiplex assay reliably differentiates between qnrA, qnrB, qnrS, and qnrVC positivity

As shown in Fig. 1, individual amplicons derived from multiplexing differ from one another by 60 to 100 bp and do not generate any unspecific bands. All the PCR fragments were resolved into visible bands in agarose gel electrophoresis.

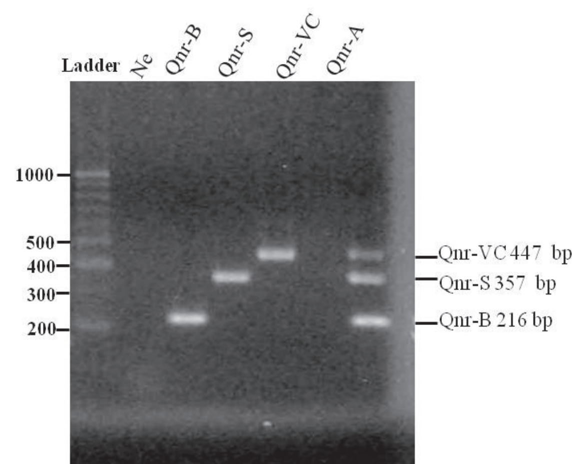

Fig. 1. Multiplex PCR assays (SHPT@QNR) for screening quinolone resistance genes (qnrB, qnrS, qnrVC and qnrA).

Screening patient samples for quinolone resistance utilising the SHPT@QNR assay

After optimising the amplifying abilities of the SHPT@QNR assay (Fig. 1), it was used to screen the potential quinolone resistance genes (*qnrA, qnrB, qnrS, qnrVC*) from all 44 bacterial culture positive SSI samples (out of 91 suspected SSI biopsies). As seen in Table 2, 3 out of 44 SSI samples harbour the following genetic material: NKSM 33 (*qnrB, qnrVC*), *NKSM 40 (qnrB)*, NKSM55 (*qnrS*), which encode *qnrB, qnrS, qnrVC,* respectively.

Table 2. Quinolone resistance genes (*qnrA, qnrB, qnrVC*) were found in collected SSI samples. Noted: SSI-ID = code of sample.

SSI-ID	Bacterial strain	Detected Quinolone resitance gene		
		QnrA	QnrB	QnrVC
NKSM33	E. coli, P. aeruginosa	Positive	-	Positive
NKSM40	K. pneumoniae	Positive	-	-
NKSM55	P. aeruginosa	-	Positive	-

The sequencing results

The positive PCR was confirmed by direct sequencing. The obtained sequences were analysised by using blast stool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and then, they were compared with the sequence on the GeneBank. We showed that the identity of the nucleotide sequences from qnrB, qnrVC and qnrS PCR products were 99%, 99% and 100%, respectively. The result is shown in Fig. 2.

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Klebsiella aerogenes strain AR_0062 plasmid tig00000053_pilon, complete sequence	279	279	97%	2e-71	99%	CP026757.1
☐	Klebsiella pneumoniae strain FDAARGOS_447 plasmid unnamed3, complete sequence	279	279	97%	2e-71	99%	CP023950.1
☐	Enterobacter hormaechei plasmid pMTY11043_IncHI2 DNA, complete genome, strain: TUM11043	279	279	97%	2e-71	99%	AP018352.1
☐	Klebsiella pneumoniae strain QH38-1 QnrB (qnrB) gene, complete cds	279	279	97%	2e-71	99%	KY769120.1
☐	Klebsiella pneumoniae strain QH4-1 QnrB (qnrB) gene, complete cds	279	279	97%	2e-71	99%	KY769118.1
☐	Pseudomonas aeruginosa strain Pa25 class I integron ANT(2")-Ia, VIM-2 (blaVIM-2), ANT(2")-Ia,	700	700	81%	0.0	99%	JX861889.1
☐	Vibrio cholerae non-O1/non-O139 strain DRV181 class 1 integron, partial sequence	700	700	81%	0.0	99%	HM015625.1
☐	Vibrio cholerae strain VC627 class I integron integrase (intI1) gene, partial cds, and streptomycin	700	700	81%	0.0	99%	EU436855.2
☐	Vibrio cholerae O1 biovar El Tor strain MCV09 transposon SXT QnrVC3 (qnrVC3) and dihydrofol	695	695	81%	0.0	99%	HM015627.1
☐	Escherichia coli strain J53 transposon SXT, partial sequence	695	695	81%	0.0	99%	FJ968160.1
☐	Vibrio parahaemolyticus strain B3-5-1 QnrVC1 gene, partial cds	684	684	78%	0.0	99%	KU363013.1
☐	Shigella flexneri strain M11560 quinolone resistance pentapeptide repeat protein QnrS1 (qnrS) ge	544	544	97%	8e-151	100%	KU848190.1
☐	Uncultured bacterium clone RS_qnrs_OTU3 quinolone resistance protein (qnrS) gene, partial cds	544	544	97%	8e-151	100%	KU604991.1
☐	Uncultured bacterium clone RS_qnrs_OTU1 quinolone resistance protein (qnrS) gene, partial cds	544	544	97%	8e-151	100%	KU604989.1

Fig. 2. The sequencing results qnrB, qnrVC, qnrS PCR amplicons.

Discussion

Hospital-acquired bacterial infections paired with antibiotic resistance is a major contemporary public health threat, especially in developing countries like Vietnam, where the medical infrastructure is sub-standard and utilisation of antibiotics is barely regulated. So far, local specific epidemiological data on hospital infection and acquired bacterial antibiotic resistant property were only sporadically reported [7, 8]. In this situation, relevant diagnostic methodologies for monitoring bacterial infections are needed. Together with bacterial infectivity, nosocomial infections related antibiotic resistance is also of public concern. It raises a quest to look for suitable tracking tools. From the experience revealed during the clinical performance in our institution, we suspect the existence of such microorganisms carrying the extended spectrum betalactamese or carbapenemese capability as well as quinolone resistance. However, once the patients have received prophylaxis, broad range antibiotic pressure might inhibit the colonial growth in bacterial culture testing. This is why culture-based antibiotic resistance assays, such as minimum inhibitory concentration tests, often can't be carried out in a clinical setting. That leads to emerging

antibiotic resistance, which is covered up until it has been deeply entrenched in the bacterial population.

The recent emergence of the so-called nucleic acid-based detection methodology has opened up possibilities of not only quickly tracking the presence of different microbial pathogens, which is only present in limited numbers, but also the genes encoding for antibiotic resistance. A challenge establish PCR in the clinic is the diversity of genetics materials coding for antibiotic resistance. Since a single PCR reaction can only detect one genotype, the number of PCR reactions proportionally increases with the diversity level of antibiotic resistance, putting a strain on resources such as time, manpower and money. The multi-primer format is an option here. However, the increase in the number of primers would force them to compete with each other and/or create a risk of getting unspecific signals. In this study, we designed primers complementary to the conserved regions of relevant antibiotic resistance gene families, therefore, reducing the primer number and ending up with only four primer pairs, which still possess the capacity of detecting 51 different genotypes of quinolone resistance gene families.

Conclusions

In short, we reported the establishment of a four-primer-pairs optimised multiplex PCR assay that is significant for screening common gene families that cause quinolone resistance for bacteria.

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Conflict of interests

The authors declare no conflict of interests in this study.

REFERENCES

[1] E.S. Kim, and D.C. Hooper (2014), "Clinical importance and epidemiology of quinolone resistance", *Infect. Chemother.*, **46(4)**, pp.226-238.

[2] P.M. Hawkey, and A.M. Jones (2009), "The changing epidemiology of resistance", *J. Antimicrob. Chemother*., **64,** Suppl. 1, pp.i3-10.

[3] PP. Nordmann, and L. Poirel (2005), "Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae", *J. Antimicrob. Chemother.*, **56(3)**, pp.463-469.

[4] A. Robicsek, G.A. Jacoby, and D.C. Hooper (2006), "The worldwide emergence of plasmid-mediated quinolone resistance", *Lancet Infect. Dis.*, **6(10)**, pp.629-640.

[5] A. Paauw, et al. (2007), "Failure to control an outbreak of qnrA1-positive multidrug-resistant Enterobacter cloacae infection despite adequate implementation of recommended infection control measures", *J. Clin. Microbiol.*, **45(5)**, pp.1420-1425.

[6] J. Liu, et al. (2015), "The dissemination mode of drug-resistant genes in Enterobacter cloacae", *Indian J. Med. Microbiol.*, **33**, pp.S87-92.

[7] D. Nguyen, et al. (2001), "Incidence and predictors of surgical-site infections in Vietnam", *Infect. Control Hosp. Epidemiol.*, **22(8)**, pp.485-492.

[8] K.V. Nguyen, et al. (2013), "Antibiotic use and resistance in emerging economies: a situation analysis for Vietnam", *BMC Public Health*, **13**, p.1158.