Chapter 8

PCR Technique for the Microbial Analysis of Inanimate Hospital Environment

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Abstract

Discipline of molecular ecology and molecular techniques such as polymerase chain reaction (PCR) offers a possibility to study and reveal the microbial diversity in environmental settings with complicated mixed communities, non-culturable organisms, interfering contaminants and low levels of target DNA. Hospital environment represents a new ecological niche for clinically important nosocomial pathogens and antibiotic-resistant microorganisms, which have been commonly found on various hospital surfaces. Accurate characterization of microbial communities depends on several factors, starting with sample collection and conditional enrichment step. In the step of nucleic acid isolation and purification, the DNA, as a dominant signature molecule, is extracted followed by removing co-extracted impurities. PCR target sequences are often 16S rDNA gene, functional gene probes or species-specific probes, depending on the objective of the study. Furthermore, properly prepared PCR amplicons can serve as a basis for characterizing microbial community. The PCR technique is a powerful tool for the analysis of microbial diversity of environmental ecosystems. In a hospital environment, advantages of detecting pathogens and antibiotic-resistant bacteria need to be pointed out.

Keywords: microorganisms, hospital environment, inanimate surfaces, DNA extraction, PCR

1. Introduction

Characteristics of the hospital environments are very specific where inanimate environment can be colonized with a wide range of microorganisms [1, 2]. The cultured microorganisms represent only a small fraction of natural microbial communities, hence the microbial diversity

in terms of species richness and species abundance is grossly underestimated [3]. Therefore, the discipline of molecular ecology and molecular techniques such as polymerase chain reaction (PCR) offers a possibility to study and reveal the real-microbial population complexity and a possibility to overcome limitations of culture-based approaches. Due to the power of the PCR to amplify small amounts of DNA, organisms occurring in small numbers in an environment are now detectable [3]. Special challenges in environmental settings are complicated mixed communities, interfering contaminants and low levels of target DNA [4]. The specifics of environmental samples are low to medium concentration of target cells, low sample homogeneity and high degree of PCR inhibition [5].

Many types of pathogenic microorganisms have been found on various common hospital surfaces. Most common nosocomial bacteria present and detected on inanimate hospital surfaces, using specific marker genes, are presented in **Table 1**.

Microorganism	Locality	Marker gene
Clostridium difficile	Bed, sink, toilet, wall, rails, call button, stretcher [6–9]	tcd_D, tcd_E, tcd_C, cdu_2, cdd_3 [10]
Klebsiella pneumoniae	Bed frame, over-bed table, bedcovers, drains, sinks, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope [11–13]	bla_{TEM}, bla_{CTX-M} [14] bla_{SHV} [15] bla_{KPC} [16]
Staphylococcus aureus	Air, bed, mattress cover, bathroom floor, bed linen, chairs, table, floor, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope [13, 17–20]	*spa* [21] fem_A [22, 23]
Acinetobacter baumannii	Bed rails, sinks, tables, curtains, door handles [24]	bla_{OXA-23}, bla_{NDM-1} [25] omp_A [26] bla_{OXA}, bla_{VIM}, bla_{MBL} [27]
Pseudomonas aeruginosa	Bed, tables, ward sinks and surgical equipment, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope, water [13, 28]	las_A [29]
Escherichia coli	Bed, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope, water [13]	bla_{TEM}, bla_{CTX-M} [14] bla_{SHV} [15] bla_{KPC} [16]
Legionella pneumophila	Drinking water [30]	*mip* [30]
Vancomycin resistant enterococci [VRE]	General areas in patients' rooms and toilets, light switch [31]	van_A [31]
ESBL Enterobacteriaceae	Mechanical ventilator, showers, beds, wall, sinks, toilet, hospital room [32, 33]	bla_{SHV}, bla_{CTX-M}, bla_{CMY}, bla_{IMP}, bla_{VIM} [32, 33]

Table 1. Most common nosocomial bacteria present and detected on inanimate hospital surfaces.

Hospital environment also represents a new ecological niche for clinically important antibiotic-resistant microorganisms. Along with identification through the amplification of conserved genomic sequences, PCR can also be used to detect antimicrobial resistance or virulence genes [34].

In molecular approaches, for studying microbial population complexity, DNA is the dominant signature molecule that phylogenetically dissects microbial communities and substantially increases our insight into microbial diversity. This method does not provide a clear distinction between viable and non-viable organisms, so may not be an accurate reflection of the microbial load present on a surface [34]. On the other hand, accurate determination of the total bacterial load is important in many microbiological applications but cannot be obtained with traditional bacterial cultivation methods. These classical incubation methods based on phenotypic detection of microorganisms are also time-consuming and can work poorly with slow growing or viable but non-culturable (VBNC) organisms [35]. The PCR method clearly has potential in environmental studies where there may be low numbers of viable but non-culturable microorganisms [34] that can still be active cells and can maintain their infectivity in the case of pathogenic bacteria [36–38].

2. Sample collection

Accurate characterization of microbial communities depends upon several factors, starting with the sample collection step that is often ignored as a source of problem [3] in afterwards analysis. Three major obstacles are the sample volume, sample site accessibility and sample transport.

Sampling sites in hospitals can be divided in two large groups:

- Inanimate surfaces (stethoscope, hospital textiles, beds, sinks…)
- Hospital water (drinking water, waste water)

For each of the mentioned group, the appropriate sampling method is required.

2.1. Swabbing and elution

The swab-rinse sampling technique was first described in 1916 by Manheimer and Ybanez [39]. Current recommendations for surface sampling suggest the use of a pre-moistened flocked nylon swab [40], followed by a dry flocked nylon swab for soaking up any remaining liquids. Ekrami et al. [13] used cotton tipped sterile swabs moistened in sterile brain-heart infusion broth. Swabs for environmental surface sampling have been used in numerous studies, and they allow the recovery of microorganisms from hard-to-reach surfaces such as behind taps [41], drains [6] and bed rails [34, 42]. The swab can be transferred into eluting solution (e.g. 0.9% NaCl with 0.2% Tween 80) [43, 44] and vortexed to obtain the substrate for DNA isolation.

2.2. Contact plate sampling

For direct surface sampling, RODAC (replicate organism detection and counting) plate is a common choice [45]. Small Petri dishes are filled in order to provide a convex surface, optionally with a nutrient or selective growth medium. The plate is then pressed onto any flat surface and incubated. The resulting colony count can be expressed as cfu/area [46]. The efficiency of this method depends on the evenness of the surface tested [47]; therefore, Rabuza et al. [48] concluded that the RODAC plate method used for sampling microorganism on textiles has certain limitations due to the rough, uneven three-dimensional (3D) fabric surface. Regarding the use of molecular techniques, each selected colony of interest can be used for DNA isolation and subsequent PCR analysis.

2.3. Elution

When sampling hospital textiles, as a part of inanimate environment that can contribute to the transmission of healthcare associated infections, the elution method is the most effective. This method is based on the principle of eluting microorganisms from textiles; therefore, microorganisms that have penetrated into the deeper structure of the 3D structure of the textile material are also collected. Microorganisms trapped in the 3D structure of the material will not be detected by the RODAC plate method, neither by swabbing, but they can be captured by elution, either destructive or non-destructive [48].

2.4. Water samples filtration and concentration

Since a fundamental limiting factor in the assessment of microbial quality of waters, and especially drinking water, is often the very low number of each organism present, most samples needs to be concentrated, usually by sterile filtration. Bacteria are generally recovered on membrane filters with porosities of 0.22–0.45 μm. Subsequently, membrane filters may be incubated on the solid media or soaked in the liquid media [49].

2.5. Enrichment in growth medium

Generally, the purpose of environmental monitoring is to establish an aerobic colony count of bacteria from a surface, which can be processed without enrichment. This provides a direct enumeration of the level of microbial contamination of a surface [34]. On the other hand, enrichment steps can be used to increase detection in the case of identifying specific multi-resistant or virulent pathogens, which may be the cause of an outbreak. In this case, the number of organisms is not strictly required, but rather their presence or absence [34]. Typically, the number of pathogenic microorganisms is low [50] and their recovery is low because they are under stressed conditions. Therefore, the chances of detecting pathogenic bacteria will be greater by using an enrichment step. Usually enrichment step is also used prior to detection by PCR. Special attention must be paid to false positive results, since PCR does not discriminate between viable and non-viable organisms [49]. Again, disadvantages of DNA-based methods may be partly overcome by including a pre-enrichment step that allows organisms to multiply before gene probe tests are applied [51].

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3. Nucleic acid isolation and purification

When collecting hospital environmental samples by methods described above, protocols for DNA isolation from water samples or pure cultures are usually successfully applied [52], but to date none have been accepted as a standard procedure [4]. The extracted DNA is a mixture of DNA referred as a community DNA, which is ideally a representative of all populations within the sample community; however, in reality, the extraction efficiency of different types of microorganisms can vary widely [52].

DNA/RNA isolation and purification must be achieved through methods efficient enough for releasing DNA from the cells; too rigorous conditions could lead to highly fragmented nucleic acids [3]. DNA or RNA, which is not released from the cells, will not contribute to the final analysis of diversity. RNA extraction is a special case because of the possible RNases effect in the procedures [3] and also short half-life of mRNA [52]. Since DNA is mostly found in low concentrations and DNA solution also contains high concentration of saline, this is of concern in later disturbance of PCR. The most popular application for both cases is simple alcohol precipitation, where many varieties and also commercially available kits exist [53].

To remove co-extracted impurities one or more purification steps are often necessary. However, the number of purifications performed should be minimized as much as possible since large portions of the extracted DNA may be lost during the purification process [52].

4. PCR target sequence

Depending upon the objective of the study, gene probes to target various genes can be designed. Generally, the gene probes can be divided into the phylogenetic probes, to obtain information about taxonomy and phylogeny of microorganism; functional gene probes, to search for the unique activity of the microbial community; species-specific probes.

4.1. Phylogenetic probes

PCR target sequence is often 16S rDNA gene due to the presence of variable and conserved regions used as a phylogenetic marker [54]. Full length 16S rDNA gene can be amplified either directly or after reverse transcription of rRNA with a set of primers binding to conserved regions of the 16S rRNA/rDNA [3]. On the other side, 16S rDNA gene often fails to discriminate between species and strains level; therefore, the 23S rRNA gene and the ITS regions are also well employed [4].

Clifford et al. [55] described a set of 16S rRNA real-time PCR primers, designed to have the same optimal annealing temperature, and displaying high specificity for four clinically important pathogens (e.g. *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*).

The amplification of 16S rDNA gene can also be useful for initial estimation or pre-screening of the microbial diversity by denaturing gel electrophoresis of amplified 16S rDNA products [3].

4.2. Functional gene probes

The target gene may code the production of a unique enzyme, where the positive gene probe indicates that the environmental sample contains the genetic potential for that particular activity [52]. In hospital environmental samples, this special activity usually refers to the antibiotic resistance. Szczepanowski et al. [56] designed and synthesized 192 resistance-gene-specific PCR primer pairs to detect plasmid-borne antibiotic-resistance genes in wastewater treatment plant bacteria. Perreten et al. [57] described rapid and efficient screening of Gram-positive bacteria for the presence of up to 90 of the most prevalent and transferable antibiotic resistance genes using microarray technology.

4.3. Species-specific probes

Primers can also be species specific, as for detecting environmental pathogens. The target sequences that are unique to a particular microbial species allow screening of an environmental sample for the presence of that specific microorganism [52]. There is a vast majority of literature describing species-specific primers for pathogen microorganisms usually find in the hospital environment and on top of that, primers described for identification of microorganisms in clinical samples are working equally well.

4.4. mRNA

Messenger mRNA is turned over rapidly in living bacterial cells, with very short half-lives inside the cell [58, 59] and has therefore been proposed as marker for cell viability [51]. mRNA is also desirable to target when detecting those microbial cells that are in a viable but non-culturable (VBNC), i.e. a dormant state in the environment, since live cells are considered those capable of cell division, metabolism (respiration) or gene transcription (mRNA production). There are several reports on the existence of many microorganisms, including human pathogens, in the environment in a VBNC state that are shown to be potentially infectious when suitable conditions prevail [36, 60–62].

5. Inhibition of PCR amplification

Materials co-extracted with nucleic acids strongly inhibit DNA modifying. Sometimes dilution of the DNA template could be useful, but very low DNA concentrations may influence the PCR efficiency. Therefore, it is desirable to avoid the effects of PCR inhibitors in the amplification reactions. Although the methods described in chapter 'Nucleic acid isolation and purification' can remove the majority of the environmental contaminants and are useful for various molecular biological studies; there is no standard protocol for removing all possible

inhibitors that can be applied for all types of environmental samples [60]. In addition, various biotic and abiotic components of environment, such as blood, urine, feces, tissue, skin, bleach and detergent, can act as the source of PCR inhibitor [63–65], affect lysis efficiency and may interfere with subsequent DNA purification and enzymatic steps [3].

Finally, it should be noted that community DNA extracts may also contain non-microbial DNA, usually origination from humans (patients, hospital workers and visitors) [52] and co-extracted non-target DNA can also inhibit the PCR [66, 67].

6. PCR-based microbial complexity analyses

Properly prepared PCR amplicons can serve as a basis for further analysis of microbial populations.

Culture-independent methods based on amplification and sequencing of 16S rRNA genes allow identification of thousands of different bacteria in a single sample [68–70] when combined with high-throughput DNA sequencing, and hundreds of samples can be multiplexed simultaneously. Therefore, 16S rRNA gene has become a mainstay for characterizing microbial community structure [70, 71]. Hewitt et al. [72] used culture-independent next-generation sequencing to survey bacterial diversity in neonatal intensive care unit surfaces with amplification of the bacterial small subunit [16S] ribosomal RNA gene sequence using 'universal' barcoded primers. They found averaging approximately 100 bacterial genera per surface containing many known opportunistic pathogens, as well as abundant groups whose pathogenic potential and ability to resist antibiotic treatment are poorly understood [72].

Oberauner et al. [73] used the 16S rRNA pyrosequencing approach to study the intensive care units (ICU) environmental microbiome. The phylogenetic spectrum combined species associated with the outside environment, taxa closely related to potential human pathogens and beneficials as well as included 7 phyla and 76 genera [73]. A similar methodology was obtained by Poza et al. [74], who amplified a hypervariable region of the bacterial 16S rRNA gene to explore the bacterial diversity at inanimate surfaces of the ICU wards. Detected microbiota contained a total of 3000 operational taxonomic units. The identified representatives were 16 canonical bacterial phyla, members of the phyla Firmicutes (mainly *Staphylococcus* and *Streptococcus*) and Actinobacteria (mainly *Micrococcaceae, Corynebacteriaceae* and *Brevibacteriaceae*), the phylum Proteobacteria (mainly by members of the families Enterobacteriaceae, Methylobacteriaceae and Sphingomonadaceae), the phyla Proteobacteria, Bacteroidetes, Deinococcus-Thermus and Cyanobacteria, Proteobacteria (mainly due to the high abundance of Enterobacteriaceae members) [74]. 16 S rDNA PCR and sequencing was also employed in study of Xu et al. [75], where 53 isolates from environmental water-associated sites in a haematology unit, and the outer surfaces of cleaning lotion containers sited throughout a tertiary referral hospital were investigated. Sequence analysis was able to identify 51 isolates, mostly Gram-positive bacteria. Nine different genera were identified from the haematology unit and 13 from the cleaning lotion containers [75].

For the estimation of the total microbial population, purified PCR 16S rRNA amplicons can be separated by denaturing high-performance liquid chromatography (DHPLC), with afterward sequencing of chosen fractions (outstanding peaks on DHPLC chromatograms). Described methodology was used by Rozman for estimation of the total microbial population complexity on hospital textiles, where 63 bacterial genera/species were identified, *Acinetobacter* spp., *Corynebacterium* spp., *Staphylococcus* spp., *Sphingomonas mucosissima* and *Stenotrophomonas maltophilia* being mostly abundant [76].

7. Conclusion

DNA molecules can survive in the environment for long periods of time [49] therefore the PCR technique is a powerful tool for the analysis of microbial diversity of environmental ecosystems that can solve many questions in the area of microbial ecology and microbial community structure. Offered advantages of detecting pathogens and antibiotic-resistant bacteria or resistant genes in environmental samples need to be pointed out. PCR has been available for three decades and has become a gold standard to use in microbiology; moreover, several modified PCR assays are the essential tool for use in environmental microbiology.

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