Molecular Approaches to Detection of Bacteria in Critical Care Patients

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Introduction

For over 100 years clinical medicine has relied on culture-based techniques to define infections in patients. However, over the past 30 years, it has become clear that culture-independent methods more completely describe both microbial diversity and community dynamics and the importance of such interactions in states of health and disease has been revealed [1, 2]. Furthermore, the use of culture-based techniques has clouded our understanding of the pathogenesis of human infections. The concept that one species causes infection by entering the host, defeating the host's defense system and multiplying to a threshold that allows it to cause injury is probably only applicable for a small subset of microbes, e.g., bioterror agents. The new emerging paradigm in microbial pathogenesis is that many organisms, such as *Streptococcus pneumoniae*, already exist in bacterial communities of the oro- and nasopharynx of most healthy individuals and that a change in their virulence gene expression and/or an increase in numbers permitting dissemination cause symptoms of infection [3]. The molecular signals that bring about these shifts in pathogen physiology are not fully understood; however, given the importance of bacterial cell-to-cell signaling (quorum sensing) it is possible that shifts in bacterial community composition may lead to emergence and dominance of pre-existing pathogenic species within the community. This hypothesis is supported by the finding that within hours of their admission to the intensive care unit (ICU), critically ill patients exhibit dramatic changes in the bacterial communities colonizing their oro- and nasal pharynx [4, 5]. These shifts in community composition are multifactorial and are significant for the pathogenesis of nosocomial infections, particularly those of the lungs. However, to date, changes in bacterial species composition have largely been described by culture-dependent techniques that both inadequately document the bacterial population composition and insufficiently describe community dynamics.

Bacterial Communities in Humans

Gastrointestinal Microbial Communities

As adults, approximately 90% of the cells present in our bodies are of microbial origin [6] and only a small fraction of these can be detected by traditional culture techniques [7]. It is now apparent that human health depends on the microbial population present in the body; this is most clearly demonstrated by data relating health to intestinal microbial flora. The intestines are colonized rapidly following birth and this microbial community varies in an age-, diet- and health-dependent manner [8].

The gastrointestinal microbiome plays a substantial role in host metabolism by enhancing and maximizing energy production from food, contributing to beneficial biosynthetic pathways (e.g., essential amino acids and vitamins) and, through decontamination, reducing exposure to toxic substances [9]. Interestingly, recent studies suggest that the gut microbiota is altered in obese individuals [10] and in patients with cardiovascular disease [11]. Further, the bacterial communities of infants with allergic diseases appears to be different to that of non-allergic infants and it has been demonstrated that changes in the intestinal microbiota of these infants decrease allergies [12, 13]. The results of these studies are striking and suggest that a similar paradigm may hold true for many other disease states. However, to date there have been no culture-independent investigations into the microbial communities associated with critically ill patients.

Microbial Communities of the Oro- and Naso-Pharynx

In addition to colonization of the gastrointestinal tract, the oral pharynx of neonates becomes colonized (most likely inoculated during birth), and this microbial community also has a significant effect on health. In adults, it has been suggested that ventilator-associated pneumonia (VAP), a subset of hospital-acquired pneumonia, occurs following nosocomial colonization of the oropharnyx, which occurs rapidly in patients hospitalized in ICUs [14–16]. Multiple factors cause such oropharyngeal colonization, including desiccation of the mucosa, decreased salivary secretion, mechanical injury induced by nasogastric and endotracheal tubes, and decreased immunoglobulin A content [17–19]. Dental plaque, which exists on the subgingival and supragingival surfaces of the teeth [20–24], also appears to be a source for microbes that colonize and infect ICU patients [17–19]. Sequential sampling of dental plaque from ICU patients has demonstrated that more than 50% of patients acquiring a respiratory infection exhibit prior colonization by the pathogenic organism at a dentogingival site [25]. In fact, El-Sohl et al. documented that respiratory pathogens isolated from dental plaque were genetically identical to those recovered from bronchoalveolar lavage (BAL) fluid in patients from nursing homes [18]. It appears, therefore, that many patients are not newly colonized in the hospital, but rather bacteria identified in their respiratory samples originate from the oral microbial population particularly from their dental plaque (Fig. 1). Conditions in the ICU, especially oral intubation, may permit specific pathogens to proliferate and dominate the microbial community in addition to providing them with a protected conduit to the lungs.

Several clinical studies have attempted to prevent perioperative pneumonia by perturbing oral flora. Most of the investigations used prophylactic chlorhexidine oral rinse pre- and peri-operatively and demonstrated a significant decrease in nosocomial pneumonia [25–28]. DeRiso et al. [26] documented a significantly decreased incidence of nosocomial lung infections in patients undergoing open-heart surgery who received twice-daily 0.12% chlorhexidine oral rinse as part of a double-blind, placebo-controlled trial. Patients who received the rinse had a 5% rate of nosocomial respiratory infection compared to 14% in the non-treated group. In a separate study, systemic antibiotic use and mortality were also significantly decreased in those patients who received oral chlorhexidine treatment; 1.2% mortality in the treatment group compared to 5.6% in the untreated group [29]. In another trial that was not double blinded, a 52% reduction in the prevalence of nosocomial pneumonia occurred with chlorhexidine rinses in patients undergoing heart surgery [30]. It, therefore, appears that antiseptic rinsing (due to its effect on the oral micro-

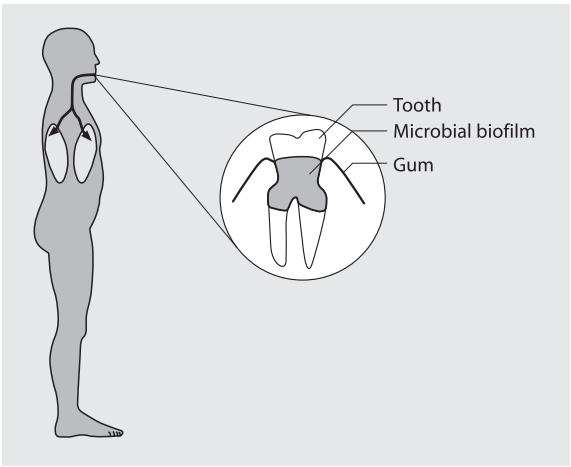

Fig. 1. Species populating sub- and supra-gingival microbial biofilms can act as a source of microbial infection of the respiratory system. Increased incidence of infection is observed in orally intubated, mechanically ventilated patients.

bial community) has been successful in decreasing the incidence of nosocomial infection in cardiac surgical patients.

Most recently, patients requiring mechanical ventilation for 48 hours or more were enrolled in a randomized, double-blind, placebo-controlled trial with three arms: chlorhexidine, chlorhexidine and colistin, or placebo [29]. Trial medication was applied every 6 hours to the buccal cavity. Oropharyngeal swabs were obtained daily and quantitative cultures performed. Endotracheal colonization was monitored twice weekly. The daily risk of VAP was reduced in both treatment groups compared to the placebo treated group. Both treatments led to a significant reduction in Gram-positive organisms. However, only the chlorhexidine and colistin combination treatment led to a significant reduction in both Gram-positive and -negative organisms. In the group that received this treatment, endotracheal colonization was reduced more compared to the group treated with chlorhexidine alone; however, both treatments were equally effective in VAP prevention [29]. This investigation again documented the importance of the oral microbial population in the pathogenesis of VAP. While this investigation reported a decrease in the percent of positive culture results in the treatment groups, it failed to document specifically which bacterial species were affected by each treatment and the oral microbial community dynamics associated with a reduced incidence of VAP. The positive results reported warrant a more comprehensive (culture-independent) evaluation of alterations in microbial diversity affected by such treatments, including assessment of the total microbial community present and how specific treatments may cause shifts in microbial community dynamics that affect patient health.

It is generally accepted that most of the microbes resident in the oral cavity exist as biofilms. Bacterial biofilms are composed of microcolonies of cells non-randomly distributed in a matrix composed of expopolysaccharide [20, 31, 32]. This protective layer provides a permeability barrier against antimicrobials, thus increasing resistance of bacteria within the biofilm. Physiological heterogeneity is known to exist within biofilms; antibiotics may kill actively growing bacteria in the very outer region of a biofilm but slow-growing cells embedded deep within the matrix remain impervious to such treatments [33]. It has also been shown that bacteria in biofilms exhibit differential gene expression compared to those grown as free-swimming or planktonic cultures [34]. An excellent example of this phenomenon is increased

expression of efflux pumps by bacterial biofilms [35]. These pumps normally export cell-cell signaling molecules involved in coordinating the activities of the bacteria in the biofilm; however, they can also export antimicrobials that enter the bacterial cell [36]. This serves to reduce the effective intracellular concentration of antimicrobial and provides an additional resistance mechanism to cells in the biofilm.

Dental plaque located both above and below the gingival margin, represents a mixed-species biofilm in which non-random coaggregation of specific bacterial species is known to occur. It has been established that at least six specific microbial groups or complexes exist within subgingival plaque [37]. Interestingly, several studies have revealed that the strains of bacteria observed in both healthy subjects and those with periodontitis appeared similar, but the absolute numbers and proportions of the periodontal pathogens were significantly higher in diseased individuals. While these studies expand our understanding of the microbial community dynamics that underlie periodontal disease, it remains unclear how these biofilms of mixed microbial populations contribute to VAP or nosocomial pneumonia. To date, investigations into the effects of antiseptic rinses on the oral microbial community have not evaluated biofilms or the majority of the oral floral, but have reported only the small number of bacterial species that can be cultured.

Culture-Independent Assessment of Microbial Communities

Due to fastidious growth requirements of the majority of bacteria, standard culture methods do not adequately document bacterial number or diversity [2, 38]. Even attempts to replicate specific bacterial environments by supplying specific essential nutrients do not significantly increase the number of culturable bacteria; 80% of microbes identified using molecular techniques cannot be cultured [2, 7, 38]. More recently, culture-independent techniques have been used to define the presence of microbial species in a variety of environments. The most commonly used method for members of the bacterial and archael domains makes use of the ubiquitous 16S rRNA gene [39]. Members of both domains possess conserved sequences within their 16S rRNA genes, which flank regions of sequence variability. One approach using this gene is to construct fluorescently labeled probes homologous to the 16S rRNA gene of the species of interest. This technique, termed FISH (Fluorescent *in situ* Hybridization), has been used widely for culture-independent detection of specific bacterial species [40, 41]. However, this approach is limited in the number of species that can be interrogated due to constraints on the number of fluorescent labels that can be employed in any one experiment and novel species cannot be identified using this technique since probe design necessarily anticipates the species present. However, the technique remains a useful method for culture-independent direct interrogation of samples.

A more wide-ranging approach, also based on the 16S rRNA gene, is to construct clone libraries. Initially, a pair of 'universal' primers is designed, based on the sequence of the conserved regions of the 16S gene. This permits amplification of the full length 16S rRNA gene from all microbes in a given sample. This amplicon pool is then cloned into vectors to generate a clone library. Individual clones are subsequently sequenced and analysis of the entire ~1500 bp 16SrRNA gene in each clone permits 16S rRNA sequences to be clustered into groups (where a threshold of sequence similarity is established ~98% identity) and the identification and relative abundance of species present in a microbial community established [2]. One advan-

tage of clone libraries is the ability to identify novel species. Cloning and sequencing of the 16s rRNA gene requires homology in the 'universal' regions of the gene to be used as a priming site for DNA polymerase but unique species can be revealed by sequencing the entire gene. Thus, the presence of novel bacterial and archaeal species can be determined in a culture-independent manner. This technique has been used for a number of years in the field of environmental microbial ecology and has provided insights into the microbial populations and dynamics in a number of environmental samples [42–44]. More recently the approach has also been used to document the microbial communities associated with the periodontal cleft and intestine of human subjects [45, 46]. Additionally, this approach has demonstrated that the number of microbes identified by culture represents a very small fraction of those actually present as determined by culture-independent techniques [2]

However, generation of 16S clone libraries is time-consuming and expensive due to the need for extensive sequencing to detect lower abundance species in samples dominated by a small number of specifies. Recently, a novel microarray-based approach has been developed that permits parallel sampling of all known bacterial species (as of March 2004) in a single assay. This microarray, termed the PhyloChip, uses taxonomic-specific clusters of oligonucleotide probes to detect specific organisms [47]. As with clone library generation, DNA is extracted from a sample and the 16S rRNA gene amplified by polymerase chain reaction (PCR). However, this amplicon pool is fragmented, labeled with biotin and hybridized to the microarray. Arrays are washed and the presence of bacterial species detected by scanning the array for fluorescence (Fig. 2).

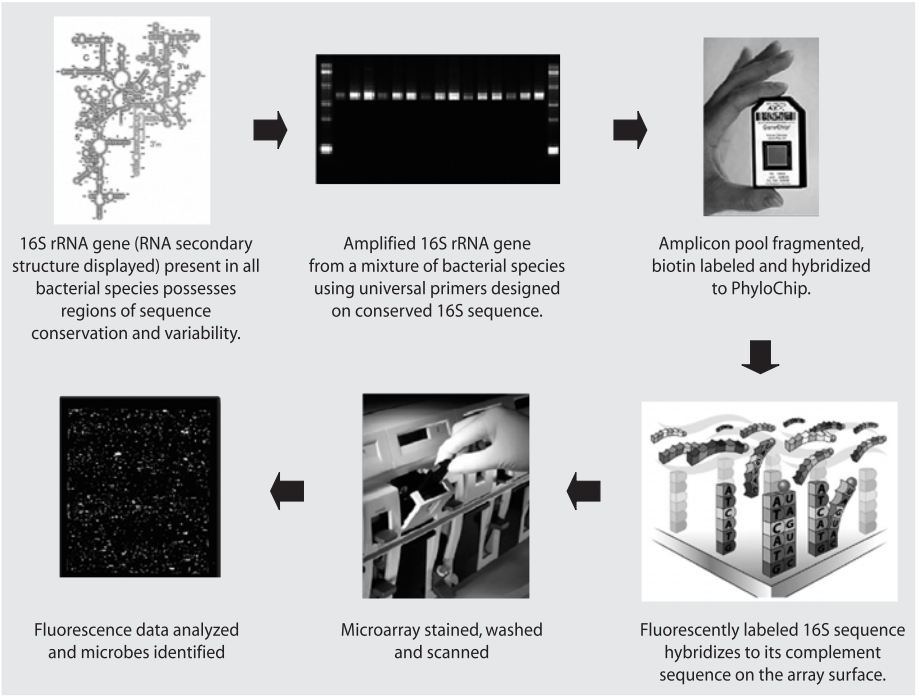

Fig. 2. Schematic of 16S rRNA gene amplification and subsequent microbe identification using the novel PhyloChip.

The PhyloChip is advantageous in that it is rapid, permits massive parallelism, and detects low abundance species even in the presence of dominant organisms in a microbial community.

Culture-independent techniques such as FISH, 16S rRNA clone libraries, and the PhyloChip, represent alternative approaches for microbial detection and diversity determination in a clinical setting [1, 46, 47]. Compared to current clinical culture methods, clone library and PhyloChip techniques provide a more comprehensive picture of microbial diversity and provide tools for increased understanding of how perturbations of microbial communities contribute to states of health and disease.

The Use of 16S rRNA to Detect Novel Organisms

As mentioned above in addition to using 16S rRNA for microbial community determination, this gene has also been useful in detecting the presence of novel organisms. In the 1990s patients with acquired immunodeficiency syndrome (AIDS) were found to have abnormal collections of small blood vessels, bacillary angiomatosis, in their skin and visceral organs. *Rochalimaea henselae*, the organism responsible for bacillary angiomatosis, was found by amplifying part of the 16S rRNA gene from tissue samples obtained from these patients [48]. Similarly, *Ehrlichia chaffeensis*, a new species associated with tick bites, was found using 16S rRNA amplification and sequencing of DNA extracted from leukocytes obtained from infected patients [49]. In 1991, using this technique on a small bowel specimen taken from a patient with Whipple's disease and *Tropheryma whipplei*, the etiologic agent of this disease was discovered [50]. Given that so many idiopathic diseases currently exist, application of culture-independent methods to such disorders appears fundamental to increasing our understanding of these disease processes.

Conclusion

The utility of molecular approaches to bacterial detection and description of bacterial community dynamics includes rapid generation of results, more comprehensive analysis of microbial communities and community dynamics in clinically relevant sites, and the ability to monitor microbial community alterations during antibiotic therapy. Monitoring bacterial communities during therapeutic administration would enable the efficacy of such treatments to be assessed rapidly in patient samples. Indeed, these molecular tools may redefine what truly constitutes 'infection' and provide a much better understanding of the contribution of microbial community structure to pathogenesis. Given the copious use of antibiotics by critical care practitioners, improved understanding of these concepts is central to improved patient care. The continued use of standard culture techniques prevents a more complete understanding of microbial dynamics associated with disease and during therapy that contribute to poor patient outcome. Over the coming years, as culture-independent techniques become more widely used clinically, our comprehension of disease pathogenesis and effective measures for treatment will improve dramatically.

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