Detection, identification and quantification of microorganisms in selected infections

Rudkjøbing, Vibeke Børsholt

Publication date:
2012

Document Version
Early version, also known as pre-print

Detection, identification and quantification of microorganisms in selected infections

A dissertation submitted in partial fulfillment of the requirements for obtaining the degree of

DOCTOR OF PHILOSOPHY

by

Vibeke Børsholt Rudkjøbing

Section of Biotechnology

Department of Biotechnology, Chemistry and Environmental Engineering

Aalborg University

AALBORG UNIVERSITY
DENMARK

Aalborg

September 2012

Contents

Preface

This dissertation is submitted in partial fulfillment of the requirements for obtaining the degree of Doctor of Philosophy (PhD). The dissertation consists of an introduction summarizing the project-related literature and 6 scientific papers included as appendices.

The PhD project was carried out between July 2009 and September 2012 at the section of Biotechnology in the Department of Biotechnology, Chemistry and Environmental Engineering at Aalborg University, Denmark.

At this stage there are many people I would like to thank, first and foremost my supervisors Per Halkjær Nielsen and Trine Rolighed Thomsen. Thank you for the opportunity to work in such an exciting field, and for the support and excellent guidance throughout this study. I would particularly like to thank Trine, without whose encouragement, infectious enthusiasm and uncanny people skills I might never have gotten to this point. Thank you for the many talks, both scientific and personal, and the great times we have had together on campus, at conferences and out in the real world.

Such an interesting cross-disciplinary project would not have been possible without my many collaborators, both domestic and international, and I am deeply grateful for the time and energy you have devoted to our joint projects. Also, thank you to all the highly skilled and friendly people I met during my stay at the Center for Genomic Sciences in Pittsburgh –it was truly inspirational and exciting.

I would also like to thank everyone in the Environmental Biotechnology group; you have made my years at Aalborg University so enjoyable that it at times didn't feel like work. In particular, my office roommates, our great technicians and the Polish and Australian people for the laughs and talks about of life, science and nothing in particular. A special thank you to Yijuan Xu - my ally in a sea of sludge people - for the great times, collaborations and discussions we have had over the years, and Henrik Kjeldal for being a great person to grumble about life with.

My deepest thanks to my family and friends for their support, patience and show of interest, and for supplying plenty of opportunities to think about something else than bacteria, sickness and the thesis. Last but not least I would like to thank my boyfriend Klaus for supporting and bearing with me throughout this process (and for picking up my slack). Thank you for keeping me grounded and pulling me back when I got way too geeky, and for your ability to laugh with and at me.

Vibeke Børsholt Rudkjøbing Aalborg, September 2012

Abstract in English

Infectious diseases are a major cause of morbidity and mortality worldwide. This problem is not as predominant in industrialized countries due to improved sanitation, food availability, health care systems and treatment strategies (including vaccines and antimicrobial therapy). Infections are, however, still problematic, not only to the infected patients, but to the society at large due to the socioeconomic costs. The continued problems caused by microorganisms despite the advent of antimicrobial treatments are both due to emergence of multi-resistant microorganisms, but also because microorganisms can employ a biofilm strategy. Biofilm formation is increasingly being linked to chronic infections, where the biofilm matrix enables the microorganisms to persist despite immune response and antimicrobial therapy. Further adding to the problems in handling infections is the realization that the culture-dependent methods employed for decades to identify the causative pathogens may have some insufficiencies.

The purpose of this PhD study has been to evaluate if alternative methods to culture-dependent techniques could be used to investigate the microbial communities in infections and provide clinically relevant information within a short period of time.

The usefulness of various alternative methods (including molecular methods and microcopy-based visualization) was evaluated based on testing of samples from patients suffering from selected acute and chronic infections. Acute infections were exemplified by necrotizing soft tissue infections (NSTIs), and chronic infections were exemplified by infections of the lungs and sinuses of cystic fibrosis (CF) patients, chronic venous leg ulcers and prosthetic joints.

A general finding of this thesis was that molecular methods identified additional microorganisms compared to the findings by culture. It was, however, also found that various molecular methods might give different results, indicating that the further studies are warranted to determine the ultimate method for identification of microorganisms in clinical samples.

In NSTIs the added value of using molecular methods were particularly found in the ability to identify microorganisms in samples obtained from patients where administration of antimicrobial agents might result in false-negative results by culture-dependent methods. Furthermore, since the disease is both fulminant and potentially lethal, the reduced turnaround time that can be obtained by some molecular methods might make the use of such methods highly relevant.

Investigations of samples from CF patients in this PhD project have added to the knowledge of the infections that afflict this patient group. Lung infections are the primary cause of premature deaths of the patients and investigation of microbial communities indicated that a link existed between low microbial diversity and high pathogenicity, since end-stage patients were found to be infected by a single dominant pathogen. Non-end-stage patients were found to have polymicrobial lung infections; however, the biofilm aggregates in the lung airways were largely monomicrobial and spatially segregated. In the sinuses of CF patients molecular methods could identify a more diverse microbial community than culture, consisting both of CF pathogens, environmental species and anaerobes. The microorganisms in sinuses have been implicated in recurrent lung infections after successful antimicrobial eradication and establishment of lung infections in lung-transplanted CF patients. The ability to identify all microorganisms in the sinuses may therefore be clinically relevant, although the effect of the microbial diversity in the sinuses is presently not fully understood.

Molecular investigations of chronic venous leg ulcers indicated that the microbial communities in such wounds were highly diverse, and that the distribution of microorganisms within the wound varied, both in terms of community composition and abundance of individual species. This finding has implications on the appropriate sampling method of such wounds, since a single biopsy sample might not represent the entire microbial community.

In suspected prosthetic joint infections it was found that although culture-dependent and molecular methods might give concordant results in some cases, the presence of biofilms on prosthesis surfaces might be the reason for cases where molecular methods could identify additional microorganisms. The study also indicated that the routine culture conditions used for examination of this infection type at clinical microbiology departments were insufficient since they did not allow for growth of fastidious microorganisms such as *Propionibacterium acnes*.

In addition to the increased knowledge of the investigated infection types, the results of this PhD project have shown that molecular methods can be used to derive clinically relevant information that may improve outcome for infected patients. Furthermore, the results have contributed in convincing medical professional of the added value that can be obtained by using such methods.

Future studies will hopefully lead to a definition of a method that can identify all microorganisms in a sample at a reasonable price and with a short turnaround time, and thus diminish the problem of different results obtained by different molecular methods. However, the ability to test antimicrobial susceptibility means that culture-dependent methods will not be completely abandoned, and the optimal method in a clinical microbiology setting might therefore be one that combines culture-dependent antimicrobial susceptibility testing with molecular methods to achieve reliable results within a short period of time. Further studies are required to elucidate the function and effect of the diverse microbial communities in infections, which can hopefully be used to combat infections more efficiently in the future.

Dansk resume (abstract in Danish)

Infektionssygdomme er en af de ledende årsager til sygdom og dødsfald på verdensplan. I industrialiserede lande er dette dog blevet mindsket, hvilket skyldes både øget hygiejne, tilgængelighed af mad samt forbedret ygehusvæsen og behandlings muligheder (heriblandt vacciner og antimikrobiel behandling). Til trods for dette er infektioner stadig problematiske, ikke kun for den syge patient, men også for samfundet som helhed grundet de samfundsøkonomiske omkostninger der er forbundet med infektioner. Trods udbredelsen af antimikrobiel behandling kan bekæmpelsen af infektioner være problematisk, hvilket både skyldes udvikling af multiresistente mikroorganismer, men også at mikroorganismer kan leve i benyttende biofilm. Dannelse af biofilm gør at mikroorganismer kan overleve både kroppens immunforsvar samt antimikrobiel behandling, og et stigende antal kroniske infektioner bliver i dag forbundet med biofilm dannelse. Yderligere må det erkendes at de dyrkningsbaserede metoder, som i årtier er blevet brugt til identifikation af sygdomsfremkaldende mikroorganismer, har en række problemer.

Formålet med dette PhD projekt har derfor været at vurdere om andre metoder end dyrkningsbaseret identifikation kan bruges til at undersøge mikroorganismerne der indgår i infektioner, og give klinisk relevant information i løbet af kort tid.

Nytteværdien af forskellige alternative metoder (herunder molekylære metoder samt mikroskopibaseret visualisering) blev vurderet på grundlag af forsøg udført på prøver fra patienter, som led af udvalgte akutte og kroniske infektioner. Nekrotiserende bløddelsinfektioner blev brugt som illustration af akutte infektioner, mens kroniske infektioner blev eksemplificeret af lunge- og bihule infektioner hos cystisk fibrose patienter, af kroniske venøse bensår samt af ledinfektioner i forbindelse med proteser.

Dette PhD projekt har vist at molekylære metoder kan identificere yderligere mikroorganismer i forhold til dyrkningsbaserede metoder. Resultaterne indikerede imidlertid også at forskellige molekylære metoder kunne give forskellige resultater, hvilket er et tegn på at yderligere studier er nødvendige for at kunne definere den bedste metode til at identificere mikroorganismer i kliniske prøver.

For nekrotiserende bløddelsinfektioner ligger merværdien ved brug af molekylære metoder især ved muligheden for at identificere mikroorganismer i prøver fra patienter hvor antimikrobiel behandling måske kan resultere i falsk-negative svar ved dyrkning. Derudover er muligheden for hurtigt at opnå svar ved brug af molekylære metoder yderst relevant for denne type infektioner, idet sygdommen er både fulminant og potentielt dødbringende.

Undersøgelserne af cystisk fibrose patienter i dette PhD projekt har øget den nuværende viden om de infektioner, der kan forekomme i denne patient gruppe. Lungeinfektioner er den primære årsag til for tidlige dødsfald blandt patienterne. Ved at undersøge den mikrobielle sammensætning i lungerne fandtes en mulig forbindelse mellem lav mikrobiel diversitet og høj patogenicitet, da lungerne fra patienter med terminal lungeinfektion var domineret af en enkelt patogen art. Patienter med ikke-terminal kronisk lungeinfektion var generelt inficerede med mange forskellige mikroorganismer. Selvom lungeinfektionerne overordnet set var polymikrobielle, var biofilm aggregaterne i luftvejene monomikrobielle og ikke i fysik kontakt med hinanden. I bihulerne hos cystisk fibrose patienter fandt molekylære metoder en mere forskelligartet sammensætning af mikroorganismer i forhold til de dyrkningsbaserede metoder. Denne diversitet blev udgjort både af bakterier der er kendte som sygdomsfremkaldende i cystisk fibrose, bakterier der stammer fra det omgivende miljø samt

anaerobe bakterier. Mikroorganismer i bihulerne har været associerede med de tilbagevendende lungeinfektioner, der forekommer hos cystisk fibrose patienter efter vellykket antimikrobiel bekæmpelse af lungeinfektioner, samt inficering af transplanterede lunger i cystisk fibrose patienter. Muligheden for at identificere alle mikroorganismer i bihulerne er derfor måske klinisk relevant, selvom funktionen af den mikrobielle diversitet i bihulerne endnu ikke er fult forstået.

Molekylære undersøgelser af kroniske venøse bensår indikerede, at den mikrobielle sammensætning i disse sår er meget forskelligartet og at fordelingen af mikroorganismer indeni sårene varierede - både med hensyn til den mikrobielle sammensætning og hyppigheden af individuelle arter. Disse fund har direkte indflydelse på prøvetagningsproceduren for denne type sår, idet en enkelt biopsi prøve sandssynligvis ikke kan repræsentere hele det mikrobielle samfund i såret.

I prøver fra patienter med mistænkt proteseinfektion blev det vist, at selvom resultaterne fra dyrkningsbaserede metoder og molekylære metoder kunne være overensstemmende, var de molekylære metoder til tider i stand til at identificere mikroorganismer som ikke blev fundet med de dyrkningsbaserede metoder. Dette kan skyldes dannelse af biofilm på protesens overfald. Studiet indikerede desuden også, at de vækstbetingelser der blev benyttet i kliniske rutine undersøgelser af denne type prøver, ikke var tilstrækkelige til at kunne detektere langsomt voksende bakterier, som for eksempel *Propionibacterium acnes*.

Udover at bidrage til en øget viden om mikroorganismerne der findes i de udvalgte infektionstyper, har dette PhD projekt vist at molekylære metoder kan bruges til at opnå klinisk relevant information, som kan forbedre udfaldet for patienter. Desuden har projektet været med til at overbevise sundhedspersonale om den merværdi der kan opnås ved brug molekylære metoder.

Fremtidige studier vil forhåbentlig medføre, at der kan defineres en metode til hurtig identifikation af alle mikroorganismer i en prøve, hvilket kan mindske problemet med at forskellige metoder giver forskellige resultater. Imidlertid vil dyrkningsbaserede teknikker ikke blive opgivet helt, da disse er de eneste som giver mulighed for at teste mikroorganismers modtagelighed overfor antimikrobiel behandling. Det er derfor muligt, at den optimale metode i klinisk mikrobiologi består af dyrkningsbaseret antimikrobiel modtagelighedstest i kombination med molekylære metoder for at opnå pålidelige resultater i løbet af kort tid. Yderligere studier er påkrævet for at opklare hvilken funktion og effekt de diverse mikrobielle fund har i infektioner, og denne viden kan forhåbentlig omsættes til en mere effektiv bekæmpelse af infektioner i fremtiden.

List of supporting papers

I **Rudkjøbing, V.B.**, Thomsen, T.R., Melton-Kreft, R., Ahmed, A., Eickhardt-Sørensen, S.R., Bjarnsholt, T., Nielsen, P.H., Ehrlich, G.D., Moser, C. (in prep). Comparing culture and molecular methods for the identification of microorganisms involved in necrotizing soft tissue infections.

II **Rudkjøbing, V.B.**, Thomsen, T.R., Alhede, M., Kragh, K.N., Nielsen, P.H., Johansen, U.R., Givskov, M., Høiby, N., Bjarnsholt, T. (2011). True Microbiota Involved in Chronic Lung Infection of Cystic Fibrosis Patients Found by Culturing and 16S rRNA Gene Analysis. *Journal of Clinical Microbiology* 49, 4352-4355.

III **Rudkjøbing, V.B.**, Thomsen, T.R., Alhede, M., Kragh, K.N., Nielsen, P.H., Johansen, U.R., Givskov, M., Høiby, N., Bjarnsholt, T. (2012). The microorganisms in chronically infected end-stage and non-end-stage cystic fibrosis patients. *FEMS Immunology & Medical Microbiology* 65, 236–244.

IV **Rudkjøbing, V.B.**, Aanaes, K., Wolff, T., von Buchwald, C., Johansen, H.K., Thomsen, T.R. (submitted). Microorganisms involved in sinus infection of cystic fibrosis patients determined by culture and molecular-based methods. *PLoS ONE*.

V Thomsen, T.R., Aasholm, M.S., **Rudkjøbing, V.B.**, Saunders, A.M., Bjarnsholt, T., Givskov, M., Kirketerp-Møller, K., Nielsen, P.H. (2010). The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. *Wound Repair and Regeneration* 18, 38–49.

VI Xu, Y., **Rudkjøbing, V.B.**, Simonsen, O., Pedersen, C., Lorenzen, J., Schønheyder, H.C., Nielsen, P.H., Thomsen, T.R. (2012). Bacterial diversity in suspected prosthetic joint infections: An exploratory study using 16S rRNA gene analysis. *FEMS Immunology & Medical Microbiology* 65, 291–304.

1 Human infections

Infectious diseases are a major cause of morbidity and mortality worldwide (particularly in developing countries) and was estimated to be responsible for 26% of the deaths in the world in 2001 (Pinheiro *et al.*, 2010). This is largely due to the burden of infectious diseases in developing countries, whereas the problem has been greatly reduced in industrialized countries. This is attributed to many factors including improved sanitation, food availability and living conditions along with development of antimicrobial therapy and vaccines and improved health care systems (Pinheiro *et al.*, 2010). The use of vaccines and antimicrobial therapy has led to a certain degree of control over acute infections; however, this approach has left the health care system with a new set of problems (Donlan and Costerton, 2002; Costerton *et al.*, 2011). Some of the emerging major contributors to morbidity, mortality and increased healthcare costs are the ever increasing number of multi-resistant microorganisms, hospital acquired infections and chronic biofilm infections. In the United States it is estimated that 65 – 80 % of all human infectious diseases are caused by the biofilm phenotype, with up to 17 million new biofilm infections and 550,000 deaths each year (Potera, 1999; Donlan and Costerton, 2002; Wolcott and Dowd, 2011; Wolcott *et al.*, 2012). The socioeconomic cost of infections is high, for instance hospital acquired infections alone have been estimated to amount to about 2 % of the Danish hospital costs (Pedersen and Kolmos, 2007).

Although infections are a recurring problem, it is clear that presence of microorganisms do not lead to disease in the majority of cases. Humans are continuously in contact with microorganisms; in fact the total number of microorganisms in the human body is at least 10 times greater than the number of human cells (Highlander *et al.*, 2011). Most of these microorganisms are commensals or opportunistic pathogens that only cause problems if the immune system is weakened or if they gain access to a normally sterile part of the body. Dedicated or primary pathogens are not a part of the normal human microbiota, and can cause disease in otherwise healthy persons, since they are highly specialized in gaining entry and surviving inside human hosts (Alberts *et al.*, 2002). The body deploys a multitude of defense mechanisms to protect itself from microorganisms. These can be broadly divided into three categories: physical barriers preventing entry to the tissues, the innate immune system and the adaptive immune system. The physical barrier is comprised by strong barriers such as the skin, hair, and nails, and more vulnerable internal surfaces consisting of mucosal membranes. The barriers protect against infection by means of their physical and chemical properties and utilization of diverse flora of microorganisms densely populating the surface of some of the barriers (Alberts *et al.*, 2002; Highlander *et al.*, 2011; Ichinohe *et al.*, 2011). If the barriers are breached, the various cells of the immune system are responsible for containment and eradication of infection. The overall effect of the innate immune system is to create a state of inflammation. Here vascular dilation results in leaks of blood plasma into the connective tissue, inviting white blood cells to move from the blood into the tissue to eradicate microorganisms. This also leads to destruction and remodeling of the tissues (Kimbrell and Beutler, 2001; Jensen and Moser, 2010). The adaptive immune response comes later than the innate immune response and is characterized by a higher degree of specificity. It recognizes species or even strain specific antigens, as opposed to the innate immune system that recognizes broad range molecular patterns (Moser and Jensen, 2010).

Potential pathogens may enter the body by various routes including the internal barriers, through seeding from a reservoir or directly through a breach in the skin, for instance by bites or accidental or surgical trauma (Ala'Aldeen, 2007; Olsen and Musser, 2010; Hansen *et al.*, 2012). After the pathogen has gained entry, it must establish a stable population which normally requires adhesion to

host cell surfaces or molecules (Figure 1). The adhesion leads to activation of complicated signaling pathways in both the microorganism and the host (Finlay and Cossart, 1997; Alberts *et al.*, 2002; Anderson *et al.*, 2006; Ala'Aldeen, 2007). The effect is a dramatic event where the immune system tries to clear the infection and the microorganism uses numerous mechanisms to evade eradication (Monack *et al.*, 2004).

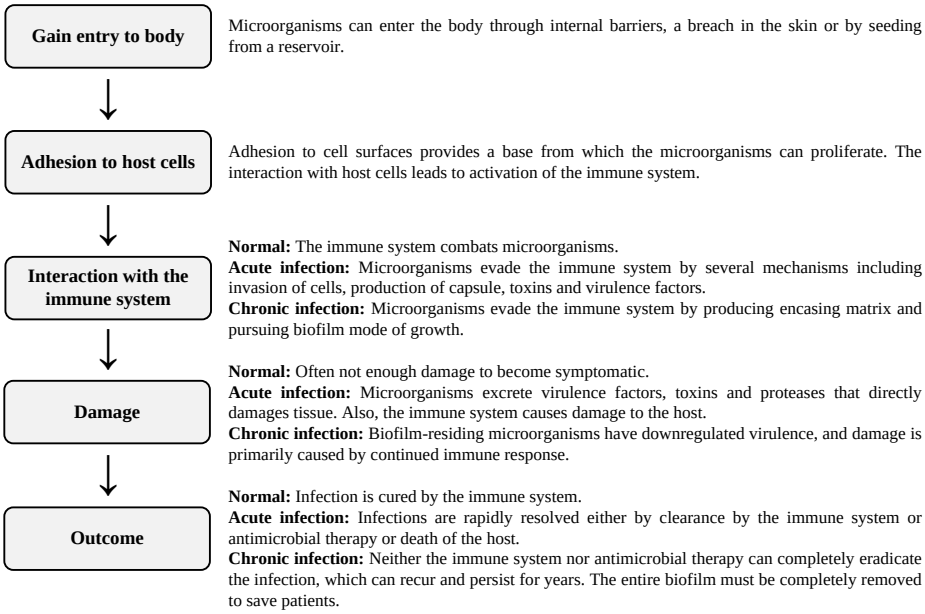


Figure 1: Overview of the stages of disease development after microorganisms have gained entry to the human body.

There seems to be two fundamentally different types of infection: acute infections, which appear to be the result of microorganisms pursuing a planktonic phenotype, and chronic infections that persist in the host due to formation of biofilm (Figure 1) (Furukawa *et al.*, 2006; Wolcott and Dowd, 2011). Biofilm formation is an ancient prokaryotic adaptation that allows microorganisms to survive in hostile environments (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Wolcott *et al.*, 2012). Historically, studies of pathogenesis have focused on acute infections, but recently biofilm infections have been garnering much attention (Furukawa *et al.*, 2006; Wolcott and Dowd, 2011). Acute infections are generally aggressive infections with vast tissue destruction, but of short duration due to a quick resolution either by clearance by the immune system or by death of the host (Furukawa *et al.*, 2006; Wolcott and Dowd, 2011). The microorganisms in chronic biofilm infections are generally confined to a particular location, contained by the host defenses, although dissemination occurs through detachment and shedding of planktonic cells and aggregates by various mechanisms (Parsek and Singh, 2003; Hall-Stoodley and Stoodley, 2005; Furukawa *et al.*, 2006; Wolcott and Dowd, 2011). Unlike acute infections the microorganisms in biofilms exhibit a slower growth rate, and chronic infections can persist for years (Donlan and Costerton, 2002). Many bacterial species that produce chronic infections can also cause acute invasive infections (Parsek and Singh, 2003). It

seems that microorganisms can choose whether to cause an acute infection, growing and spreading rapidly in the host, or adopting a chronic biofilm infection strategy (Furukawa *et al.*, 2006).

In acute infections the evasion of the immune system includes invasion of host cells, production of toxins, protective capsules, and virulence factors involved in inhibition of host-derived molecules and binding of phagocytic cells (Finlay and Cossart, 1997; Cunningham, 2000; Anderson *et al.*, 2006; Barer, 2007; Fuchs *et al.*, 2012). For chronic biofilm infections the evasion of the immune system is accomplished by the extracellular polymeric substance (EPS) matrix that encases a structured community of aggregated microorganisms (Figure 1) (Costerton *et al.*, 1999; Parsek and Singh, 2003; Hall-Stoodley *et al.*, 2004).

The symptoms of infection are direct manifestations of both the immune response and damage of the involved tissue, and have to reach a certain level for the individual to become symptomatic. The damage done to the host may be inflicted directly from the pathogens or by the individuals own immune response (Figure 1) (Alberts *et al.*, 2002; Ala'Aldeen, 2007). The microorganisms involved in acute infections can utilize a wide arsenal of virulence factors and toxins to directly induce damage to the host tissue or initiate apoptosis. The microorganisms can then feed on the host tissue by secreting proteases that digest the tissue (Finlay and Cossart, 1997; Wolcott and Dowd, 2011). The formation of biofilm seems to have an oppressive effect on expression of certain toxins, and the microorganisms involved in chronic infections show a moderated virulence (Parsek and Singh, 2003; Furukawa *et al.*, 2006). The exact processes by which biofilm-associated microorganisms directly cause disease in the human host are poorly understood. Suggested mechanisms include detachment of cells or cell aggregates and production of some endotoxins (Donlan and Costerton, 2002). In many cases the damage that is inflicted on the patient stems from the individuals own immune defense due to an excessive or prolonged innate response (Ala'Aldeen, 2007).

In most infections the adaptive immune system will eventually win the fight, and infection be cleared. Acute infections can often be cleared by a single course of treatment, after which it will not return. However, if the infection is not cleared, the continued presence of microorganisms will provoke a continued inflammation. In chronic infections the EPS matrix of the biofilm ensures that the microorganisms persist despite presence of inflammation, adaptive immune response, and even antimicrobial treatment (Monack *et al.*, 2004). The microorganisms residing in biofilms have a dramatically lower susceptibility to antimicrobial agents compared to their planktonic counterparts. The mechanisms responsible for this are thought to be delayed or impaired penetration of some antimicrobial agents through the biofilm matrix or the different physiology and growth states that are displayed by the microorganisms in the biofilm (Donlan and Costerton, 2002; Wolcott and Dowd, 2011). Even if most of the microorganisms in a biofilm are eradicated, the biofilm can be reconstituted in the exact same host niche, so that the infection reappears after successful antimicrobial therapy (Wolcott and Dowd, 2011).

Correct identification of the microorganisms involved in infections and evaluation of their antimicrobial susceptibility is an important part of medicine to determine an optimal treatment strategy. The gold standard for identification of pathogens is largely based on routine culture-dependent techniques performed at clinical microbiology departments. Determination of pathogenic microorganisms has been largely based on a set of criteria proposed by Robert Koch in 1890. Over the years these postulates have been reworded and extended, and can be summarized as: 1) the microorganism must be found regularly in diseased individuals (but not healthy individuals), 2) it can be isolated and grown in pure culture, 3) inoculation of the microorganism will cause disease in

healthy individuals (experimental animals) and the same organism must then be re-isolatable from the experimentally diseased individual (Highlander *et al.,* 2011; Nelson *et al.,* 2012). The use of pure cultures and phenotypic identification methods is often time consuming and most patients will therefore receive empirical antimicrobial treatment before the pathogens have been identified. It is possible that the administered treatment is sufficient, in which case the culture report from the clinical microbiology department is used for confirmation, but the report may also indicate that adjustment of the treatment is necessary (Slack, 2007; Turnidge *et al.,* 2011). Although culture-dependent methods are the gold standard in clinical microbiology, there are some technical limitations to the methods if antimicrobial treatment has been administered, if the microorganisms exist in a viable but non-culturable state, or if the *in vitro* conditions do not meet the requirements of the microorganisms (Amann *et al.,* 1995; Vartoukian *et al.,* 2010). Additionally, acute and chronic infections present different challenges to the diagnosis of pathogens by culture-dependent methods. For acute infections routine culture-dependent methods may often be able to identify the infecting microorganisms, however, the time required for this identification can be too slow compared to the progression of some diseases. For chronic infections caused by biofilms the use of culture-dependent methods may be difficult, and often leads to false-negative culture results. A consequence of Koch's postulates has been an adaptation of a monomicrobial view of infections. However, biofilm infections are often polymicrobial, which means that the strategy of pathogen isolation and investigation of pure cultures may be counterintuitive and unable to clarify the complexity of biofilm infections (Burmølle *et al.,* 2010; Nelson *et al.,* 2012; Wolcott *et al.,* 2012). Also, it can be difficult to prove that biofilm residing microorganisms and polymicrobial infections in general are etiological agents of disease according to Koch's postulates, since interaction between different microorganisms is not taken into account in the postulates (Donlan and Costerton, 2002).

2 Objectives of the PhD project

Based on the reported limitations of culture-dependent methods, the overall aim of this PhD project was to evaluate the possibility of using alternative molecular methods as supplement or replacement for culture-dependent methods in clinical microbiology. Since multiple molecular methods have been developed, this study has been focused on techniques that are commonly used within other fields of microbiology, and their ability to obtain clinically relevant information. To achieve this goal the specific aims were to:

- compare the ability to detect and identify microorganisms within a short period of time by standard culture-dependent methods used at clinical microbiology departments with commonly used molecular methods.
- use various molecular methods to obtain information on diversity, relative abundance and spatial distribution of microorganisms in selected human infections.

The methods were tested on samples from acute infections as exemplified by necrotizing soft tissue infections (NSTIs) and chronic infections, as exemplified by infections of the lungs and sinuses of cystic fibrosis (CF) patients, chronic venous leg ulcers and prosthetic joint infections. Besides culture-dependent methods, the tested methods included sequencing (by Sanger and next generation sequencing), a pathogen detection platform (Ibis T5000 biosensor), quantitative PCR (qPCR) and fluorescence in situ hybridization (FISH).

3 Identification of microorganisms in disease

In order to obtain a microbial diagnosis, suitable samples must be collected and submitted to appropriate tests. There are several elements to consider regarding acquisition and handling of samples (Box 1). It is important that several samples are collected from the infection site in order to obtain reliable results since it has been shown that several infections (particularly infections involving biofilms) exhibit a heterogeneous spatial distribution of microorganisms throughout the infection site (**article III, V and VI**) (Burmølle *et al*., 2010).

Box 1. Sampling considerations

Time considerations

- ***Samples should be collected as soon as possible after onset of disease.***
- ***Samples for culture should be collected before antimicrobial treatment is initiated.***

Sample site

- ***Samples must be collected to represent infection site avoiding microorganisms from the surrounding area.***
- ***Sampling of appropriate samples using suitable collection methods depends on infection*** **(**Table 1**), *but should be done using aseptic techniques and disinfection where possible.***
- ***Multiple samples should be collected from within the site of infection if possible.***

Transportation

- ***Suitable transport conditions should be used, depending on the type of test to be performed.***

The type of sample collected depends on the anatomic site of infection, which together with sample volume and accessibility of infected material influences the choice of collection method (Table 1). After samples have been collected, they are either processed on site or transported to an appropriate laboratory. Since it is possible that microorganisms may perish or be overgrown by other species during the transport, it is important that transport is rapid and that the viability of any pathogen is protected (Slack, 2007).

Table 1: Common clinical samples used for diagnosis of pathogens in clinical microbiology.

Infection	Anatomic site	Appropriate sample	Collection method
Cystic fibrosis	Lower respiratory tract	Sputum	Expectoration
		Bronchoalveolar lavage fluids	Aspiration
		Endotracheal aspirates	Aspiration
Chronic venous leg ulcers	Superficial wound	Pus or irrigation fluid	Aspiration
		Purulence from beneath dermis	Swab, biopsy
Necrotizing soft tissue infections	Deep wound	Blood culture[a]	Aspiration
		Ulcer edge[a]	Needle aspiration
		Purulence from infection site	Biopsy
		Tissue from infection site	Biopsy
Prosthetic joint infections	Prosthesis	Peri-implant tissue[b]	Biopsy
		Synovial fluid[b]	Aspiration
		Biofilm from removed prosthesis	Sonication
Sinusitis	Sinus	Secretions from aspiration or wash	Aspiration
		Biopsy material	Biopsy

Appropriate clinical samples based on (Baron and Thomson, 2011) except [a] (Stevens *et al.*, 2005) and [b] (Della Valle *et al.*, 2010).

Samples for standard culture-dependent techniques should be kept in suitable transport media and treated directly after arrival at the laboratory. Samples for molecular methods are most often also kept in transport media for direct processing, but can also be frozen (Baron and Thomson, 2011). Freezing of samples might be done with or without a stabilization reagent, depending on the extraction protocol (for instance RNA*later*® solution for RNA extraction). Samples for molecular

methods need to be subjected to nucleic acid extraction prior to analysis. This is a critical pre-analytic step for all molecular methods and may require some optimization since extraction methods that work for one pathogen in a particular sample type may not work for another pathogen or another sample type (Nolte and Caliendo, 2011).

Overall, the range of different methods for identification of microorganisms consists of phenotypic identification, molecular identification and visualization methods. The methods can be group into those that require growth and subsequent isolation of pathogens into pure cultures, and methods where complex microbial communities can be directly analyzed without the necessity of obtaining monomicrobial cultures before analysis. Although the latter can be used to analyze both complex and monomicrobial communities, the use of some methods on pure culture isolates may be excessive compared to the information that can be obtained (Figure 2).

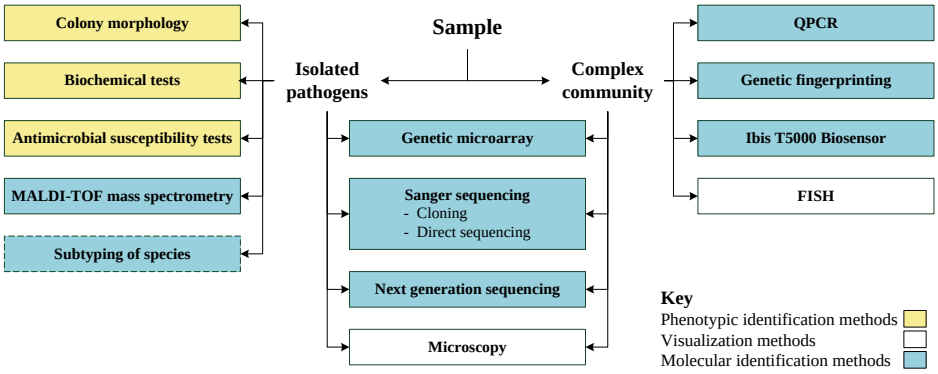


Figure 2: Overview of methods for identification of microorganisms in samples obtained from infected patients. The methods are either based on culture and isolation of pathogens or independent of pure culture isolation. The latter can also be applied to pure cultures, but this use of some of the methods may be excessive compared to the obtainable information. The methods are classified as either phenotypic, visualization or molecular methods according to the key. Subtyping of species is included in this overview although it is not strictly speaking an identification method.

Culture-dependent methods have been the backbone of the approved diagnostic methods in the healthcare systems since the first use of culture media for recovery of bacteria from human disease sites (Atlas and Snyder, 2011). However, in other disciplines of microbiology such as study of microorganisms in natural and industrial ecosystems, the detection and identification of microorganism is now entirely based on methods targeting microbial RNA or DNA. A wide array of molecular methods have been developed (the most common are included in Figure 2), driven by a need for faster and more accurate methods with reduced hands-on-time (Barken *et al.*, 2007; Costerton *et al.*, 2011). Implementation of these methods in clinical microbiology has been slow and is still not complete. In the USA such methods are generally only approved by the Food and Drug Administration (FDA) for detection of a small number of pathogens that are difficult to culture (Costerton *et al.*, 2011). One of the reasons for the continued use of culture-dependent methods as gold standard is the possibility of assessing antimicrobial susceptibility of isolates.

3.1 Methods based on culture and isolation of pathogens

In clinical microbiology a number of classical tests are used for identification of medically important microorganisms. These are typically based on growth of microorganisms in a predetermined, artificial environment that is designed to mimic the conditions of the natural habitat of the microorganisms. Common culture media contains water, growth factors, and sources of carbon and nitrogen and may be liquid, semi-solid, or solid (Atlas and Snyder, 2011). Typically the tests used at clinical microbiology departments do not give a full identification of all isolated microorganisms; most laboratories use simple and incomplete methods of identification depending on the level of information required. Such shortcuts are taken to achieve timely reporting of relevant pathogens and the choice of analytical approaches is constrained by cost. For example, typing of microorganisms is not performed in the daily routine, but only used in special cases (Slack, 2007).

3.1.1 Isolation of pathogens

Individual microorganisms are isolated from complex samples by use of solid media, where the colonies can be distinguished from each other based on their properties (Figure 3). Inoculation of the media requires different techniques depending on the sample type; fluids may have to be centrifuged, swabs can be rolled directly onto plates, tissue and bone should be minced, and processing of prosthetic material may require sonication before inoculation (Baron and Thomson, 2011; Larsen *et al.*, 2012). A wide variety of media are available, each with a specific use. Samples from sites that are normally sterile may be investigated with media designed for propagation of all possible microorganisms, while other media can be used to promote growth and identification of specific microorganisms while restricting growth of others (Atlas and Snyder, 2011). Based on the site of infection, suspected pathogens and the doctor's requests, appropriate medium and incubation conditions are chosen. Samples for anaerobic culture have special growth conditions, and since these grow more slowly than aerobic and facultative microorganisms, at least five days of incubation is necessary before it can be reported as negative (Baron and Thomson, 2011). In cases such as *Propionibacterium acnes*, a longer incubation of up to two weeks may be necessary (**article VI**) (Larsen *et al.*, 2012).

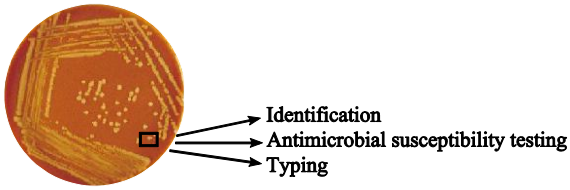


Figure 3: Streaking of solid media plate enables isolation of distinct colonies that can be further investigated in order to obtain identification of pathogen, test antimicrobial susceptibility and determine the subtype of the species (if this is indicated).

If more than one colony type is present, subcultures of each are made to ensure a pure culture of the unknown microorganisms for further characterization and identification (Atlas and Snyder, 2011). Also, the potential pathogens must be differentiated from members of the normal microbiota. This is largely based on recognition of usual contaminants and pathogens of the particular sample site according to Koch's postulates. Identification of pathogens can be aided by correlating culture results with microscopy evaluations and the relative quantities of each isolate. However, in samples from presumably sterile anatomic sites potential pathogens occur in any quantity (Baron and Thomson, 2011).

It may be possible to classify the microorganisms based on growth on specific selective media, nutrient requirements, colony morphology and odor (Atlas and Snyder, 2011; Petti *et al.*, 2011). However, it is often necessary to perform additional tests to determine species identity and antimicrobial resistance patterns (Figure 2 and Figure 3).

3.1.2 Biochemical tests

Biochemical tests are performed to determine the biochemical profiles of isolated microorganisms, which can enable classification of the microorganisms. There are several biochemical tests available; overall they are all based on the interaction of the isolates with substrates. Generally, the reactivity of the tests is based on pH reaction, enzyme profile, antigen-antibody binding, carbon source utilization, or volatile and non-volatile acid detection, which can be detected by color change or chromatographic changes (Carpenter, 2011; Petti *et al.*, 2011).

Traditionally, the biochemical tests have been tube-based and the results of the tests were compared to charts of expected biochemical reactions. Due to the demand for faster methods, several manual testing kits and instrument based semi-automated or automated methods have been developed (Petti *et al.*, 2011). Commonly used biochemical tests include catalase-, hemolysis-, indole- and oxidase tests among others (Atlas and Snyder, 2011). A specific type of biochemical tests is immunoassays, where antibodies are employed to detect specific molecules in the sample. There are several different types of immunoassays using different strategies to detect the binding of antibodies to their target molecules. One of the most commonly used techniques is enzyme-linked immunosorbent assay (ELISA). Here an enzyme will catalyze a substrate into a detectable (typically colored) product, and such assays has the advantage that they allow for automation of the process, and many platforms are available that can perform a wide repertoire of tests (Carpenter, 2011).

3.1.3 MALDI-TOF mass spectrometry

Mass spectrometry (MS) can been used to determine the chemical identity of materials by using ionization radiation to disrupt the sample material thus forming charged compounds that can be identified according to their mass-to-charge ratio. This principle can be used to identify microorganisms by using matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOF MS), which is increasingly being implemented at clinical microbiology departments as an alternative to biochemical testing (van Veen *et al.*, 2010; Nolte and Caliendo, 2011; Vandamme, 2011). The method can be used directly on intact whole cells (Holland *et al.*, 1996; Krishnamurthy and Ross, 1996), but cell wall disruption and protein extraction may be necessary in some cases to enrich proteins and peptides if whole-cell MALDI-TOF MS analysis is inconclusive (Sauer and Kliem, 2010; van Veen *et al.*, 2010).

Identification by MALDI-TOF MS is based on the following characteristics: 1) spectral fingerprints vary between microorganisms, 2) among the compounds detected in the spectrum, some peaks (molecular masses) are specific to genus, species, and sometime to subspecies, 3) obtained spectra are reproducible as long as the bacteria are grown under the same conditions (Carbonnelle *et al.*, 2011). The procedure thus provides a unique mass spectral pattern for the microorganisms based on which the identity can be determined (Seng *et al.*, 2009; Sauer and Kliem, 2010; Carbonnelle *et al.*, 2011). The patterns can be analyzed efficiently in high throughput using various algorithms (Freiwald and Sauer, 2009; Sauer and Kliem, 2010).

MALDI-TOF MS is referred to as a molecular method in this thesis although it strictly speaking is a chemotaxonomic method, since microorganisms are classified based chemical markers. The method

requires that the investigated microorganisms are from pure cultures to ensure sufficient amounts of cells and because mixed mass spectra currently cannot be resolved (Freiwald and Sauer, 2009).

3.1.4 Antimicrobial susceptibility tests

Determination of antimicrobial susceptibilities of significant isolates is one of the principal functions of the clinical microbiology laboratory (Jorgensen and Ferraro, 2009; Turnidge *et al.*, 2011). The main objective of susceptibility testing is to predict the outcome of treatment with an antimicrobial agent, and to guide clinicians in the selection of the most appropriate agent (Turnidge *et al.*, 2011).

There are several options with regard to methodology and selection of agents for susceptibility testing. The selection of agents depends on the likelihood of encountering resistant microorganisms, which agents are commonly prescribed by physicians, and in particular which species are being tested for susceptibility (Turnidge *et al.*, 2011). There are several methodologies available; overall these can be categorized as disk diffusion and dilution methods. Disk diffusion methods are used to categorize microorganisms as susceptible, intermediate or resistant. The method uses commercially prepared filter paper disks impregnated with an antimicrobial agent at a specified concentration. The disks are applied to the surface of an agar plate inoculated with the microorganism, and after incubation the plates are evaluated to see if zones of growth inhibition appear around the disks. The zones are connected to the susceptibility of the microorganism and diffusion rate of the microbial agent through the medium (Jorgensen and Ferraro, 2009; Patel *et al.*, 2011). Disk diffusion testing has an inherent flexibility in drug selection and is low in cost (Turnidge *et al.*, 2011). Dilution methods (such as broth and agar dilution and antimicrobial gradient strips) are used to determine the minimum inhibitory concentration, which is the lowest concentration of microbial agent that will inhibit growth over a defined period of time. This is determined by exposing microorganisms to serial dilutions of the antimicrobial agent (Patel *et al.*, 2011; Turnidge *et al.*, 2011). Dilution methods have the advantage that they produce a quantitative result and may be useful in testing some anaerobic or fastidious microorganisms (Jorgensen and Ferraro, 2009; Turnidge *et al.*, 2011). Furthermore, automated instruments have become available for susceptibility testing. Depending on the system these may have limited flexibility in agent selection and may not detect subtle resistance mechanisms, but can generate results faster than conventional methods (Turnidge *et al.*, 2011).

3.1.5 Subtyping of species

In some cases identification of subtypes of microorganisms is desired, for instance, in epidemiological studies. Subtyping is not a method for microbial identification, but rather for differentiating bacterial isolates beyond the species level. A wide array of methods can be used to achieve this end, the choice of which depends on the intended application and the wanted level of differentiation. Commonly used methods include phenotypic-based methods (such as serotyping and phage typing), different types of genetic fingerprinting typically following PCR amplification of certain genes and gene sequencing. One of the first DNA sequence-based subtyping methods was multilocus sequence typing (MLST), which can be used for distinguishing and relating bacteria on the intra- and interspecies level (Gerner-Smidt *et al.*, 2011). The method characterizes bacterial isolates based on the sequences of internal fragments (450-500 bp) of typically seven house-keeping genes scattered around the genome, referred to as loci (Maiden *et al.*, 1998; Enright and Spratt, 1999). For each locus, a sequence that varies in even a single nucleotide is assigned a distinct allele number, and the combination of the alleles of the seven loci constitutes the sequence type of each isolate. MLST ignores the total number of differences in the sequences of each allele, and sequences

are given different allele numbers whether they differ at a single nucleotide site or at many sites (Enright and Spratt, 1999). The use of multiple loci is essential to achieve the resolution required to provide meaningful relationships among strains (Maiden *et al.*, 1998).

One of the strengths of MLST is the availability of international databases on the internet containing data derived from thousands of isolates of the major pathogenic species. Since the method is based on sequencing the results can readily be compared to these databases (Maiden *et al.*, 1998; Enright and Spratt, 1999; Gerner-Smidt *et al.*, 2011). The use of housekeeping genes means that the found sequence types are stable over time, since these genes are typically under little selective pressure and the accumulation of changes therefore is relatively slow. This might, however, lead to limited discriminatory power, and more rapidly evolving genes may therefore be used instead. (Enright and Spratt, 1999; Gerner-Smidt *et al.*, 2011).

MLST is primarily used on isolates, however, recent work has indicated that the method potentially can be used directly on clinical samples (sputum from CF patients) (Drevinek *et al.*, 2010).

3.2 Methods that do not require isolation of pathogens

Several methods exist that do not require growth of microorganisms and can be used to directly investigate complex samples, including microscopy and molecular methods.

The development of the polymerase chain reaction (PCR) using two primers, thermostable polymerase and thermal cycling (Saiki *et al.*, 1988) was a milestone in biotechnology and a profound advance within molecular diagnostics. The method allows for fast amplification of a nucleic acid target. PCR has many applications and several techniques have been developed for the analysis of the resulting amplification products (Box 2).

Box 2. Analysis of PCR products

Real time analysis

- ***Fluorescence quantification*** PCR products are quantified in real time by addition of a fluorescence reporter to the amplification reaction.

Post amplification handling

- ***Fingerprinting*** PCR products are analyzed based on band pattern arisen from methods such as gel- or capillary electrophoresis.
- ***Sequencing*** PCR products are analyzed based on the nucleic acid sequence.
- ***Mass spectrometry*** Base composition of PCR products are inferred from precise mass determination.
- ***Hybridization*** Presence of specific nucleic acid sequences is determined by target-probe hybridization.

One of the key molecules for identification of microorganisms is ribosomal RNA (rRNA) genes that have variable and conserved regions, which are utilized in broad-range phylogenetic analysis (Barken *et al.*, 2007). The conserved region constitutes target sites for primers, while the variable regions form the basis for phylogenetic analysis, and the identification of microorganisms is thus based on ancestry (Amann *et al.*, 1995; Coenye and Vandamme, 2003; Vandamme, 2011). Besides broad-range molecular methods it is possible to use molecular methods that are target-specific. These do, however, require some degree of prior knowledge of infecting microorganisms, but may be faster to perform and have increased sensitivity (Maiwald, 2011). PCR-based methods are numerous and commonly used in many settings. However, these methods do not offer the opportunity to investigate the spatial distribution of microorganisms, which is possible by microscopy methods.

3.2.1 Microscopy

The microscope has played an important role in biology and medicine since the first description of microscopic life forms (Wiedbrauk, 2011). In clinical microbiology microscopic examination can be used to obtain different goals; 1) evaluate the quality of the sample, 2) observe presence of potential pathogens and 3) provide presumptive identity of potential pathogens (Baron and Thomson, 2011).

Several types of microscopes have been developed; the most frequently used in the clinical setting is the compound light microscope. Other microscopes that are used in the clinical microbiology laboratory include dark-field microscopes, phase-contrast microscopes and fluorescence microscopes (Wiedbrauk, 2011). While dark-field and phase contrast microscopes can be used to directly observe microorganisms in clinical material, it is otherwise usually necessary to alter the sample to improve contrast and aid differentiation of microorganisms from sample material. This can be accomplished by adding positively charged color stains, which will bind to the negatively charged surface of most microorganisms. Examination of samples using stains is a rapid way to obtain a presumptive bacteriological diagnosis (Baron and Thomson, 2011). There are two basic types of stains: simple stains which color all objects in the same manner (allowing for enumeration of organisms and some determination of shape and size) and differential stains which are used to detect differences in structure among microorganisms. The most commonly used differential stain is the Gram stain (Atlas and Snyder, 2011).

Gram stain

The differential Gram staining procedure uses crystal violet and safranin stains. Gram positive cells will retain the crystal violet stain, whereas the stain can be washed away from Gram negative cells, which are subsequently stained by the safranin counter stain. The method thus enables classification of Gram positive and Gram negative bacteria based on differences in their cell wall structure. Identification of the Gram negative and Gram positive microorganisms is primarily based on morphology, and is a crude method that often needs to be confirmed by other methods (Atlas and Snyder, 2011).

Microscopy of Gram-stained smears is the best routine method to distinguish between contaminants and microbes present at the infection site. The infection site should demonstrate many polymorphonuclear leucocytes and few squamous epithelial cells. Presence of squamous epithelial cells would suggest contamination with members of the normal microbiota (Baron and Thomson, 2011; Bjarnsholt *et al.*, 2011).

3.2.2 FISH

Since the first description FISH more than two decades ago (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Amann *et al.*, 1990), the technique has become one of the most widely used approaches to study microorganisms directly in natural systems without prior cultivation and isolation. The principle of FISH is based on hybridization of fluorescently labeled oligonucleotide probes to ribosomal rRNA. A typical FISH protocol includes four steps; 1) fixation and permeabilization of the sample, 2) hybridization, 3) washing steps to remove unbound probes and 4) detection of cells that contained the target sequence and therefore retained the probe and became fluorescently labeled. The detection of fluorescently labeled cells is typically achieved by microscopy, and is possible due to the large number of ribosomes in active cells. The probes are relatively small (generally between 15 and 30 nucleotides) which should enable them to cross permeabilized cell walls and access the binding site (Giovannoni *et al.*, 1988). However, some cell types require additional treatment by enzymes or

chemicals to ensure sufficient permeabilization (Nielsen *et al.*, 2009). Based on the composition of the probe it is possible to specifically target a narrow phylogenetic group or any other higher phylogenetic hierarchical group (Amann *et al.*, 2001). Efficiency of probe binding depends on the hybridization and washing conditions and the three-dimensional structure of rRNA since not all sequences are equally accessible for the probes. Loop and hairpin formation as well as rRNA-protein interactions hinder hybridization, leading to differential sensitivity of oligonucleotide probes (Giovannoni *et al.*, 1988; Moter and Göbel, 2000).

Using FISH it is possible within a relatively short time to obtain knowledge of phylogenetic characteristics, microbial community structure and spatial and relative distribution of individual microorganisms in their natural habitat (Nielsen *et al.*, 2009). However, the signal intensity of the hybridized probes can sometimes be below the detection limit. To resolve this several variations of the FISH protocol has been developed. These include use of helper oligonucleotide probes, signal amplification with reporter enzymes and peptide nucleic acid (PNA) probes (Kerstens *et al.*, 1995; Nielsen, 1999; Fuchs *et al.*, 2000). PNA probes have a non-charged peptide backbone to reduce electrostatic repulsion, which can otherwise impede binding. The use of PNA probes have been reported to allow stronger hybridization and the protocols for hybridization are much faster than for oligonucleotide probes (Egholm *et al.*, 1993; Bjarnsholt *et al.*, 2009; Thomsen *et al.*, 2012). The reduced background fluorescence and hands-on time makes the use of PNA-FISH more suitable for investigation of clinical samples than conventional FISH.

3.2.3 QPCR

An increasing number of published clinical studies have shown the usefulness of qPCR for diagnosis of microbial pathogens. The increased use of qPCR is caused by the simple, sensitive and fast nature of the method (Espy *et al.*, 2006; Barken *et al.*, 2007; Wittwer and Kusukawa, 2011). Because amplification and analysis of PCR product occurs in the same step (real-time analysis) the risk of contamination is minimized and turnaround time improved (Espy *et al.*, 2006; Nolte and Caliendo, 2011; Wittwer and Kusukawa, 2011). The principle of qPCR is relatively simple; it is a PCR reaction with addition of fluorescence reporter (either intercalating fluorescent dyes that bind to double stranded DNA or specific probes labeled with fluorescent dyes) that can be measured using precision optics. The results can be used quantitatively based on the assumption that there is a linear relationship between quantity of input template and the amount of generated product and therefore signal, which is measured during the exponential phase of amplification. Based on this relationship qPCR measures how rapidly fluorescence signals exceed a threshold; the fewer cycles it takes to cross the threshold the higher the initial template concentration (Bustin, 2004; Nolte and Caliendo, 2011).

Although the results from qPCR can be quantitative, this term should be interpreted with caution, taking into account that the results are logarithmic and that variation of measurements changes with concentration (Bustin, 2004). At best a 0.5 $\log_{10}$ variance (corresponding to a threefold difference) is documented to exist between repeats of the same initial template concentration. This is important to bear in mind during evaluation of results, so that small differences do not take on assumed relevance (Wolk and Hayden, 2011).

A number of FDA-approved and commercial qPCR assays for detection of viruses, bacteria, fungi, and parasites have become available. Viruses remain the most common target for qPCR in the clinical microbiology laboratory; however, the applicability of qPCR is much wider (Wolk and

Hayden, 2011). Packages of qPCR assays that enable screening of multiple microorganisms commonly found in specific diseases are focuses for development and commercialization.

3.2.4 Genetic fingerprinting

One of the most basic ways to verify PCR products or interpret polymorphic DNA fragments (such as those that arise from 16S rRNA PCR on complex samples), is the use of genetic fingerprinting (Bassam *et al.*, 1992). Polymorphic DNA fragments may differ in length or sequence, which can be interpreted by simple procedures such as gel electrophoresis or capillary electrophoresis of fluorescently labeled fragments (van Belkum, 1994; Frye and Healy, 2011; Gerner-Smidt *et al.*, 2011). In this way a genetic fingerprint can be obtained, either directly on the DNA fragments or after treatment with enzymes (restriction length polymorphism, terminal-restriction fragment length polymorphism (T-RFLP) and amplified fragment length polymorphism) (Marzorati *et al.*, 2008; Gerner-Smidt *et al.*, 2011). Fingerprints are recorded as banding patterns which can be obtained by several gel-based electrophoresis methods, where the fragments are separated according to length (standard gel electrophoresis, pulse field gel electrophoresis) or sequence (denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis) (van Belkum, 1994; Marzorati *et al.*, 2008; Gerner-Smidt *et al*., 2011).

For gel-based electrophoresis detection methods, interpretation of results is performed either visually or by using a gel documentation system to scan and record the gel images. (Frye and Healy, 2011).

3.2.5 Sanger sequencing

Determination of the nucleotide sequences of DNA molecules by the chain-termination method (named the Sanger sequencing method) has been one of the most influential innovations in biological research. The key principle of Sanger sequencing is the use of modified nucleotides; dideoxynucleotide triphosphates (ddNTPs). The method uses primers that anneal to single-stranded target DNA, and incorporate nucleotides by the aid of DNA polymerase. When a modified nucleotide is incorporated strand elongation cannot continue, so four reactions are made, each with a different ddNTP. The product will be four mixtures of partial sequences of varying length, which can then be separated according to size by gel electrophoresis according to the original method. Since the ddNTP are labeled (originally with radioisotopes) the pattern of products can be read, and the nucleotide sequence determined (Sanger *et al.*, 1977). The method has been improved and streamlined by use of fluorescent labeling and capillary electrophoresis. This has enabled use of a single reaction with 4 differently labeled ddNTPs, and changed the output format from a sequences ladder to fluorescent peak trace chromatograms (Nolte and Caliendo, 2011). Software translates these traces into DNA sequence, while also generating error probabilities for each base-call. After gradual improvement over the years Sanger sequencing can now achieve read-lengths of over 1000 bp, and per-base accuracies as high as 99.999% (Shendure and Ji, 2008).

A condition for the Sanger sequencing method to work is that the target sequence is pure to avoid mixed chromatograms that are difficult to interpret. To obtain pure products fingerprinting techniques or cloning can be used before sequencing. Sanger sequencing has historically only been used directly on PCR products when it can be assumed that a sample contains a single species of microorganism (Maiwald, 2011).

Separation of PCR products by cloning

Separation of nucleic acid fragments by cloning is a technique that can be used before sequencing of polymorphic DNA fragments. The so-called clone libraries are created by inserting the individual DNA fragments into cloning vectors, which are subsequently transformed into bacterial cells. Each of the resulting cells should contain one plasmid with a single DNA fragment. Following proliferation of the transformed cells, sufficient copies of the plasmids are produced for sequencing (Cohen *et al.,* 1973).

To obtain reliable results that represent the original sample an adequate number of clones should be sequenced. What constitutes "adequate" depends on the complexity of the sample since clone libraries have some biases towards low-abundance targets (Higuchi *et al.,* 2011; Maiwald, 2011). Equations to estimate if the number of clones is adequate have been developed, such as Good's coverage estimator and the Chao estimator.

Construction and sequencing of clone libraries is labor intensive, time consuming and costly, and the method is therefore not appropriate for implementation at clinical microbiology departments. Methods to circumvent construction of clone libraries while still enabling sequencing of complex samples are therefore highly relevant to fully utilize the potential of sequencing.

Direct sequencing of PCR products

Identification of pure cultures based on 16S rRNA gene sequencing has been well established and in some cases implemented at clinical microbiology departments, but the ability to perform direct sequencing of selectively amplified molecular marker genes fragments from complex clinical samples is of increasing interest (Kommedal *et al.,* 2008; Nolte and Caliendo, 2011).

In an effort to apply Sanger sequencing directly to mixed microbial community, a web-based application has been developed by iSentio (Bergen, Norway) for interpretation of chromatograms of mixed PCR products from multiple species. The application is build on an algorithm that sort out the ambiguous signals from mixed chromatograms in order to identify the different contributing bacteria. The application only reports identities having a similarity that reaches an empirically set cutoff, and in cases where more than one species from the same genus are found, only the highest scoring species are reported. Using these constraints mixed chromatograms from polymicrobial samples containing up to three different species can be sequenced directly in a time efficient manner (Kommedal *et al.,* 2008, 2009).

3.2.6 Next generation sequencing

Although the throughput of Sanger sequencing has been advanced by the development of capillary electrophoresis and algorithms to resolve complex chromatograms, the experience of sequencing the human genome showed that the method was not able to efficiently analyze complex diploid genomes at low cost (Lander *et al.,* 2001; Wheeler *et al.,* 2008; Higuchi *et al.,* 2011). To resolve the issues of throughput, price and requirements of cloning that are inherent to the Sanger sequencing method, several next generation sequencing platforms have been developed that offer great improvements in terms of total sequence production and reduction of cost and time (Barken *et al.,* 2007; Wheeler *et al.,* 2008; Higuchi *et al.,* 2011; Liu *et al.,* 2012).

Although there are differences in the strategies used by the different next generation sequencing platforms, their workflow are conceptually similar and differ from Sanger sequencing in a number of ways. They all feature clonal amplification of libraries that are prepared by *in vitro* ligation, which

obviate the need for laborious cloning of DNA library into bacteria. Also, the sequencing occurs by synthesis, meaning that the DNA sequence is determined by the addition of nucleotides to the complementary strand rather than the strategy of chain termination used in Sanger sequencing. Finally, the DNA templates are spatially segregated and sequenced simultaneously in a massively parallel manner, unlike Sanger sequencing that requires a physical separation step (e.g. transfer of individual templates to wells in a microtiter plate) (Shendure and Ji, 2008; Higuchi *et al.*, 2011; Liu *et al.*, 2012).

There are several applications of next generation sequencing regardless of the platform, including mapping of structural rearrangements, epigenetics (by analysis of DNA methylation or immunoprecipitation followed by sequencing), transcriptome analysis and RNA sequencing (Shendure and Ji, 2008; Liu *et al.*, 2012). In the context of microbial identification the most relevant application of next generation sequencing is amplicon sequencing and whole genome sequencing (Mardis, 2008; Liu *et al.*, 2012). These applications have conceptually different approaches, and are either based on PCR amplification or fragmentation of DNA before sequencing (Figure 4).

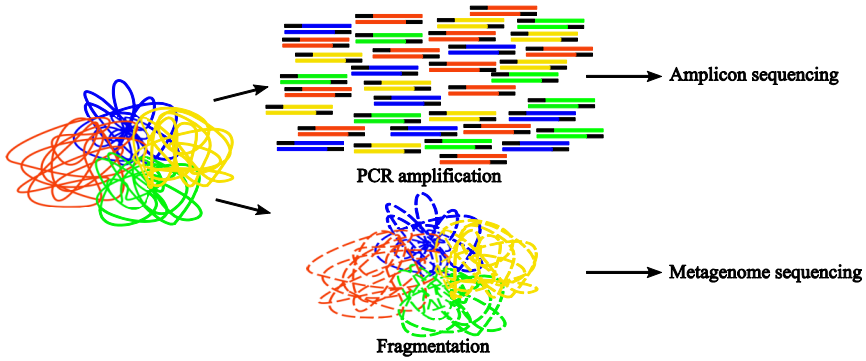


Figure 4: Conceptual differences between amplicon and genome sequencing. For amplicon sequencing PCR is used to amplify target region before next generation sequencing, while genome sequencing uses fragmentation of genomic DNA before next generation sequencing. If genomic DNA originates from a complex sample sequencing of all the genomes is referred to as metagenome sequencing.

Compared to Sanger sequencing the read-length that can be obtained by next generation sequencing may be short. This can be problematic for assembly of sequences into full genomes after genome sequencing. However, the potential utility of short-read sequencing has been tremendously strengthened by the availability of whole genome assemblies for major model organisms, as these effectively provide a reference against which short reads can be mapped (Shendure and Ji, 2008). The short reads does, however, influence the ability to perform *de novo* assembly.

The sequencing field is advancing rapidly, and evolution of next generation sequencing platforms has brought huge advancements in performance accuracy, applications, consumables, manpower requirement and obtainable read length (from 250 bp to 700 bp for the Roche 454 system, 35 bp to 75 bp for the ABI SOLiD system and 36 bp to 150 bp for the Illumina system) (Shendure and Ji, 2008; Liu *et al.*, 2012). Furthermore, new technologies are being developed, including compact sequencers and third generation sequencing platforms. Although no consensus definition of third generation sequencing has been established yet, two main characteristics have been described. These include an ability to perform sequencing with no prior PCR (single-molecule sequencing), and capture of signal

(either fluorescent or electric current) in real time instead of the strategy of "washing and scanning" used in next generation sequencing. These characteristics mean that sequencing analysis can be performed very rapidly but still in high throughput and that read lengths are greatly increased. Third generation platforms include the PacBio RS by Pacific Bioscience, and the Oxford Nanopore sequencer (Schadt *et al.*, 2010; Liu *et al.*, 2012). The compact sequencers (among others the Ion Torrent platform) sit between next generation sequencing and third generation sequencing since they require "washing and scanning". The Ion Torrent performs sequencing by synthesis by measuring changes in pH due to release of hydrogen ions as part of the base incorporation process. This methodology dramatically accelerates the time to result and reduces costs but does not result in longer read lengths (Schadt *et al.*, 2010). For clarity reasons the term NGS will be used to cover all these platforms throughout the rest of the thesis.

3.2.7 Ibis T5000 biosensor

In contrast to most other methods, the Ibis T5000 biosensor uses high-performance mass measurements to analyze nucleic acid sequences. The Ibis T5000 biosensor is based on a multilocus approach, and uses PCR to amplify broadly conserved regions, including ribosomal sequences and housekeeping genes, by use of "intelligent primers" (Hofstadler *et al.*, 2005; Vandamme, 2011). The choice of primers follows a strategy of redundancy, and is made up by several broad-range and clade-specific primers, so that one type of microorganism should be targeted by more than one primer set (Hofstadler *et al.*, 2005). The PCR products that are in the 80–140 bp size range, are desalted and analyzed by electrospray ionization (ESI) MS. The resulting spectral signals are then processed to determine the accurate masses of both strands of all the PCR products. This allows sufficient accuracy to determine the base composition of each amplicon based on the discrete masses associated with different combinations of the four nucleotide bases. The base compositions from multiple primer pairs are used to "triangulate" the identity of the organisms present in the sample (Hofstadler *et al.*, 2005; Ecker *et al.*, 2006). The method is commercially available in the form of the Ibis T5000 biosensor and Abbott PLEX-ID instruments (Ecker *et al.*, 2006, 2008; Jacob *et al.*, 2012). These instruments have several pre-prepared assays, in the form of 96 well plates, offering broad range identification of bacteria, fungus and virus, targeted identification of a specific microorganism or characterization of microorganisms based on subtyping and drug resistance determination (Ecker *et al.*, 2006, 2008; Eshoo *et al.*, 2009; Grant-Klein *et al.*, 2010). The applicability of the instruments are wide and can be used both for identification and characterization of a broad range of pathogens and for molecular genotyping, by following the same general principle as MLST but detecting variation based on base composition and not sequence (Ecker *et al.*, 2008, 2009; Hall *et al.*, 2009; Gerner-Smidt *et al.*, 2011).

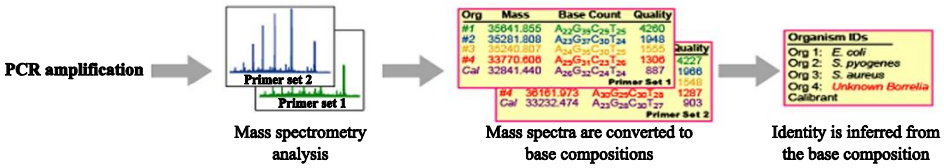


Figure 5: Illustration of the principle of the Ibis T5000 biosensor, where PCR products are analyzed by mass spectrometry. The mass spectra that are obtained for the various primer sets are converted into a base count for each PCR strand, which forms the basis for determination of species identity by triangulating the results from multiple primer sets and comparing them to an integrated database. The picture was modified from (Ecker *et al.*, 2006)

To analyze a sample, extracted nucleic acids are transferred into wells of the microtiter plate that each contains one or more primer pairs. The following PCR amplification produces a mixture of PCR products, representing the complexity of the original sample. The Ibis T5000 and Abott PLEX-ID contain robotics that handle the desalting of the samples in 96-well plate format and sampling into the ESI mass spectrometer, which leads to a minimized hands-on time. The software associated with the instruments identifies the microorganism by first determining the masses and associated base compositions from the mass spectrometry data, and then comparing the results across primer pairs (Ecker *et al.,* 2006). To do this a key element of the system is utilized; a curated database that associates base counts with primer pairs for thousands of microorganisms (Ecker *et al.,* 2006; Nolte and Caliendo, 2011).

The Ibis T5000 biosensor has the potential of providing the relative amount of each of the detected microorganisms in a sample by use of an internal calibrant. This is a nucleic acid sequence that is similar to the primer target sites and is amplified during PCR of the sample. The generated calibrant amplicon has a deletion that uniquely distinguishes it from the amplicons that are produced from the sample. Since the concentration of the calibrant in each PCR is known, the calibrant can be used to obtain quantitative results, but also functions as an internal positive control (Ecker *et al.,* 2008).

3.2.8 Genetic microarrays

The basic principle of genetic microarray is build upon hybridization experiments to screen for specific DNA sequences from a sample, performed in a small and highly parallel format. In a genetic microarray hundreds to thousands of nucleotide probes are bound to a solid surface, typically glass, in precise patterns. The sample nucleic acids can hybridize to these probes, and ultimately the array-bound sample can be detected (Gerner-Smidt *et al.,* 2011; Nolte and Caliendo, 2011). The target nucleic acid can be either RNA or DNA, and a variety of sample preparation methods exist for different array types. Common for the methods is either amplification of the target while tagging or incorporating biotinylated or fluoresceinated nucleotides, or staining of nucleic acids using fluorescence dyes (Southern, 2001; Nolte and Caliendo, 2011). In order for the target to bind to the probes the amplicons must be single stranded to ensure hybridization to the immobilized probes with complementary sequences (Southern, 2001). After hybridization of sample to the microarray, binding of targets can be detected, and the amount of hybridized sample can be quantified based on signal intensities (Barken *et al.,* 2007).

The genetic microarray platform has many types of applications depending on the probes attached to the surface. These include transcriptome analysis (e.g. comparison of mutants and wild-type strains, or strains under different growth conditions), analysis of gene expression, identification of single nucleotide mutations, detection of species specific sequences, virulence genes and genes encoding antimicrobial resistance, and discovery and characterization of pathogens (Barken *et al.,* 2007; Nolte and Caliendo, 2011). Gene expression analysis by microarrays has been used to study several pathogens and been implemented for cases such as viral hepatitis infections (Miller and Tang, 2009). Furthermore, arrays have been described for the detection of some pathogenic prokaryotes, eukaryotes and viruses (Wang *et al.,* 2002; Wilson *et al.,* 2002). Despite these experiences and the advantage of high throughput analysis offered by the method, the genetic microarrays have so far had little direct impact on diagnostic microbiology (Barken *et al.,* 2007; Miller and Tang, 2009).

4 Comparison of methods

It is clear that many methods exist for detection, identification and subtyping of microbial species. No matter which method is used for investigation of microorganisms, the key to success or failure lies with proper transport conditions, storage conditions and general handling of the primary sample. Furthermore, the first steps taken during analysis are often pivotal for the outcome. The results from culture-dependent methods are dependent on the choice of nutrients and incubation conditions applied during the first inoculation of samples. Similarly, the results that can be obtained by the molecular methods depend on the choice of protocol for extraction of nucleic acids. The sensitivity of the molecular analyses is directly affected by the quality of nucleic acids used as input. To represent the true microbial community in a sample the extraction protocol must be unbiased and simultaneously remove inhibitors. Also, since the sensitivity is influence by the ratio between target and background nucleic acids, selective DNA extraction may be suitable for clinical samples that often contain nucleic acids from the host that can reach dominating concentrations (**article III**), which may interfere with PCR. Elimination of these unwanted nucleic acids (for instance by MolYsis™ pretreatment) may improve sensitivity (**article VI**) (Gebert *et al.*, 2008), but it is possible that nucleic acids from some microorganisms are also eliminated (Horz *et al.*, 2010).

Another consideration for analysis by molecular methods is the possibility that extracted DNA originates from non-viable microorganisms, for instance, from cells that have been eradicated by antimicrobial treatment, which is not clinically relevant. To avoid analysis of these molecules it is possible to use RNA-based methods or pretreatments such as propidium monoazide photo-induced cross-linking of extracellular DNA and DNA in cells with compromised cell membranes (Nogva *et al.*, 2003; Rudi *et al.*, 2005). At present no ideal method for nucleic acid extraction has been found, and the optimal extraction protocol may vary depending on the sample type. Extraction issues mean that although the molecular methods have high analytical sensitivity, the sensitivity for detection of microorganisms within a sample may be reduced. Theoretically, culture has a higher sensitivity than molecular methods since only a single cell needs to be present to result in growth. However, the reality is that some microorganisms are not detected by the standard culture-dependent methods used at clinical microbiology departments. Lack of detection can be caused by exposure to antimicrobial agents, biofilm formation, entry into a viable but non-culturable state, slow growth rates, improper handling of anaerobes or requirement of as yet undiscovered growth conditions (often referred to as unculturable, although this term may be misleading and due to insufficient culture optimization) (Vartoukian *et al.*, 2010).

It is possible to investigate microorganisms directly in the sample by microscopy, which is not possible by culture-dependent methods or molecular methods. Direct investigation of sample material means that no concentration of targets occurs, and introduction of biases associated with these steps can thereby be avoided. However, microscopy-based methods have high detection limits.

Besides the general issues that are connected with culture-dependent methods, microscopy and molecular methods, the individual methods have different advantages and limitations (Table 2) that influence which method might be more suitable to use for investigation of microorganisms in different settings and sample types.

Table 2: Overview of the methods for identification of microorganisms covered in this thesis and their advantages and limitations (methods that require pure cultures are indicated by *). The turnaround time of the methods does not take pre-analytical handling into account, based on references: a, Seng et al. (2009); b, Savelkoul and Peters (2011); c, Frye and Healy (2011); d, Liu et al. (2012); e, Ecker et al. (2008) and f, Miller (2011).

	Principle	Advantages	Limitations	Time
Phenotypic identification methods				
Isolation of pathogens	Microorganisms are grown on media according to nutrient requirements.	▪Gold standard in clinical microbiology. ▪Allow for antimicrobial susceptibility testing. ▪Require few specialized instruments.	▪Strongly affected by transport conditions. ▪Antimicrobial treatment hampers growth. ▪Standard culture conditions may be insufficient	1-14 days[a]
Biochemical tests*	Isolates are incubated with various reagents; based on interaction with these identification can be made.	▪Widely standardized and implemented. ▪Availability of convenient test format and automated systems.	▪Costly, depending on type and number of tests performed. ▪May not distinguish closely related species. ▪Based on anticipation of certain pathogens.	5-48 hours[a]
Antimicrobial susceptibility tests*	Isolates are incubated with antimicrobial agents and inhibition of growth is evaluated.	▪Very clinically important information. ▪Only establish method to determine susceptibility. ▪Highly standardized.	▪Cannot predict systemic effectiveness. ▪Test concentration may be unrealistic. ▪Susceptibility patterns can vary for subtypes of species.	1-2 days[a]
Visualization methods				
Microscopy	Identification is based on observations of morphological traits, often assisted by staining reactions.	▪Cost efficient. ▪Evaluation of sample quality. ▪Investigation of spatial distribution. ▪Can aid interpretation of culture results.	▪Limited specificity. ▪Condition of cells influences the results. ▪Microorganisms that are small or lack cell wall can be problematic.	<1 hour[a]
FISH	Microorganisms are identified based on hybridization of fluorescently labeled probes to ribosomal sequences.	▪Same advantages as normal microscopy, but has greater specificity. ▪Can be used in conjunction with staining.	▪Depend on physiological and activity state. ▪Only species that are targeted are detected. ▪Probe design can be problematic. ▪Insufficient probe penetration and hybridization can cause low signal intensity.	1.5-4 hours[b]
Molecular methods				
MALDI-TOF MS*	Isolates are identified based on mass spectra that provide specific protein mass patterns.	▪Cost efficient. ▪Software enables rapid data analysis. ▪Customizable databases. ▪Standardized and validated. ▪May be used on positive blood cultures.	▪Requires monomicrobial samples. ▪Same bacteria can give different mass spectra depending on culturing conditions. ▪Depend on correctness of the database. ▪Protein extraction might be necessary.	<1 hour[a]
MLST*	Typing of isolates is based on sequencing of multiple loci. Different sequences are assigned a distinct allele, and sequence type is based on alleles from all loci.	▪Data is unambiguous and portable. ▪Standardized schemes are available. ▪Availability of large international reference databases.	▪May lack discriminatory power. ▪Intolerant to sequencing errors. ▪Multiple PCRs increase risk of contamination.	As Sanger sequencing + data anlysis

Table 2 continued

	Principle	Advantages	Limitations	Time
qPCR	PCR is monitored by use of fluorescence reporters. Amplification results in increase in fluorescent signal which can be measured.	▪No post amplification handling. ▪Can give quantitative results. ▪Highly sensitive and wide dynamic range. ▪Availabillity of standardized assays.	▪Sensitive to inhibitors. ▪Copy numbers may not portable. ▪Has inherent variability. ▪Only targeted species will be found.	4-8 hours[b]
Genetic fingerprinting	PCR products are evaluated based on differences in sequence or length by electrophoresis.	▪Time and cost. ▪Simultaneous comparison of numerous samples.	▪Limited resolution. ▪Difficulties in data interpretation. ▪Limited comparability across gels.	1-2 days[c]
Sanger sequencing	Fluorescently labeled chain terminator nucleotides are incorporated during strand synthesis, and the sequence can be determined by electrophoresis.	▪Data is unambiguous and portable. ▪High quality raw sequences. ▪Long read-lengths can be obtained. ▪Vast databases are available.	▪Generally requires pure target sequences. ▪Costly. ▪Low throughput. ▪Databases may contain uncurated sequences. ▪Universal similarity cut-offs are not defined.	<3 hours[d]
Clone libaries	DNA fragments are inserted into plasmids that are transformed into bacteria cells.	▪Polymorphic DNA fragments can be individually analyzed.	▪Laborintensive and costly. ▪Representation of entire community requires "adequate" clone picking.	3 days + data analysis
Direct sequencing	PCR products from low diversity samples can be directly sequenced. Mixed chromatograms may be resolved by an algorithm (Ripseq).	▪Improved turnaround time compared to cloning. ▪Not labor intensive. ▪Automated dataanalysis.	▪Only verified for up to three different species per PCR. ▪Identification depends on correctness of the database.	As Sanger sequencing.
NGS	Massively parallel sequencing, where libraries are prepared *in vitro* and sequencing occurs by synthesis.	▪Data is unambiguous and portable. ▪High throughput. ▪Might be used for typing of strains. ▪May be used without prior PCR. ▪Continuous rapid improvements in the field.	▪May give short read lengths. ▪Require extensive bioinformatic analysis. ▪Dependent on correctness of databases. ▪Universal similarity cut-offs are not defined. ▪Sample batching may be necessary.	Up to 4 days[d] + data analysis
Ibis T5000 Biosensor	Mass spectrometry-derived base composition signatures obtained from PCR amplification of multiple loci is used to identify most organisms present in a sample.	▪Portable base composition data. ▪Primer redundancy and combination of broad and narrow range targets. ▪Targets some antimicrobial resistance genes. ▪Highly automated. ▪Can give quantitative results.	▪Novelty of method means that is has not been widely tested and cost is high. ▪Treshold setting influence results. ▪Dependent on database accuracy. ▪Interpretation of mass-spectra is "black-box".	4-6 hours[e]
Microarrays	Specific nucleotide sequences are detected based on hybridization to probes affixed to the microarray.	▪High-throughput multiplexing. ▪Can screene for species or species types. ▪Low-volume and sensitive analysis. ▪May be used without prior PCR. ▪Can give quantitative results.	▪Only targeted sequences can be detected. ▪Laborious array design. ▪Costly due to fluorescence staining of traget nucleic acids.	8 hours[f]

4.1 The optimal method?

The best method for identifying microorganisms should provide sensitive, universal, flexible and quantitative results even in complex samples within a short period of time, preferably with potential for automation. Based on these criteria, the phenotypic identification methods are not optimal, both due to lack of growth of some microorganisms, but also because the identification of microorganisms by these methods can be laborious, time-consuming and costly depending on the number of tests that must be performed (Table 2). Furthermore, phenotypic markers might show variability due to environmental cues such as the condition of the culture, subculture and storage (Sauer and Kliem, 2010). The time and cost is to a large extend linked with biochemical testing, and the increasing implementation of MALDI-TOF MS as an alternative to these tests have been reported to reduce costs of bacterial identification by 68-89% and allow much faster identification of microorganisms (Seng *et al.*, 2009; Gaillot *et al.*, 2011). Although MALDI-TOF MS is mostly used for identification of isolates, it has been shown to be applicable to pellets from positive blood culture bottles (after lysis of erythrocytes); however, polymicrobial infections cannot be resolved (Prod'hom *et al.*, 2010). Since culture-dependent methods are not optimal for identification of all microorganisms a definition of new gold standard method is required. However, the best method will vary depending on the goal of the investigation, and the best method in a research setting differs from the best method in a clinical laboratory, since the latter is restrained by time and cost consideration to a larger extend than research. Furthermore, in research it is important to investigate all microorganisms in samples while clinical microbiology is more focused on the treatment of the infections.

4.1.1 From a research perspective

Investigation of all microorganisms and their interactions in a complex sample can be obtained by a number of different methods. For identification of the microorganisms, sequencing (by Sanger sequencing and NGS) and subsequent comparison with reference sequences in databases is particularly relevant since these results are generally unambiguous. Despite the general agreement of the importance and applicability of sequencing, there is no universal consensus on the degree of sequences divergence permissible within a species or genus, and the cut-off value is variable and depends on the gene target and the microorganism. Sanger sequencing has the distinct disadvantage that pure targets are required, which necessitate use of either fingerprinting or clone library construction. While fingerprinting may have insufficient resolution, construction of clone libraries is very time consuming and labor intensive (Table 2). Although it might be possible to perform Sanger sequencing directly on DNA from a mixed microbial community by using the Ripseq algorithm to interpret mixed chromatograms, the number of species that can be resolved in a single sample is limited. Massively parallel amplicon sequencing by NGS platforms overcome these problems and the continued and rapid developments in this field have improved sequence quality, read-length, bioinformatic data analysis and reduced costs to a degree that makes the use of such methods very attractive compared to Sanger sequencing.

Currently studies of microbial communities are primarily based on molecular marker genes. A typical target for bacterial identification is the 16S rRNA gene, whereas fungal identification does not have such a "gold standard gene", though internal transcribed spacer regions and 28S rRNA genes have been shown to be useful for fungal identification (Petti *et al.*, 2011). The use of a single marker molecule limits the ability to identify all microorganisms in a sample, since the different DNA sequences are competing for the same reagents during PCR. Therefore, species present in low concentrations are at risk of not being detected. Also, design of universal primers that targets all

bacteria is not possible (Baker *et al.*, 2003). For instance, "universal" 16S rRNA primers designed for qPCR and frequently used in the literature, can underestimate the total number of bacteria present in a sample (**article III**). The ability to perform sequencing without prior PCR is therefore highly relevant in order to study the true microbial diversity in samples, and can be achieved by using NGS. Performing whole genome- and metagenome sequencing might offer insights into the potential properties of microorganisms in infections and yield information that is clinically relevant. The work that is required to translate the sequence information into clinically relevant information (and validate the findings) is at present too exhaustive to be applicable in clinical microbiology departments. As more microbial genomes and molecular marker genes are sequenced, the value of using microarrays to navigate microbial genomes in a high-throughput manner is increased. The value of the microarrays lies in the ability to screen multiple samples, and can be used complimentary to NGS applications. It is, however, possible that NGS will eventually out-compete microarrays.

The use of microscopy-based methods can enable investigation of the actual microorganisms, rather than their genetic material, and thus be used to obtain relevant information (both in a research setting but also in a clinical setting). Using FISH specific microorganisms can be targeted and visualized, which can be helpful for detection of the specific target, for investigation of spatial distribution in clinical samples or observation of atypical morphotypes. FISH has the advantage that it offers increased specificity compared to microscopy with or without staining, where identification of microorganisms is based on morphology and chemical or physical properties (Table 2).

4.1.2 From a clinical perspective

One goal for clinical microbiology is the ability to screen for panels of probable pathogens, and many methods are available to achieve this, including FISH, qPCR and microarrays. FISH has the advantage of low cost and limited equipment requirements and can be the fastest way to detect microorganisms, although it may require experienced personnel to locate the microorganisms in the samples by microscopy. However, the limited sensitivity means that it is only suitable for some samples. QPCR may be performed faster than FISH and has a higher sensitivity. Compared to other PCR based methods, qPCR is less prone to contamination since there is no post-amplification handling, which contributes to the speed of the method. Furthermore, the results can be used quantitatively (Table 2). These advantages have meant that qPCR has become the main stay for diagnosis of viral infections. Both FISH and qPCR require assumptions to be made regarding the identity of pathogens. The choices of primers and probes dictate which microorganisms can be detected; and although multiplexing might possible, the capability for this is limited. The principle of microarrays is similar and microorganisms are detected by probe hybridization, but use of this method reduces the problem of assumption-based detection due to the high degree of multiplexing offered by the method (Table 2). Microarrays have been used directly on extracted nucleic acids, pooled PCR products from multiple universal genes and products from random PCR (Gerner-Smidt *et al.*, 2011). The use of microarrays has been limited due to complexity of array design, and high price (both acquisition price and running price due to use of fluorescence staining of target nucleic acids). Compared to these methods, the Ibis T5000 biosensor has the advantage that the multilocus approach, detection redundancy and base composition determination makes it less dependent on assumptions to be made prior to analysis. Furthermore, the commercial available assays for microbial identification also include detection of some antimicrobial resistance genes, and the use of an internal calibrant makes it possible to obtain quantitative results. The relative novelty of the method does, however, mean that the price of this method is high, and validation of the approach is currently not

sufficient for implementation in clinical microbiology. Amplicon sequencing, particularly using compact NGS platforms, would similarly diminish the need for assumptions of pathogen identity to be made prior to analysis. The use of Ibis T5000 biosensor, microarrays and amplicon sequencing will allow for more complete analysis of microbial communities. This would offer a sense of confidence that the disease causing pathogens has been detected, and might also give an idea of the focus of infection that can be clinically relevant.

Quantitative results are of interest in clinical microbiology, based on the assumption that abundance of species is indicative of importance and may be used to evaluate if an antimicrobial treatment has worked. Several methods can be used to obtain quantitative results, including FISH, qPCR, microarrays and reportedly amplicon sequencing. Generally, quantitative interpretation of data obtained after broad-range PCR is problematic due to biases in the PCR amplification, and internal calibrants are necessary to obtain reliable quantitative results. Case in point, the abundance of amplicon sequences is sometime reported as reflecting the relative abundance of species in a sample (Monchy *et al*., 2011), but comparison of quantitative results by amplicon sequencing, qPCR and the Ibis T5000 biosensor indicated that this is not true (**article I**). A consideration for the use of quantitative measurements as an indicator of importance is that, although organisms might be present in low numbers they may be highly active or pathogenic and thereby potentially important players in infection, indicating that evaluation based on presence in numbers should be done cautiously.

Commonly used subtyping methods such as MLST and genetic fingerprinting might have insufficient resolution. Typing by whole genome sequencing or the Ibis T5000 biosensor offers both a higher resolution and can be done quickly, which is particularly important in epidemic outbreaks. Although microarrays can be designed for typing of species, their use is more suitable for screening for certain species types. Whole genome sequencing represents the ultimate typing method, and this use of NGS is readily applicable, but may be restrained to reference laboratories.

Evaluation of the behavior of microorganisms in a clinical sample (e.g. observation of presence in biofilms) is of great importance in clinical microbiology since this can affect the treatment outcome. To this end microscopic visualization will continue to be relevant in clinical microbiology setting, and use of FISH might improve the results. Generally, the main focus of clinical microbiology is on the treatment of infections and therefore the optimal methods in this setting would be the ones that can measure if a treatment is functional. Currently, only culture-dependent methods allow for antimicrobial susceptibility testing, and although it might be possible to use molecular methods to detect antimicrobial resistance genes, this information may not be sufficient. It is quite possible that the optimal method would be a combination of culture-dependent antimicrobial susceptibility testing with molecular analysis to ensure rapid and sensitive reporting. The initial implementation of molecular methods in clinical microbiology has been targeted to specific samples or pathogens, since a large part of added value of the methods lies in the ability to analyze culture-negative samples, particularly those arisen from administration of antimicrobial treatment.

The implementation of new methods into clinical microbiology requires extensive validation and optimization of robust standardized laboratory protocols, as exemplified by the current paradigm change from biochemical testing to chemotaxonomic-based identification (i.e. MALDI-TOF MS). This has been driven by the validation and optimization work that has been taking place since the 1990s, along with development of software packages for data analysis and establishment of comprehensive databases (Kliem and Sauer, 2012). This indicates that it might still be several years before molecular methods are implemented in clinical microbiology departments.

5 Application of molecular methods

Molecular methods have several applications within the clinical microbiology field, and several cases have been published where various molecular methods have been used on samples from different types of infections. In the following, the knowledge obtained by using molecular methods to study microbial communities in samples from patients with NSTIs, chronic wounds and CF is examined, as these types of infections were the central focus of this PhD study.

5.1 Case: NSTIs

The group of infections covered by the term NSTI is diverse and includes infections of the skin, subcutaneous tissue, fascia or muscle that are associated with necrotizing changes. NSTIs are rare but highly lethal infections, requiring rapid diagnosis and treatment (including antimicrobial therapy and aggressive surgical debridement) (Anaya and Dellinger, 2007). The gold standard for diagnosis of the microorganisms involved in NSTIs is standard culture-dependent methods, despite the rapid progression of the disease. Use of molecular methods for identification of microorganisms involved in NSTIs is limited in the literature, and the most frequent use of molecular methods is on isolates. The typical analyses are sequencing of 16S rRNA gene fragments to confirm finding of uncommon pathogens, or subtyping of species by MLST and/or PCR on virulence-, superantigen-, toxin- and adhesin genes followed by fingerprinting (Table 3).

Table 3: Overview of cases where molecular methods have been used on isolates to either confirm findings by culture or for typing of species.

Confirmation of culture by16S rRNA gene sequencing		**Typing of isolates**	
Microorganism	**Reference**	**Microorganism**	**Reference**
Bacillus cereus	(Lee *et al.*, 2010)	*Klebsiella oxytoca*	(Oishi *et al.*, 2008)
Campylobacter rectus	(Lam *et al.*, 2011)	*Klebsiella pneumoniae*	(Gunnarsson *et al.*, 2009)
Klebsiella oxytoca	(Oishi *et al.*, 2008)	*Staphylococcus aureus*	(Miller *et al.*, 2005; Morgan *et al.*, 2007; Thuong *et al.*, 2007; Changchien *et al.*, 2011)
Raoultella planticola	(Kim *et al.*, 2012)		
Shewanella algae	(Myung *et al.*, 2009)		
Sphingobacterium multivorum	(Grimaldi *et al.*, 2012)	*Streptococcus dysgalactiae* subsp. *equisimilis*	(Kittang *et al.*, 2010)
Streptococcus dysgalactiae subsp. *equisimilis*	(Kittang *et al.*, 2010; Nei *et al.*, 2012)	*Streptococcus equi* subsp. *zooepidemicus*	(Korman *et al.*, 2004)
Streptococcus equi subsp. *zooepidemicus*	(Korman *et al.*, 2004)	*Streptococcus pneumoniae*	(Ballon-Landa *et al.*, 2001)
Vibrio vulnificus	(Muldrew *et al.*, 2007)	*Streptococcus pyogenes*	(Demers *et al.*, 1993; Kaul *et al.*, 1997; Norrby-Teglund *et al.*, 1998; Erdem *et al.*, 2004; Hassell *et al.*, 2004; Jing *et al.*, 2006; Meisal *et al.*, 2008)

Besides the use of molecular methods for confirmation of culture-based findings and typing of isolates, a few studies exist where molecular methods have been used to directly identify microorganisms. These include 16S rRNA gene sequencing of isolates that could not be identified by conventional methods (Clarke *et al.*, 2010; Bempt *et al.*, 2011). A few studies used molecular methods on tissue, including PCR-based methods in cases that gave culture-negative results (Muldrew *et al.*, 2005) or as a rapid method for detection of *Streptococcus pyogenes* (Louie *et al.*, 1998). Additionally, use of FISH has been reported in two cases; to identify microorganisms commonly involved in NSTI (Figure 6) (Trebesius *et al.*, 2000) and to distinguish the more pathogenic *S. pyogenes* from non-group A streptococci (Sing *et al.*, 2001).

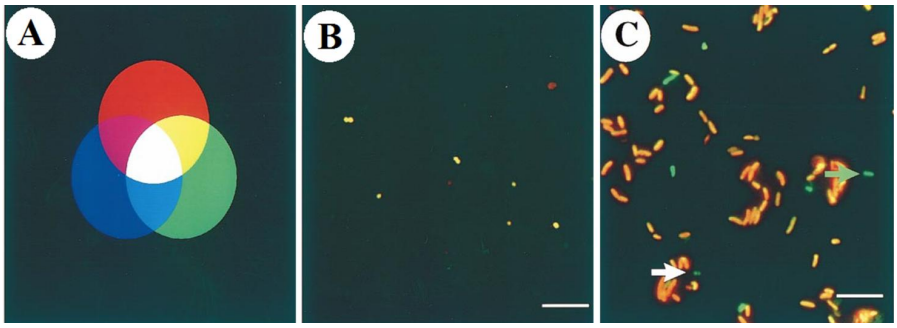


Figure 6: FISH images of NSTI samples (Trebesius *et al.*, 2000). A: The use of multiple probes means that binding of two or more different probes results in mixed colors. B: Patient 2 was found to have *S. pyogenes* (yellow, probes Strpyog-Cy3 and EUB338-FLUOS). C: Patient 3 was found to have *Pseudomonas aeruginosa* (yellow/orange, probes Pseaer-Cy3 and EUB338-FLUOS) and other bacteria (green), one with rod (green arrow) and one with a spherical morphology (white arrow). The scale bar represents 10 µm.

By using a combination of PNA FISH and DAPI staining it can be possible to visualize both microorganisms and human cells in the infected tissue from NSTI patients (Figure 7) (**article I**). Only few bacteria could be detected in the infected areas and the observed bacteria were located in clusters. Conversely the amount of inflammatory cells was high. Observation of both inflammatory cells and coagulation indicates that the human hosts are combating the infections.

The low abundances of bacteria have interesting possible explanations. It might indicate that the tissue destruction in large part may be caused by the host immune system. It may also be that the antimicrobial treatment administered to the patients before procurement of samples has rendered them metabolically inactive, and therefore difficult to detect by FISH. Another explanation might be that the pathogens have moved on from the destroyed area. It is, however, also possible that microorganisms were present but in concentrations below the detection limit. Further studies are needed to determine the cause of the observed low microbial abundance.

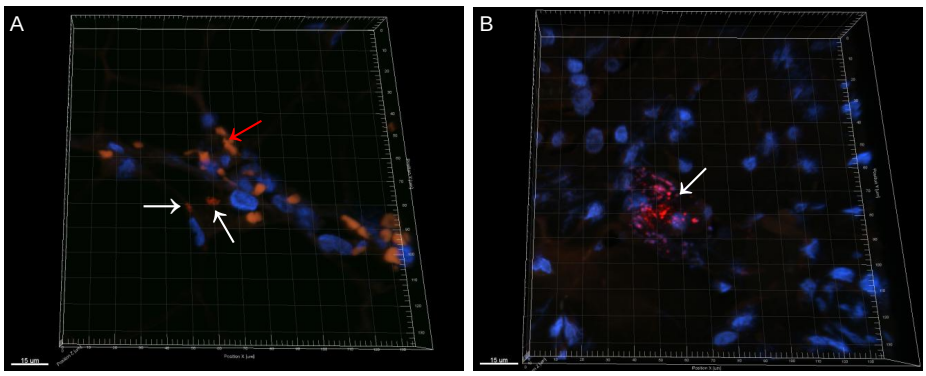


Figure 7: Visualization of samples using universal bacteria PNA FISH probe and DAPI staining (article I). Few microorganisms (bright red, indicated by white arrows) could be detected in the samples from two NSTI patients. A large amount of human host cells were observed (blue). Additionally, in the sample from patient 9B (frame A) coagulated blood could be observed (orange, indicated by red arrow), which was not observed in sample from patient 8A (frame B). The scalebars represent 15 µm.

Use of broad-range PCR based methods for detection of all microorganisms directly in NSTI samples has only been published in a single case study (Muldrew *et al.*, 2005). To investigate the applicability of such methods for rapid identification of NSTI pathogens, total DNA from samples from 10 NSTI patients were investigated by direct rRNA gene Sanger sequencing, construction of near full-length 16S rRNA gene clone libraries and the Ibis T5000 biosensor. The findings by molecular methods were compared with culture results obtained as a part of routine diagnostic of the patients (**article I**).

Identification of microorganisms based on culture-dependent methods was confirmed by all the applied molecular methods in 35% of the investigated samples. For the remaining samples, molecular methods either found partial concordance of the results (molecular methods typically identified additional microorganisms) or gave discrepant results (due to negative culture reports) (Table 4).

Table 4: Overview of the microorganisms found in 20 tissue samples obtained from 10 NSTI patients by culture-dependent methods and molecular methods (DS: direct Sanger sequencing, CL: clone libraries and Sanger sequencing and I: Ibis T5000 biosensor) (article I).

Sample	Culture	Molecular methods
Concordance of results		
1A	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
1B	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
1C	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
1D	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
2A	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
2B	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
4A*	*Streptococcus* sp.	*Streptococcus pyogenes*[DS, I], *Streptococcus didelphis*[I]
8A*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*[DS, I]
8B*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*[DS, I]
9A	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
9B	*Streptococcus pyogenes*	*Streptococcus pyogenes*[DS, CL, I]
10A	Negative	Negative[DS, CL, I]
10B	Negative	Negative[DS, CL, I]
Partial concordance of results		
3A	*Acinetobacter baumanii*	*Acinetobacter baumanii*[CL, I], *Streptococcus pyogenes*[DS]
5A	*Clostridium paraputrificum, Bacteroides fragilis*	*Clostridium paraputrificum*[CL, I], *Bacteroides fragilis*[I], *Streptococcus agalactiae*[I], *Streptococcus pyogenes*[DS], Uncultured bacterium[CL]
6A*	*Streptococcus pyogenes, Escherichia coli*	*Streptococcus pyogenes*[DS, I], *Escherichia coli*[I], *Mycoplasma hominis*[DS], *Bacteroides fragilis*[I], *Staphylococcus epidermidis*[I], *Staphylococcus hominis*[I], *Cladosporium cladosporioides*[I]
7A*	Fungus	*Candida albicans*[I], *Mycoplasma* spp.[DS, I], *Fusobacterium necrophorum*[DS, I]
Discordance of results		
4B	Negative	*Streptococcus pyogenes*[DS, CL, I]
7B	Negative	*Mycoplasma salivarium*[DS, CL], *Mycoplasma* spp.[I], *Fusobacterium necrophorum*[CL, I]
8C*	Negative	*Streptococcus pneumoniae*[DS, I]

Samples marked with * were analyzed by 16S rRNA amplicon sequencing to evaluate the findings of the other methods.

The findings in six samples were evaluated by amplicon sequencing of 16S rRNA gene fragments using the Roche 454 pyrosequencing platform (Figure 8). These samples were from all three groups of results (concordant, partial concordant and discordant). For four of the samples (patient 4A, 8A,

8B and 8C) the amplicon sequencing confirmed the findings by the other molecular methods. For the remaining two samples, amplicon sequencing revealed additional microorganisms (in patient 6A these were *Peptoniphilus* sp. and *Porphyromonas* sp., in patient 7A it was *Prevotella* sp. and *Sharpea* sp.). The findings of *Candida albicans* and *Cladosporium cladosporioides* by Ibis T5000 biosensor could not be confirmed, since these were not targeted by 16S rRNA gene based methods.

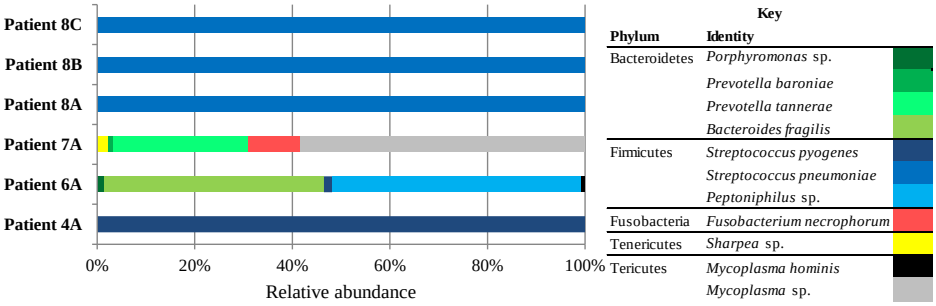


Figure 8: The taxonomic groups identified by 16S rRNA amplicon sequencing. The stacked graph illustrates the relative abundance of each taxon (color coded according to the key).

These results indicate that use of molecular methods may be valuable diagnostic tools for identification of microorganisms in NSTIs. 15% of the samples were found to be culture-negative although microorganisms could be identified with the molecular methods. Interestingly, all these samples originated from patients where other samples taken from different places in the infection sites resulted in identification of microorganism by culture. Furthermore, the molecular methods also identified microorganisms that were not registered in the culture reports for 20% of the samples, which together with the reduced turnaround time that can be obtained by some of the molecular methods may be relevant for the fulminant NSTIs.

5.2 Case: Chronic wounds

Chronic wounds are a major and ever increasing healthcare problem and a contributing factor in hundreds of thousands of annual deaths (Dowd *et al.*, 2008; Sen *et al.*, 2009). There are three major types of chronic wounds defined by the predisposing condition of the patient: venous leg ulcers, diabetic foot ulcers, and pressure ulcers (Dowd *et al.*, 2008). The wound environment is ideal for microbial growth due to tissue necrosis which provides ample nutrients (Bowler *et al.*, 2001).

Some studies exist where the microbial findings by culture-dependent methods are compared with the findings by molecular methods, such as 16S rRNA gene analysis (by fingerprinting and Sanger sequencing, cloning and Sanger sequencing, and amplicon sequencing using NGS platforms) and Ibis T5000 biosensor. The studies showed that molecular methods detect a greater microbial diversity than culture no matter which molecular method was used (Figure 9). The larger diversity found by the molecular methods is both due to ability to detect microorganisms that are not found by culture, but also because the molecular methods were able to distinguish between different species or genera of microorganisms where culture-dependent methods might only be able to identify certain groups or families. The number of species found in the studies varied depending on the number of samples included in the studies, suggesting that the microbial flora of individual patients varied greatly.

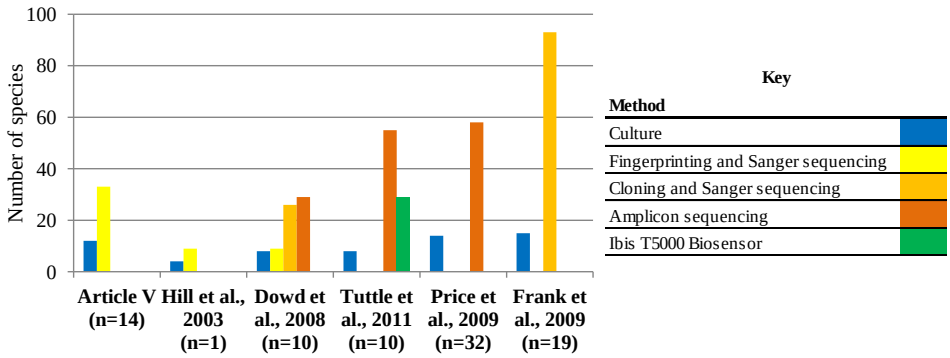


Figure 9: Overview of studies where identification of microorganisms by culture-dependent methods is compared with various molecular methods. The number of species detected by each method is seen (color coded according to the key). Article V, (Hill *et al.*, 2003), (Dowd *et al.*, 2008) and (Tuttle *et al.*, 2011) are studies of chronic venous leg ulcers, while (Price *et al.*, 2009) and (Frank *et al.*, 2009) are studies of chronic wounds with various predisposing conditions. N= the number of wounds included in the studies.

A factor contributing to the lower microbial diversity found by culture-dependent methods may be the formation of biofilms within chronic wounds, which is increasingly being appreciated as an important part of the pathogenesis. It is hypothesized that the wounds are kept chronic by the bacterial burden (Bjarnsholt *et al.*, 2008; Martin *et al.*, 2010). The first indications of presence of biofilm was obtained by PNA-FISH studies, where large microcolonies of microorganisms surrounded but not penetrated by human host cells (most likely polymorphonuclear neutrophilic leukocytes (PMNs)) were observed (Bjarnsholt *et al.*, 2008) (**article V**). Further studies using alginate-specific immunostaining, showed that the microcolonies were imbedded in a self-produced alginate matrix (Kirketerp-Moller *et al.*, 2008). Studies using FISH to investigate the distribution of microorganisms and host immune cells, have been published (Fazli *et al.*, 2009, 2011), along with studies of competition between different species within *in vitro* biofilms (Malic *et al.*, 2009; Pihl *et al.*, 2010). These competition studies showed that *Pseudomonas aeruginosa* could outcompete other microorganisms, especially if the *P. aeruginosa* strains showed reduced expression of certain virulence factors, which has been linked with enhanced persistence in the chronic wound environment (Malic *et al.*, 2009; Pihl *et al.*, 2010).

Physiological conditions can vary spatially within a wound and create diverse microenvironments that may support different microbial communities. For instance, the surface of a wound seems to support different microbial flora than the deeper wound layers (Fazli *et al.*, 2009; Malic *et al.*, 2009). Furthermore, studies investigating the spatial distribution of microorganisms within individual wounds using molecular methods have indicated that microbial flora also varies across the entire area of the wounds (**article V**) (Andersen *et al.*, 2007; Wolcott *et al.*, 2009; Price *et al.*, 2011; Wolff *et al.*, 2011). These studies used sampling of small discrete locations within wounds, as illustrated in Figure 10.

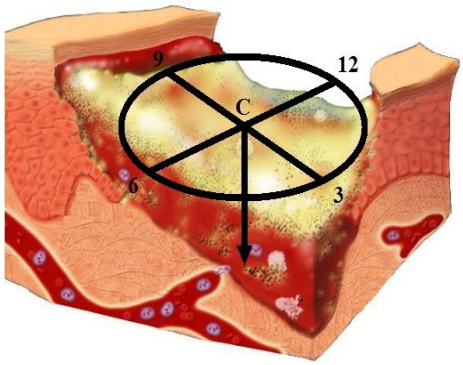


Figure 10: Sampling procedure to investigate spatial localization of microorganisms by molecular methods. Picture obtained from (Cunningham *et al.*, 2010), and edited to illustrate sampling procedure from article V. Samples used by Andersen *et al.* (2007), Wolcott *et al.* (2009) and Price *et al.* (2011) included center of the wound (C), and two opposing edges of the wound (e.g. position 3 and 9).

In addition to showing different microbial flora at discrete sites inside wounds, it was shown that the relative abundance of individual microorganisms also varied at different sites in the individual wounds (**article V**) (Wolcott *et al.*, 2009). The findings indicate how important appropriate sampling is to fully characterize the global wound ecology. However, an ideal sampling method has not been established yet and is a matter of continued debate. It has been suggested that swabs of wound surface are inaccurate. Culture-based investigations have shown that the yield of anaerobes in swabs are lower than that found by biopsies (Davies *et al.*, 2007) and that anaerobes in the wound beds may be implicated in mediation of chronicity of the wounds (Wall *et al.*, 2002; Stephens *et al.*, 2003). A study using qPCR to investigate swabs and biopsies taken during the healing process showed that the number of microorganisms decreased in biopsy samples as the wounds healed, whereas no significant decrease could be found in swab samples, indicating that positive swabs may indicate just the presence of colonizing microorganisms (Gentili *et al.*, 2012).

The identity of microorganisms present in wounds and the effect on wound healing is difficult to assess. Studies comparing the microbial communities in healing and non-healing chronic venous leg ulcers have been performed using 16S rRNA fingerprinting and Sanger sequencing (Davies *et al.*, 2004) and 16S rRNA amplicon sequencing and the Ibis T5000 biosensor (Tuttle *et al.*, 2011). Although both studies found differences in the incidence of specific microorganisms between the two patient groups, the findings in the two studies were not concordant. The evidence from the literature on various chronic wounds is that all wounds are colonized with microorganisms, and differentiating colonizers from invading microorganisms is difficult. Individual wounds have unique profiles of microorganisms, and the spatial distribution of the microorganisms within individual wounds is heterogeneous. This suggests that each wound has to be carefully evaluated, with sampling covering all of the wound area, and that no single pathogen is likely to be the causative agent of such infections.

5.3 Case: CF

"*Anyone who reviews the literature on cystic fibrosis and isn't confused, is confused*"
-Dr. Efraim Racker, 1985

CF is an intensively studied disease, which has increased the knowledge of the disease and resulted in an increase in life expectancy for CF patients. However, the immense literature on the subject can also tend to confuse the understanding of the infections that occur in CF patients. The genetic disorder that causes CF affects several organs. The most severe effect is on the lungs, and respiratory failure accounts for most premature deaths of CF patients (Quinton, 1999; Conese *et al.*, 2003; Parsek and Singh, 2003). Nasal and sinus inflammation is a frequent condition in patients with CF and most CF patients are thought to have sinus infections (Gysin *et al.*, 2000; Rasmussen *et al.*, 2012). However, since the symptoms of sinus infections are less problematic than the symptoms of lung infections, the reported incidences are relatively low (Mainz et al. 2009; Robertson et al. 2008)

5.3.1 Lung infections

The classical understanding of CF pathogens is concerned with a few microorganisms that dominate the culture findings. These belong to the *Pseudomonas*, *Staphylococcus*, *Burkholderia* and *Haemophilus* genera, while other microorganisms (including fungus, Gram negatives, *Achromobacter*, *Streptococcus* and *Mycobacterium* spp.) are less common findings (Figure 11).

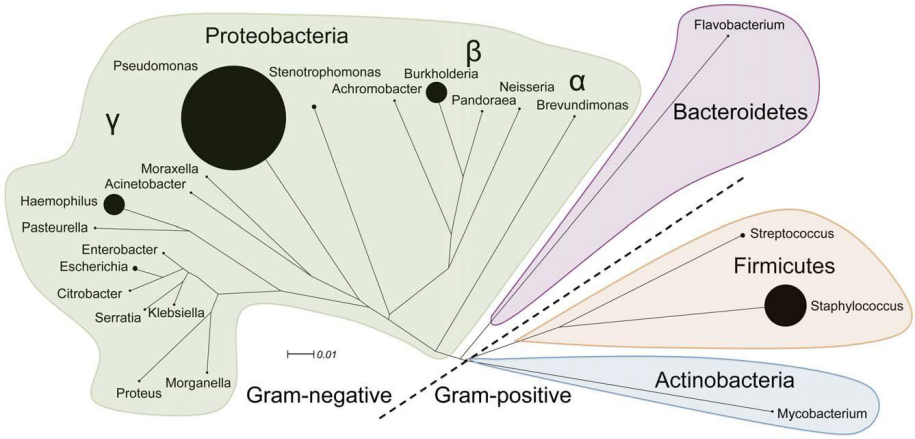


Figure 11: Bacterial genera recovered from CF sputum during 28 years of culture-dependent identification at the Southern Alberta Cystic Fibrosis Clinic (Alberta, Canada). The proportional abundance of each genus is depicted with solid circles (Sibley *et al.*, 2011).

The CF patients experience repeated lung infections that eventually develop into chronic infections. In the classical view of the development of disease, the key pathogens *Staphylococcus aureus*, *Haemophilus influenzae* and *Streptococcus pneumoniae* are considered mainly as causing recurring intermittent infections, whereas *P. aeruginosa* and members of the *Burkholderia cepacia* complex mainly are considered as causes of chronic infections (Høiby, 1982; Gilligan, 1991). It is now generally accepted that chronic infections of CF lungs persist due to formation of biofilms. The early indications of the involvement of biofilm was the isolation of mucoid strains of *P. aeruginosa* from CF patients (Doggett *et al.*, 1964) and microscopy studies of CF lung tissue showing that the microorganisms reside in EPS-enclosed microcolonies within the airway lumen (Lam *et al.*, 1980;

Worlitzsch *et al.,* 2002). Since then, the biofilms produced in the lungs of CF, particularly by *P. aeruginosa,* have become one of the most intensively studied clinical biofilms (Costerton *et al.,* 1999). Findings by FISH suggest that the spatial distribution of *P. aeruginosa* within sputum depends on whether the strain was mucoid or non-mucoid. Aggregated *P. aeruginosa* cells appear to be mainly the mucoid type (Yang *et al.,* 2008). These aggregated microorganisms are protected from the phagocytic action of PMNs, leaving the biofilm surrounded by PMNs, which has been shown for *in vitro* biofilms, and confirmed in lung tissue samples by FISH (Bjarnsholt *et al.,* 2009). Establishment of biofilms and hence chronic infections may be delayed by administration of aggressive antimicrobial therapy, which can eradicate intermittent infections caused by planktonic pathogens. However, once a chronic infection has been established it cannot be eradicated (Høiby *et al.,* 2005).

Various molecular methods have been used to investigate the microorganisms involved in CF lung infections. Until 2003 molecular methods were primarily used on isolated microorganisms and aimed towards typing of pathogenic strains or detection and identification of specific microorganisms, typically isolates that were difficult to identify by phenotypic investigations. Molecular methods are still being used on isolates for typing of strains (e.g. Segonds *et al.,* 2009; Grinwis *et al.,* 2010; Mortensen *et al.,* 2011; Nicoletti *et al.,* 2011; Waters *et al.,* 2012) and identification of microorganisms by methods such as MALDI-TOF MS (Degand *et al.,* 2008; Fernández-Olmos *et al.,* 2012; Marko *et al.,* 2012; Masoud-Landgraf *et al.,* 2012) and 16S rRNA gene sequencing. 16S rRNA gene sequencing has been used in cases of misidentification of isolates or inconclusive culture reports. For instance, the phenotypic identification of Gram negative, oxidase-positive rods (including non-typical *P. aeruginosa*) and anaerobes carries a high risk of misidentification due to their marked phenotypic diversity (Qin *et al.,* 2003; Wellinghausen *et al.,* 2005; Tunney *et al.,* 2008; Kidd *et al.,* 2009; Field *et al.,* 2010). Other cases where 16S rRNA gene analysis has proven useful are in the identification of unusual microorganisms such as *Inquilinus* sp. (Chiron *et al.,* 2005), *Herbaspirillum* sp. (Spilker *et al.,* 2008), *Pandoraea sputorum* (Pimentel and MacLeod, 2008), *Segniliparus rugosus* (Butler *et al.,* 2007), *Brevundimonas diminuta* and *Ochrobactrum anthropican* (Menuet *et al.,* 2008).

Over the past decade, the use of molecular methods without prior isolation by culture, has led to the view that CF airways contain complex polymicrobial communities (Zemanick *et al.,* 2011). The early studies of diversity in sputum samples relied on clone hybridization or genetic fingerprinting methods (van Belkum *et al.,* 2000; Kolak *et al.,* 2003; Rogers *et al.,* 2003, 2004, 2006). Of the fingerprinting methods only T-RFLP is still frequently used today (Rogers *et al.,* 2009; Spasenovski *et al.,* 2009; Sibley *et al.,* 2011; Stressmann *et al.,* 2011, 2012), but most studies of the microbial community in CF lungs use sequencing of the 16S rRNA genes (by cloning and Sanger sequencing or amplicon sequencing) (Bittar *et al.,* 2008; Armougom *et al.,* 2009; Guss *et al.,* 2011; Sibley *et al.,* 2011; Stressmann *et al.,* 2011; van der Gast *et al.,* 2011; Delhaes *et al.,* 2012; Zhao *et al.,* 2012). Although it is often stated in the literature that molecular methods identify more species than the classical CF pathogens found by culture-dependent methods, few studies directly compare findings by culture and molecular methods on samples from the same patients (Figure 12).

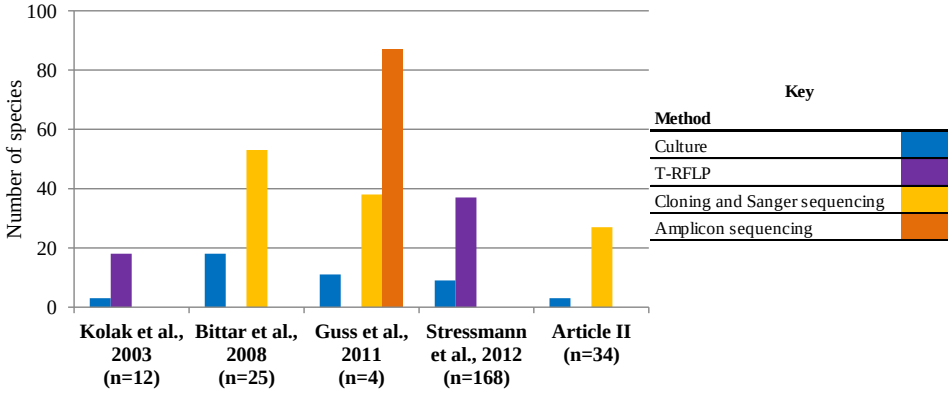


Figure 12: Comparison of studies where detection of species by culture is compared with various molecular methods targeting the 16S rRNA gene. The number of species detected by each method is seen (color coded according to the key). The studies by Kolak *et al.* (2003), Bittar *et al.* (2008), Guss *et al.* (2011) and Stressmann *et al.* (2012) were of CF sputum samples, while Article II was of tissue and sputum excised from explanted CF lungs. N= the number of samples included in the studies.

Direct comparison of the results by culture and molecular methods clearly shows that the latter identified a larger number of microorganisms. It is, however, possible that this discrepancy can be minimized by using more extensive culture approaches (Sibley *et al.*, 2011). Generally, the mean number of species per sputum sample is reported to be around 3 fungi (Delhaes *et al.*, 2012) and 5-7 bacteria (Harris *et al.*, 2007; Bittar and Rolain, 2010; Rogers, Skelton, *et al.*, 2010; Stressmann *et al.*, 2011; Delhaes *et al.*, 2012). However, some studies have found a higher number of species using T-RFLP (Sibley *et al.*, 2008), microarrays (Klepac-Ceraj *et al.*, 2010), and amplicon sequencing (Guss *et al.*, 2011; Zhao *et al.*, 2012).

Routine culture-dependent detection of supposed CF pathogens is carried out on sputum and throat samples produced by the CF patients. Such secretions are nearly always contaminated by microbes from the pharynx and mouth, where aerobic, facultative, and anaerobic bacteria are part of the normal flora, but these contaminants are normally filtered from the culture results (Høiby and Frederiksen, 2000). These types of samples are the most frequently used in the molecular studies of microbial communities involved in CF lung infections, and these results are typically not filtered to remove possible contaminants, which may explain some of the discrepancies in the results by the two types of methods (Goddard *et al.*, 2012). Some authors claim that the potential contamination of sputum and throat samples is not significant (Rogers *et al.*, 2006) or that contaminating saliva can be removed from the sputum sample by a series of washing steps (Rogers *et al.*, 2006; Guss *et al.*, 2011). In a study comparing samples from explanted lungs with throat and sputum samples from the same patients before lung transplantation, differences in microbial communities were observed. The throat and sputum samples contained non-typical microorganisms, which were not found in the explanted lungs (Goddard *et al.*, 2012). Likewise, a study using 16S rRNA gene amplicon sequencing to study the microbial diversity in chronic obstructive pulmonary disease of non-CF patients, indicated that sputum and throat samples were not representative of the lower bronchial mucosa flora (Cabrera-Rubio *et al.*, 2012). Further indications of the problems connected with the use of sputum samples for investigation of lower airway infection is the finding of a heterogeneous distribution of microorganisms in different areas of the lungs, as determined by 16S rRNA gene amplicon sequencing of tissue from different areas of CF lungs (Goddard *et al.*, 2012; Willner *et al.*,

2012). Investigation of the microorganisms in sputum samples using PNA FISH, have additionally shown that the different microorganisms cluster together in distinct, largely monomicrobial aggregates (**article III**) (Figure 13). This further add to the spatial diversity found in the CF lungs, and since the monomicrobial aggregates were not found to be in direct physical contact with each other the biofilm infections may be monomicrobial despite the overall polymicrobial nature of the infection, which can have implications on treatment of CF lung infections.

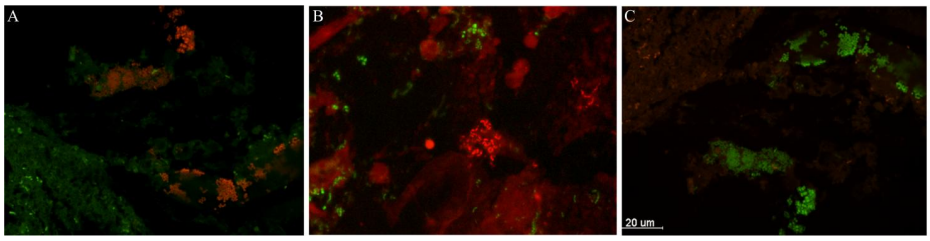


Figure 13: PNA FISH images of bacterial aggregates in expectorated sputum of non-end stage CF patients (article III). The aggregates within the sputum were largely monomicrobial and segregated from each other. A: *S. aureus* (red) and other bacterial species (green), B and C: *P. aeruginosa* (red), and other bacteria (green).

Visualization of microorganisms in lung tissue have shown that microorganisms are found in biofilms in the conductive zone of the lungs, and only rarely in the respiratory zone (where they exist as planktonic cells that can be eradicated by the high number of professional phagocytes) (Bjarnsholt *et al.*, 2009; Ulrich *et al.*, 2010). The lack of microorganisms in the respiratory zone has been linked to the adoption of an aggressive, chronic suppressive antimicrobial treatment strategy, where the antimicrobial agents may be able to eradicate the planktonic cells (Bjarnsholt *et al.*, 2009). Use of FISH is valuable for analysis of CF samples, since normal microscopy might not be informative for the biofilm residing microorganisms that are encountered in the CF lungs (Bjarnsholt *et al.*, 2011). To distinguish common CF pathogens in the samples, several FISH probes have been developed (Hogardt *et al.*, 2000; Wellinghausen *et al.*, 2006; Brown and Govan, 2007).

Many of the additional microorganisms identified by molecular methods are anaerobes or species that have not previously been isolated from CF sputum, making the list of microorganisms associated with lung infection of CF patients ever increasing (as reviewed elsewhere, e.g. Bittar and Rolain, 2010). The different reports of microorganisms involved in CF lung infection may not be directly comparable due to several factors. Firstly, it has been reported that the type of CFTR mutations can affect the observed microbial community, due to different effects on the lung environment by different mutations (Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010). Secondly, the microbial findings in different parts of the world may not be comparable due to differences in treatment regimes and exposure to different microbes. This has been suggested by differences in detection of *P. aeruginosa* and *B. cepacia* complex by culture in Toronto, Canada and Copenhagen, Denmark (Johansen *et al.*, 1998) and supported by investigation of bacterial communities in the lungs of American and British CF patients using molecular methods, where the majority of species were uniquely found in one geographical location (Stressmann *et al.*, 2011).

It has been suggested that certain abundant species are core to CF lung infections by adulthood (i.e. in chronic lung infections) (Stressmann *et al.*, 2011; van der Gast *et al.*, 2011). In the study by van der Gast and coworkers (2011) the core group primarily consisted of species from the oral flora, and

P. aeruginosa was the only traditional key CF pathogen assigned to this group, the remaining traditional CF pathogens were found to be a part of the satellite group. Although the finding of core species may have implications on our understanding of CF, any species present in the CF airways might be important. Interestingly, several studies have found a tendency of less complex microbial communities in the lung of older or deceased CF patients, which is correlated with deterioration of lung function (**article II** and **III**) (Cox *et al.,* 2010; Klepac-Ceraj *et al.,* 2010; van der Gast *et al.,* 2011; Goddard *et al.,* 2012; Stressmann *et al.,* 2012; Willner *et al.,* 2012; Zhao *et al.,* 2012). This loss of diversity indicates that remaining microorganisms were competitively dominant and extremely well suited to the conditions in the CF lungs due to selection during the course of long-term infection and antimicrobial selection (Cox *et al.,* 2010; Yang, Jelsbak, *et al.,* 2011; Hogardt and Heesemann, 2012). Adaptation of pathogens to the CF lung environment has been studied for *Burkholderia dolosa* (Lieberman *et al.,* 2011), *Burkholderia cenocepacia* (Madeira *et al.,* 2011), *S. aureus* (Goerke and Wolz, 2004, 2010; McAdam *et al.,* 2011), *Stenotrophomonas maltophilia* (Pompilio *et al.,* 2011) and *P. aeruginosa*. The latter has been extensively studied, also by advanced methods such as genome sequencing (Smith *et al.,* 2006; Yang, Jelsbak, *et al.,* 2011; Rau *et al.,* 2012) and microarrays (Ernst *et al.,* 2003; Huse *et al.,* 2010; Rau *et al.,* 2010; Warren *et al.,* 2011; Yang, Jelsbak, *et al.,* 2011; Yang, Rau, *et al.,* 2011).

Some concern has been raised whether the microorganisms found by molecular methods are active or if detection were due to extracellular DNA or dead cells. This is a concern for use of molecular methods in all types of samples, but particularly in the CF lungs, where the disease means that material is not cleared quickly (Rogers *et al.,* 2008). Use of reverse transcription and T-RFLP has indicated that many of the identified microorganisms were metabolically active, although differences between detected microorganisms and metabolically active microorganisms were found (Rogers *et al.,* 2005). The results of this study also indicated that although some microorganisms may be present in low numbers, they may be highly active, for instance members of the *B. cepacia* complex (Rogers *et al.,* 2005, 2009). It is, however, possible that such methods impair detection of microorganisms that are present but fail to grow due to the intense immune response and antimicrobial therapy (Goddard *et al.,* 2012). Using propidium monoazide pretreatment it has been shown that DNA from nonviable bacteria can influence the microbial flora detected by molecular methods (Rogers *et al.,* 2008; Rogers, Marsh, *et al.,* 2010; Stressmann *et al.,* 2012).

Although the main focus of CF research has been on lung infections as a cause for the inflammation that results in respiratory failure, it is debated whether inflammation might also be is directly linked to the mutations that cause the disease (Machen, 2006; Ulrich *et al.,* 2010).

5.3.2 Sinus infections

The microbiology of CF sinuses has primarily been investigated by culture-dependent methods, and the most frequently found microorganisms are known CF pathogens (Table 5).

Studies where the microbial flora of sinuses and lungs (sputum and throat samples) have been compared have shown correlation in the identified microorganisms from these two distinct anatomic sites (Dosanjh *et al.,* 2000; Holzmann *et al.,* 2004; Roby *et al.,* 2008; Mainz *et al.,* 2009). In younger CF patients the correlation tendency is not as strong (Muhlebach *et al.,* 2006; Bonestroo *et al.,* 2010). A few pathogens are primarily recovered from only one habitat. From sinuses these include coagulase-negative staphylococci, *Corynebacterium* and *Moraxella* sp., whereas pathogens more frequently recovered from the lungs include fungus and *S. maltophilia* (Mainz *et al.,* 2009).

Table 5: The microorganisms identified by culture in studies of CF sinuses (an x indicates that the microorganism was found in at least one case in the study).

Reference	α-hemolytic streptococci	*Achromobacter xylosoxidans*	*Aspergillus* sp.	*Bacteroides oralis*	*Burkholderia cepacia* complex	*Candida* sp.	*Corynebacterium* sp.	*Enterobacter* sp.	*Escherichia coli*	*Haemophilus influenzae*	*Moraxella* sp.	*Neisseria cinerea*	*Peptostreptococcus* sp.	*Propionibacterium acnes*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Staphylococcus aureus*	Other staphylococci	*Stenotrophomonas maltophilia*
(Shapiro *et al.*, 1982)	x			x					x	x			x	x	x		x		
(Dosanjh *et al.*, 2000)	x	x					x			x		x			x		x	x	x
(Holzmann *et al.*, 2004)	x														x		x	x	x
(Muhlebach *et al.*, 2006)	x									x	x				x		x		x
(Franche *et al.*, 2007)	x									x					x		x		
(Roby *et al.*, 2008)	x	x					x			x	x	x			x		x	x	
(Mainz *et al.*, 2009)	x		x			x	x	x	x	x	x	x			x	x	x	x	x
(Bonestroo *et al.*, 2010)	x							x	x	x					x		x		
(Godoy *et al.*, 2011)	x		x				x			x	x				x		x		
(Osborn *et al.*, 2011)	x	x	x		x	x				x					x		x		x
(Digoy *et al.*, 2012)	x				x				x	x					x		x	x	x
(Johansen *et al.*, 2012)		x			x										x				x
Article IV		x	x					x							x	x	x	x	

The findings by Mainz *et al.*, (2009) have been shortened to fit this table; however, the most frequently found microorganisms are included here.

The finding of a correlation between microbial flora in sinuses and lungs indicates that cross infection between the upper and lower airway occurs. This has been supported by genetic typing of isolates (primarily *P. aeruginosa* but in some cases also *S. aureus* isolates) using pulsed field gel electrophoresis (Muhlebach *et al.*, 2006; Johansen *et al.*, 2012) and single nucleotide polymorphism microarrays (Mainz *et al.*, 2009; Hansen *et al.*, 2012), where genotypically identical isolates were found in the sinuses and lungs. The sinuses may act like a protective niche for the microorganisms. This is both because the sinuses do not have a PMN dominated inflammation like the lungs (probably due to differential immunoglobulin distributions (Johansen *et al.*, 2012)), and the fact that many sinus microorganisms are not eradicated by antimicrobial therapy, although the isolates show susceptibility towards the antimicrobial agents (Shapiro *et al.*, 1982; Hansen *et al.*, 2012). Development of sinus infection early in life and findings of the same species in lung infections later in life suggest that the microorganisms migrate from the sinuses to the lower airways (Roby *et al.*, 2008). Furthermore, a longitudinal study of genetic and phenotypic profiles (including colony morphology, motility, quorum sensing, biofilm formation and antimicrobial susceptibility) of *P. aeruginosa* isolates from sinuses and lungs of CF patients, suggests that the bacteria in the sinuses diversify and evolve into phenotypes that are well adapted to the CF lungs. These bacteria intermittently colonize the lungs and may ultimately cause chronic lung infection (Hansen *et al.*, 2012). One of the adaptations that occur in the sinuses might be the ability to live under anaerobic conditions, since the microenvironments in the sinuses shown oxygen depletion due to mucous obstruction of sinuses (Aanaes *et al.*, 2011). The left and right sinuses may have different oxygen tension values, which correlates to the finding of

different microbial flora (Aanaes *et al.*, 2011) and distinct morphotype populations of strains in the two sinuses (Hansen *et al.*, 2012).

Presence of microorganisms in the sinuses may account for the recurrent lung infections after antimicrobial eradication and is a concern for lung transplant patients, since it is possible that the lung allograft become re-colonized with pathogens from the sinus reservoir. This has been supported by genotyping of isolates before and after lung transplantation which indicated that there is little or no difference in the microbial population (Walter *et al.*, 1997). Sinus surgery combined with postoperative daily nasal washing may be a way to treat chronic sinus infections and avoid re-colonization of the lung allografts (Holzmann *et al.*, 2004; Vital *et al.*, 2012), although some authors do not find an effect of such treatments (Leung *et al.*, 2008; Osborn *et al.*, 2011). Patients attending the Copenhagen CF center are offered sinus surgery to delay or prevent re-colonization of the transplanted lung within one year after transplantation.

The literature contains no studies where the microbial flora of CF sinuses has been directly evaluated by broad range PCR-based methods. The findings in **article IV** indicate that the microbial complexity in samples obtained by sinus surgery may be much greater than suggested by culture-dependent methods (Figure 14).

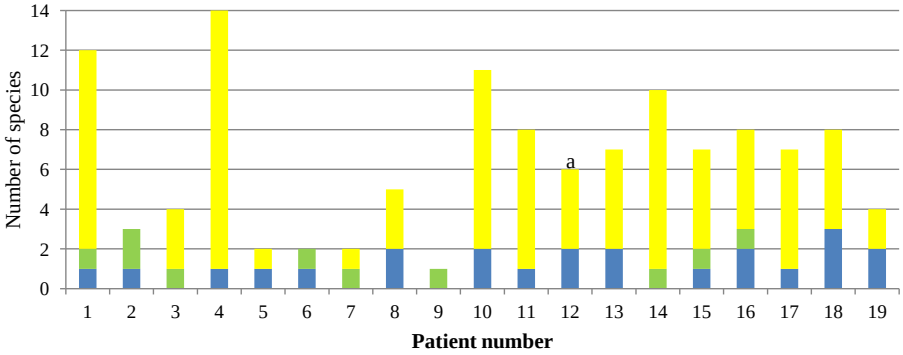


Figure 14: Overview of the number of species found in the sinuses of 19 Danish CF patients (article IV). The microorganisms were either identified only by culture (blue), only in the 16S rRNA gene clone libraries (yellow) or by both methods (green). In one case (a) the finding by culture was probably misidentified (result was *Bordetella bronchiseptica*, which has high phenotypic affinity to *A. xylosoxidans* which was found in the clone library).

Only in approximately half of the cases did culture and 16S rRNA clone libraries identify at least one common microorganism and generally the clone libraries identified a more diverse microbial community than culture. Although this may indicate that such molecular methods are more sensitive than culture-dependent methods, the culture reports did not include species that were deemed to be contaminants. Also, since the sample volume was greater for culture-dependent methods than molecular methods, it is possible that observed differences stem from variations in spatial distribution.

Although no other studies have investigated the microbial flora of CF sinuses by broad-range PCR based methods, there are several studies where such methods have been applied to chronic sinusitis samples of non-CF patients. These studies have shown a greater microbial diversity and more frequent involvement of fungus and anaerobes by using methods such as specific (multiplex) PCR,

16S rRNA gene cloning with Sanger sequencing and the Roche 454 pyrosequencing platform compared with culture (Hendolin *et al.*, 2000; Keech *et al.*, 2000; Ramadan *et al.*, 2002; Kalcioglu *et al.*, 2003; Paju *et al.*, 2003; Stephenson *et al.*, 2010). Also, studies of non-CF patients were the first to show that chronic sinusitis may be caused by biofilm formation, which has implications the understanding of sinus infections in both non-CF and CF patients (Cryer *et al.*, 2004; Perloff and Palmer, 2004; Ramadan *et al.*, 2005; Sanclement *et al.*, 2005; Sanderson *et al.*, 2006).

6 Conclusions and perspectives

The objective of this PhD project was to evaluate if alternative methods to culture-dependent techniques can give relevant information in a clinical setting, and be used as a supplement or replacement for culture-dependent methods. Identification by culture-dependent methods have well-publicized difficulties for some microorganisms, for instance cells exhibiting the effects of antimicrobial therapy and might not be suitable for a number of microorganisms due to the limited number of conditions used for testing in clinical microbiology.

Overall, the various alternative methods had different limitations and disadvantages, and their use depend on the subject matter of investigation.

It is possible to quickly screen for pathogens by microarrays, qPCR and Ibis T5000 biosensor. FISH can also be used for this purpose, but it might be time consuming to locate microorganisms in clinical samples, especially if the abundance is low. The multiplexing capabilities of particularly microarrays and Ibis T5000 biosensor mitigate the inherent problem that only targeted species will be detected. A further advantage of the Ibis T5000 biosensor is that antimicrobial resistance genes can be detected and sequence variability can be analyzed.

Sequencing of molecular marker genes and indeed entire genomes, allows for identification of microorganisms that may not be targeted by the standard culture-dependent methods. Sanger sequencing is increasingly outcompeted by NGS, in large part because the requirement of pure target DNA molecules makes Sanger sequencing too labor intensive. Furthermore, the ability to perform genome sequencing constitutes the ultimate typing method, and will surely take over for MLST and other similar subtyping methods in epidemiological studies. Continued developments and improvements of NGS platforms is associated with rapidly falling cost and turnaround time, and makes it plausible that deep amplicon sequencing and whole genome sequencing will be used in clinical microbiology laboratories in the near future.

A problem of molecular methods is the requirement of unbiased nucleic acid extraction, which is currently not possible. This is not an issue using microscopy-based techniques, where the microorganisms can be observed directly within the clinical sample, though the detection limit is generally high. Microscopy can be used to obtain clinically relevant information and is for instance the only sure way to establish presence of biofilms.

Molecular methods were found to add value to microbial analysis of all the infection types investigated in this thesis.

For NSTIs it was possible to identify microorganisms despite administration of antimicrobial agents. Although positive culture results were confirmed by the molecular methods, additional species were generally also found, and uncommon findings were made. By using a broad spectrum of molecular methods, it was found that various methods might give differing results, and indicated that direct Sanger sequencing may be more inclined to fail in identifying the microorganisms that were truly present. The infected tissue from NSTI patients were found to contain few detectable metabolically active bacteria, but showed high amount of inflammatory cells and coagulated blood, indicating an active immune responses. The more rapid diagnosis that can obtainable by some of the used molecular methods is of clinical importance in fulminant infections such as NSTI (**article I**).

For chronic CF lung infections the use of molecular methods on explanted lung tissue indicated the infections in end-stage patients were dominated by a single species and that culture analysis of

sputum samples could identify the dominant microorganisms. Comparison of the findings in transplanted end-stage patients with non-end-stage patients indicated a link between low microbial diversity and high pathogenicity. Spatial investigations of sputum from the chronically infected, but non-end-stage patients showed that although the lung infections were polymicrobial, the associated biofilm aggregates were monomicrobial and spatially segregated from other species (**article II** and **III**). The sinuses of CF patients are generally also infected, and the microorganisms residing in the sinuses have been implicated in the recurrent lung infections after successful antimicrobial treatment, and establishment of lung infections in lung-transplanted CF patients. By using molecular methods a large microbial diversity was found in the sinuses, which included common CF pathogens, environmental species and anaerobes. Comparatively the diversity found by culture-dependent methods was low. The microorganisms detected by the molecular methods were probably live cells (since DNA had been extracted using MolYsis pretreatment), but the clinical relevance of the diverse microbial communities in the sinuses is presently unclear (**article IV**).

Using molecular methods to investigate the microbial communities in chronic venous leg ulcers further underlined the ability of the methods to identify a greater diversity than culture-dependent methods, but again indicated that different methods may give different results. Interestingly, by sampling different areas inside two wounds it was found that the microbial communities in these areas varied. Furthermore, it was shown that the abundance of individual species also varied in the different areas. This finding has direct implications on the sampling procedure used for such wounds, since a biopsy taken from a single location in the wound probably does not represent the true microbial community (**article V**).

In an exploratory study of suspected prosthetic joint infections, approximately two thirds of the cases gave concordant results by culture-dependent and molecular methods. This was primarily caused by concordant negative results. In the remaining cases molecular analysis generally identified microorganisms that could not all be found by culture. One of the reasons for discrepancy was that incubation time was insufficient for growth of fastidious microorganisms such as *P. acnes*. Visualization of samples from prosthesis surface indicated that biofilm formation may be involved in prosthetic joint infections, which could influence the ability to detect microorganisms by culture-dependent methods (**article VI**).

This PhD project has shown that molecular methods can be used to derive information that is relevant in the clinical setting and may impact the outcome for patients. The findings have had a direct influence in the clinical setting, both by indicating that single biopsies from chronic wounds is not a suitable sampling method and culture-dependent analysis of prosthetic joint infections should be extended to allow for growth of for example *P. acnes*. The studies conducted in this PhD have also contributed in convincing medical professionals of the added value that can be gained by use of molecular methods. What has also become clear is that the various molecular methods can yield different results, and that further studies are needed in order to determine a universal method for identification of microorganisms in infections.

For the time being the majority of microorganisms in the clinical microbiology departments will continue to be identified using culture-dependent methods, as these are readily available, often adequate and allows for antimicrobial susceptibility testing. The latter is a crucial point, and huge incentive to continue the use of culture-dependent methods. Although it is possible to predict potential for antimicrobial resistance based on presence of resistance genes, this might not be informative about the susceptibility of the microorganisms.

From a research standpoint the possibility of investigating the entire microbial community in depth is highly desirable in order to gain a complete understanding of the infections. For this purpose the potential of performing in-depth sequencing of the metagenomes is highly relevant, and will increasingly become available as the cost of massively parallel sequencing continues to decrease. However, from a clinical standpoint the focus is primarily on the treatment of infections, and it is therefore highly possible that the best method will ultimately be a combination of culture-dependent antimicrobial susceptibility testing and molecular analysis.

While the focus of this PhD project has been on the ability of various methods to detect and identify the microorganisms in clinical samples, it is clear that the understanding of the role multifarious microbial communities in infections is lagging behind. The potential of performing metagenomic, metatranscriptomic and metaproteomic analysis might offer insights into the mechanisms that are involved in progression of disease and the interplay between microorganisms and the human host. It is possible that the future of clinical microbiology does not lie with identification of microbial species, but rather with identification and detection of particular traits, be it antimicrobial resistance or virulence profiles.

7 References

Ala'Aldeen, D.A.A., 2007. Bacterial pathogenicity, in: Greenwood, D., Slack, R.C.B., Peutherer, J.F., Barer, M.R. (Eds.), Medical Microbiology: A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control. Churchill Livingstone, pp. 154–164.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Pathogens, Infection, and Innate Immunity, in: Molecular Biology of the Cell. Garland Science, Available from: http://www.ncbi.nlm.nih.gov/books/NBK21054/.

Amann, R., Fuchs, B.M., Behrens, S., 2001. The identification of microorganisms by fluorescence in situ hybridisation. Curr Opin Biotechnol 12, 231–236.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172, 762–770.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59, 143–169.

Anaya, D.A., Dellinger, E.P., 2007. Necrotizing soft-tissue infection: diagnosis and management. Clin Infect Dis 44, 705–710.

Andersen, A., Hill, K.E., Stephens, P., Thomas, D.W., Jorgensen, B., Krogfelt, K.A., 2007. Bacterial profiling using skin grafting, standard culture and molecular bacteriological methods. J Wound Care 16, 171–175.

Anderson, G.G., Lee, Y.M., Smith, C.L., Hultgren, S.J., 2006. Mechanisms of Bacterial Adhesion and Consequences of Attachment, in: Nickerson, C.A., Schurr, M.J. (Eds.), Molecular Paradigms of Infectious Disease. A Bacterial Perspective, Emerging Infectious Diseases of the 21st Century. pp. 207–246.

Armougom, F., Bittar, F., Stremler, N., Rolain, J.-M., Robert, C., Dubus, J.-C., Sarles, J., Raoult, D., La Scola, B., 2009. Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. Eur J Clin Microbiol Infect Dis 28, 1151–1154.

Atlas, R.M., Snyder, J.W., 2011. Reagents, Stains, and Media: Bacteriology, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 272–303.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. J Microbiol Methods 55, 541–555.

Ballon-Landa, G.R., Gherardi, G., Beall, B., Krosner, S., Nizet, V., 2001. Necrotizing fasciitis due to penicillin-resistant Streptococcus pneumoniae: case report and review of the literature. J Infect 42, 272–277.

Barer, M.R., 2007. The natural history of infections, in: Greenwood, D., Slack, R.C.B., Peutherer, J.F., Barer, M.R. (Eds.), Medical Microbiology: A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control. Churchill Livingstone, pp. 165–170.

Barken, K.B., Haagensen, J.A.J., Tolker-Nielsen, T., 2007. Advances in nucleic acid-based diagnostics of bacterial infections. Clin Chim Acta 384, 1–11.

Baron, E.J., Thomson, R.B., 2011. Specimen collection, Transport and Processing: Bacteriology, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 228–271.

Bassam, B.J., Caetano-Anollés, G., Gresshoff, P.M., 1992. DNA amplification fingerprinting of bacteria. Appl Microbiol Biotechnol 38, 70–76.

Bempt, I.V., Van Trappen, S., Cleenwerck, I., De Vos, P., Camps, K., Celens, A., Van De Vyvere, M., 2011. Actinobaculum schaalii Causing Fournier's Gangrene. J Clin Microbiol 49, 2369–2371.

Bittar, F., Richet, H., Dubus, J.-C., Reynaud-Gaubert, M., Stremler, N., Sarles, J., Raoult, D., Rolain, J.-M., 2008. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS ONE 3, e2908.

Bittar, F., Rolain, J.-M., 2010. Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients. Clin Microbiol Infect 16, 809–820.

Bjarnsholt, T., Jensen, P., Fiandaca, M., Pedersen, J., Hansen, C., Andersen, C., Pressler, T., Givskov, M., Høiby, N., 2009. Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 44, 547–558.

Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P.Ø., Madsen, K.G., Phipps, R., Krogfelt, K., Høiby, N., Givskov, M., 2008. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen 16, 2–10.

Bjarnsholt, T., Nielsen, X., Johansen, U., Nørgaard, L., Høiby, N., 2011. Methods to Classify Bacterial Pathogens in Cystic Fibrosis, in: Amaral, M., Kunzelmann, K. (Eds.), Cystic Fibrosis : Diagnosis and Protocols, Volume II: Methods and Resources to Understand Cystic Fibrosis. Humana Press, Totowa, NJ, pp. 143–171.

Bonestroo, H.J.C., de Winter-de Groot, K.M., van der Ent, C.K., Arets, H.G.M., 2010. Upper and lower airway cultures in children with cystic fibrosis: Do not neglect the upper airways. J Cyst Fibros 9, 130–134.

Bowler, P.G., Duerden, B.I., Armstrong, D.G., 2001. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev 14, 244–269.

Brown, A.R., Govan, J.R.W., 2007. Assessment of Fluorescent In Situ Hybridization and PCR-Based Methods for Rapid Identification of Burkholderia cepacia Complex Organisms Directly from Sputum Samples. J Clin Microbiol 45, 1920–1926.

Burmølle, M., Thomsen, T.R., Fazli, M., Dige, I., Christensen, L., Homøe, P., Tvede, M., Nyvad, B., Tolker-Nielsen, T., Givskov, M., Moser, C., Kirketerp-Møller, K., Johansen, H.K., Høiby, N., Jensen, P.Ø., Sørensen, S.J., Bjarnsholt, T., 2010. Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol 59, 324–336.

Bustin, S.A., 2004. Quantification of Nucleic Acids by PCR, in: Bustin, S.A. (Ed.), A-Z of Quantitative PCR. International University Line, pp. 5–46.

Butler, W.R., Sheils, C.A., Brown-Elliott, B.A., Charles, N., Colin, A.A., Gant, M.J., Goodill, J., Hindman, D., Toney, S.R., Wallace, R.J., Yakrus, M.A., 2007. First Isolations of Segniliparus rugosus from Patients with Cystic Fibrosis. J Clin Microbiol 45, 3449–3452.

Cabrera-Rubio, R., Garcia-Núñez, M., Setó, L., Antó, J.M., Moya, A., Monsó, E., Mira, A., 2012. Microbiome diversity in the bronchial tract of patients with chronic obstructive pulmonary disease. J Clin Microbiol

Carbonnelle, E., Mesquita, C., Bille, E., Day, N., Dauphin, B., Beretti, J.-L., Ferroni, A., Gutmann, L., Nassif, X., 2011. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem 44, 104–109.

Carpenter, A.B., 2011. Immunoassays for the Diagnosis of Infectious Diseases, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 60–72.

Changchien, C.-H., Chen, Y.-Y., Chen, S.-W., Chen, W.-L., Tsay, J.-G., Chu, C., 2011. Retrospective study of necrotizing fasciitis and characterization of its associated Methicillin-resistant Staphylococcus aureus in Taiwan. BMC Infect Dis 11, 297.

Chiron, R., Marchandin, H., Counil, F., Jumas-Bilak, E., Freydière, A.-M., Bellon, G., Husson, M.-O., Turck, D., Brémont, F., Chabanon, G., Segonds, C., 2005. Clinical and Microbiological Features of Inquilinus sp. Isolates from Five Patients with Cystic Fibrosis. J Clin Microbiol 43, 3938–3943.

Clarke, T.M., Citron, D.M., Towfigh, S., 2010. The Conundrum of the Gram-Positive Rod: Are We Missing Important Pathogens in Complicated Skin and Soft-Tissue Infections? A Case Report and Review of the Literature. Surg Infect (Larchmt) 11, 65–72.

Coenye, T., Vandamme, P., 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiol Lett 228, 45–49.

Cohen, S.N., Chang, A.C., Boyer, H.W., Helling, R.B., 1973. Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci USA. 70, 3240–3244.

Conese, M., Copreni, E., Gioia, S.D., Rinaldis, P.D., Fumarulo, R., 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. J Cyst Fibros. 2, 129–135.

Costerton, J.W., Post, J.C., Ehrlich, G.D., Hu, F.Z., Kreft, R., Nistico, L., Kathju, S., Stoodley, P., Hall-Stoodley, L., Maale, G., James, G., Sotereanos, N., DeMeo, P., 2011. New methods for the detection of orthopedic and other biofilm infections. FEMS Immunol Med Microbiol 61, 133–140.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science 284, 1318 –1322.

Cox, M.J., Allgaier, M., Taylor, B., Baek, M.S., Huang, Y.J., Daly, R.A., Karaoz, U., Andersen, G.L., Brown, R., Fujimura, K.E., Wu, B., Tran, D., Koff, J., Kleinhenz, M.E., Nielson, D., Brodie, E.L., Lynch, S.V., 2010. Airway Microbiota and Pathogen Abundance in Age-Stratified Cystic Fibrosis Patients. PLoS ONE 5, e11044.

Cryer, J., Schipor, I., Perloff, J.R., Palmer, J.N., 2004. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec. 66, 155–158.

Cunningham, A.B., Lennox, J.E., Ross, R.J., 2010. An overview of the normal skin healing process Biofilms: The Hypertext book. Available at: http://www.cs.montana.edu/webworks/projects/stevesbook/contents/chapters/chapter004/section004/blue/page001.html

Cunningham, M.W., 2000. Pathogenesis of Group A Streptococcal Infections. Clin Microbiol Rev 13, 470–511.

Davies, C.E., Hill, K.E., Newcombe, R.G., Stephens, P., Wilson, M.J., Harding, K.G., Thomas, D.W., 2007. A prospective study of the microbiology of chronic venous leg ulcers to reevaluate the clinical predictive value of tissue biopsies and swabs. Wound Repair Regen 15, 17–22.

Davies, C.E., Hill, K.E., Wilson, M.J., Stephens, P., Hill, C.M., Harding, K.G., Thomas, D.W., 2004. Use of 16S Ribosomal DNA PCR and Denaturing Gradient Gel Electrophoresis for Analysis of the Microfloras of Healing and Nonhealing Chronic Venous Leg Ulcers. J Clin Microbiol 42, 3549–3557.

Degand, N., Carbonnelle, E., Dauphin, B., Beretti, J.-L., Bourgeois, M.L., Sermet-Gaudelus, I., Segonds, C., Berche, P., Nassif, X., Ferroni, A., 2008. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of Nonfermenting Gram-Negative Bacilli Isolated from Cystic Fibrosis Patients. J Clin Microbiol 46, 3361–3367.

Delhaes, L., Monchy, S., Fréalle, E., Hubans, C., Salleron, J., Leroy, S., Prevotat, A., Wallet, F., Wallaert, B., Dei-Cas, E., Sime-Ngando, T., Chabé, M., Viscogliosi, E., 2012. The Airway Microbiota in Cystic Fibrosis: A Complex Fungal and Bacterial Community—Implications for Therapeutic Management. PLoS One 7.

Della Valle, C., Parvizi, J., Bauer, T.W., Dicesare, P.E., Evans, R.P., Segreti, J., Spangehl, M., Watters, W.C., 3rd, Keith, M., Turkelson, C.M., Wies, J.L., Sluka, P., Hitchcock, K., 2010. Diagnosis of periprosthetic joint infections of the hip and knee. J Am Acad Orthop Surg 18, 760–770.

DeLong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells. Science 243, 1360–1363.

Demers, B., Simor, A.E., Vellend, H., Schlievert, P.M., Byrne, S., Jamieson, F., Walmsley, S., Low, D.E., 1993. Severe invasive group A streptococcal infections in Ontario, Canada: 1987-1991. Clin Infect Dis 16, 792–800; discussion 801–802.

Digoy, G.P., Dunn, J.D., Stoner, J.A., Christie, A., Jones, D.T., 2012. Bacteriology of the paranasal sinuses in pediatric cystic fibrosis patients. Int J Pediatr Otorhinolaryngol 76, 934–938.

Doggett, R.G., Harrison, G.M., Wallis, E.S., 1964. Comparison of some properties of Pseudomonas aeruginosa isolated from infections in persons with and without cystic fibrosis. J Bacteriol 87, 427–431.

Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15, 167–193.

Dosanjh, A., Lakhani, S., Elashoff, D., Chin, C., Hsu, V., Hilman, B., 2000. A comparison of microbiologic flora of the sinuses and airway among cystic fibrosis patients with maxillary antrostomies. Pediatr Transplant 4, 182–185.

Dowd, S.E., Sun, Y., Secor, P.R., Rhoads, D.D., Wolcott, B.M., James, G.A., Wolcott, R.D., 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol 8, 43.

Drevinek, P., Vosahlikova, S., Dedeckova, K., Cinek, O., Mahenthiralingam, E., 2010. Direct Culture-Independent Strain Typing of Burkholderia cepacia Complex in Sputum Samples from Patients with Cystic Fibrosis. J Clin Microbiol 48, 1888–1891.

Ecker, D.J., Drader, J.J., Gutierrez, J., Gutierrez, A., Hannis, J.C., Schink, A., Sampath, R., Blyn, L.B., Eshoo, M.W., Hall, T.A., Tobarmosquera, M., Jiang, Y., Sannes-Lowery, K.A., Cummins, L.L., Libby, B., Walcott, D.J., Massire, C., Ranken, R., Manalili, S., Ivy, C., Melton, R., Levene, H., Harpin, V., Li, F., White, N., Pear, M., Ecker, J.A., Samant, V., Knize, D., Robbins, D., Rudnick, K., Hajjar, F., Hofstadler, S.A., 2006. The Ibis T5000 Universal Biosensor: An Automated Platform for Pathogen Identification and Strain Typing. JALA Charlottesv Va 11, 341–351.

Ecker, D.J., Massire, C., Blyn, L.B., Hofstadler, S.A., Hannis, J.C., Eshoo, M.W., Hall, T.A., Sampath, R., 2009. Molecular genotyping of microbes by multilocus PCR and mass spectrometry: a new tool for hospital infection control and public health surveillance. Methods Mol Biol 551, 71–87.

Ecker, D.J., Sampath, R., Massire, C., Blyn, L.B., Hall, T.A., Eshoo, M.W., Hofstadler, S.A., 2008. Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol 6, 553–558.

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B., Nielsen, P.E., 1993. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 365, 566–8.

Enright, M.C., Spratt, B.G., 1999. Multilocus sequence typing. Trends in Microbiology 7, 482–487.

Erdem, G., Abe, L., Kanenaka, R.Y., Pang, L., Mills, K., Mizumoto, C., Yamaga, K., Effler, P.V., 2004. Characterization of a community cluster of group a streptococcal invasive disease in Maui, Hawaii. Pediatr Infect Dis J 23, 677–679.

Ernst, R.K., D′Argenio, D.A., Ichikawa, J.K., Bangera, M.G., Selgrade, S., Burns, J.L., Hiatt, P., McCoy, K., Brittnacher, M., Kas, A., Spencer, D.H., Olson, M.V., Ramsey, B.W., Lory, S., Miller, S.I., 2003. Genome mosaicism is conserved but not unique in Pseudomonas aeruginosa isolates from the airways of young children with cystic fibrosis. Environ Microbiol 5, 1341–1349.

Eshoo, M.W., Whitehouse, C.A., Nalca, A., Zoll, S., Ecker, J.A., Hall, T.A., Pennella, T.-T.D., Duncan, D.D., Desai, A., Moradi, E.K., Rudnick, K., Libby, B., Ranken, R., Sampath, R., Hofstadler, S.A., Ecker, D.J., Blyn, L.B., 2009. Rapid and high-throughput pan-Orthopoxvirus detection and identification using PCR and mass spectrometry. PLoS ONE 4, e6342.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., Smith, T.F., 2006. Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing. Clin Microbiol Rev 19, 165–256.

Fazli, M., Bjarnsholt, T., Kirketerp-Moller, K., Jorgensen, B., Andersen, A.S., Krogfelt, K.A., Givskov, M., Tolker-Nielsen, T., 2009. Nonrandom Distribution of Pseudomonas aeruginosa and Staphylococcus aureus in Chronic Wounds. J Clin Microbiol 47, 4084–4089.

Fazli, M., Bjarnsholt, T., Kirketerp-Møller, K., Jørgensen, A., Andersen, C.B., Givskov, M., Tolker-Nielsen, T., 2011. Quantitative analysis of the cellular inflammatory response against biofilm bacteria in chronic wounds. Wound Repair Regen 19, 387–391.

Fernández-Olmos, A., Morosini, M.I., Lamas, A., García-Castillo, M., García-García, L., Cantón, R., Máiz, L., 2012. Clinical and microbiological features of a cystic fibrosis patient chronically colonized with Pandoraea sputorum identified by combining 16S rRNA sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 50, 1096–1098.

Field, T.R., Sibley, C.D., Parkins, M.D., Rabin, H.R., Surette, M.G., 2010. The genus Prevotella in cystic fibrosis airways. Anaerobe 16, 337–344.

Finlay, B.B., Cossart, P., 1997. Exploitation of Mammalian Host Cell Functions by Bacterial Pathogens. Science 276, 718–725.

Franche, G.L. da S., Abreu e Silva, F., Saleh, C. de S., 2007. Bacteriology of the middle meatus aspirate in patients with cystic fibrosis. Braz J Otorhinolaryngol 73, 494–499.

Frank, D.N., Wysocki, A., Specht-Glick, D.D., Rooney, A., Feldman, R.A., St. Amand, A.L., Pace, N.R., Trent, J.D., 2009. Microbial diversity in chronic open wounds. Wound Repair Regen 17, 163–172.

Freiwald, A., Sauer, S., 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. Nature Protocols 4, 732–742.

Frye, S.R., Healy, M., 2011. Repetitive Sequence-Based PCR Typing of Bacteria and Fungi, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 199–212.

Fuchs, B.M., Glöckner, F.O., Wulf, J., Amann, R., 2000. Unlabeled Helper Oligonucleotides Increase the In Situ Accessibility to 16S rRNA of Fluorescently Labeled Oligonucleotide Probes. Appl Environ Microbiol 66, 3603–3607.

Fuchs, T.M., Eisenreich, W., Heesemann, J., Goebel, W., 2012. Metabolic adaptation of human pathogenic and related nonpathogenic bacteria to extra- and intracellular habitats. FEMS Microbiol Rev 36, 435–462.

Furukawa, S., Kuchma, S.L., O'Toole, G.A., 2006. Keeping their options open: acute versus persistent infections. J Bacteriol 188, 1211–1217.

Gaillot, O., Blondiaux, N., Loïez, C., Wallet, F., Lemaître, N., Herwegh, S., Courcol, R.J., 2011. Cost-Effectiveness of Switch to Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Routine Bacterial Identification. J Clin Microbiol 49, 4412–4412.

Gebert, S., Siegel, D., Wellinghausen, N., 2008. Rapid detection of pathogens in blood culture bottles by real-time PCR in conjunction with the pre-analytic tool MolYsis. J Infect 57, 307–316.

Gentili, V., Gianesini, S., Balboni, P.G., Menegatti, E., Rotola, A., Zuolo, M., Caselli, E., Zamboni, P., Di Luca, D., 2012. Panbacterial real-time PCR to evaluate bacterial burden in chronic wounds treated with Cutimed™ Sorbact™. Eur J Clin Microbiol Infect Dis 31, 1523–1529.

Gerner-Smidt, P., Hyytiä-Trees, E., Rota, P.A., 2011. Molecular Epidemiology, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 100–123.

Gilligan, P.H., 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin Microbiol Rev 4, 35–51.

Giovannoni, S.J., DeLong, E.F., Olsen, G.J., Pace, N.R., 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol 170, 720–726.

Goddard, A.F., Staudinger, B.J., Dowd, S.E., Joshi-Datar, A., Wolcott, R.D., Aitken, M.L., Fligner, C.L., Singh, P.K., 2012. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. PNAS 109, 13769–74.

Godoy, J.M., Godoy, A.N., Ribalta, G., Largo, I., 2011. Bacterial pattern in chronic sinusitis and cystic fibrosis. Otolaryngol Head Neck Surg 145, 673 –676.

Goerke, C., Wolz, C., 2004. Regulatory and genomic plasticity of Staphylococcus aureus during persistent colonization and infection. Int J Med Microbiol 294, 195–202.

Goerke, C., Wolz, C., 2010. Adaptation of Staphylococcus aureus to the cystic fibrosis lung. Int J Med Microbiol 300, 520–525.

Grant-Klein, R.J., Baldwin, C.D., Turell, M.J., Rossi, C.A., Li, F., Lovari, R., Crowder, C.D., Matthews, H.E., Rounds, M.A., Eshoo, M.W., Blyn, L.B., Ecker, D.J., Sampath, R., Whitehouse, C.A., 2010. Rapid identification of vector-borne flaviviruses by mass spectrometry. Mol Cell Probes 24, 219–228.

Grimaldi, D., Doloy, A., Fichet, J., Bourgeois, E., Zuber, B., Wajsfisz, A., Mira, J.P., Poyart, C., Pène, F., 2012. Necrotizing Fasciitis and Septic Shock Related to the Uncommon Gram-Negative Pathogen Sphingobacterium multivorum. J Clin Microbiol 50, 202–203.

Grinwis, M.E., Sibley, C.D., Parkins, M.D., Eshaghurshan, C.S., Rabin, H.R., Surette, M.G., 2010. Characterization of Streptococcus milleri group isolates from expectorated sputum of adult patients with cystic fibrosis. J Clin Microbiol 48, 395–401.

Gunnarsson, G.L., Brandt, P.B., Gad, D., Struve, C., Justesen, U.S., 2009. Monomicrobial necrotizing fasciitis in a white male caused by hypermucoviscous Klebsiella pneumoniae. J Med Microbiol 58, 1519–1521.

Guss, A.M., Roeselers, G., Newton, I.L.G., Young, C.R., Klepac-Ceraj, V., Lory, S., Cavanaugh, C.M., 2011. Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. ISME J 5, 20–29.

Gysin, C., Alothman, G.A., Papsin, B.C., 2000. Sinonasal disease in cystic fibrosis: Clinical characteristics, diagnosis, and management. Pediatr Pulmonol 30, 481–489.

Hall, T.A., Sampath, R., Blyn, L.B., Ranken, R., Ivy, C., Melton, R., Matthews, H., White, N., Li, F., Harpin, V., Ecker, D.J., McDougal, L.K., Limbago, B., Ross, T., Wolk, D.M., Wysocki, V., Carroll, K.C., 2009. Rapid Molecular Genotyping and Clonal Complex Assignment of Staphylococcus aureus Isolates by PCR Coupled to Electrospray Ionization-Mass Spectrometry. J Clin Microbiol 47, 1733–1741.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2, 95–108.

Hall-Stoodley, L., Stoodley, P., 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol 13, 7–10.

Hansen, S.K., Rau, M.H., Johansen, H.K., Ciofu, O., Jelsbak, L., Yang, L., Folkesson, A., Jarmer, H.O., Aanaes, K., von Buchwald, C., Hoiby, N., Molin, S., 2012. Evolution and diversification of Pseudomonas aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. ISME J 6, 31–45.

Harris, J.K., Groote, M.A.D., Sagel, S.D., Zemanick, E.T., Kapsner, R., Penvari, C., Kaess, H., Deterding, R.R., Accurso, F.J., Pace, N.R., 2007. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. PNAS 104, 20529–20533.

Hassell, M., Fagan, P., Carson, P., Currie, B.J., 2004. Streptococcal necrotising fasciitis from diverse strains of Streptococcus pyogenes in tropical northern Australia: case series and comparison with the literature. BMC Infect Dis 4, 60.

Hendolin, P.H., Paulin, L., Koukila-Kähkölä, P., Anttila, V.-J., Malmberg, H., Richardson, M., Ylikoski, J., 2000. Panfungal PCR and Multiplex Liquid Hybridization for Detection of Fungi in Tissue Specimens. J Clin Microbiol 38, 4186–4192.

Highlander, S.K., Versalovic, J.W., Petrosino, J.F., 2011. The Human Microbiome, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 188–198.

Higuchi, R., Gyllensten, U., Persing, D.H., 2011. Next-Generation DNA Sequencing and Microbiology, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 301–312.

Hill, K.E., Davies, C.E., Wilson, M.J., Stephens, P., Harding, K.G., Thomas, D.W., 2003. Molecular analysis of the microflora in chronic venous leg ulceration. J Med Microbiol 52, 365–369.

Hofstadler, S.A., Sampath, R., Blyn, L.B., Eshoo, M.W., Hall, T.A., Jiang, Y., Drader, J.J., Hannis, J.C., Sannes-Lowery, K.A., Cummins, L.L., Libby, B., Walcott, D.J., Schink, A., Massire, C., Ranken, R., Gutierrez, J., Manalili, S., Ivy, C., Melton, R., Levene, H., Barrett-Wilt, G., Li, F., Zapp, V., White, N., Samant, V., McNeil, J.A., Knize, D., Robbins, D., Rudnick, K., Desai, A., Moradi, E., Ecker, D.J., 2005. TIGER: the universal biosensor. Int J Mass Spectrom 242, 23–41.

Hogardt, M., Heesemann, J., 2012. Microevolution of Pseudomonas aeruginosa to a Chronic Pathogen of the Cystic Fibrosis Lung. Curr Top Microbiol Immunol *Epub*.

Hogardt, M., Trebesius, K., Geiger, A.M., Hornef, M., Rosenecker, J., Heesemann, J., 2000. Specific and Rapid Detection by Fluorescent In Situ Hybridization of Bacteria in Clinical Samples Obtained from Cystic Fibrosis Patients. J Clin Microbiol 38, 818–825.

Holland, R.D., Wilkes, J.G., Rafii, F., Sutherland, J.B., Persons, C.C., Voorhees, K.J., Lay, J.O., Jr, 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10, 1227–1232.

Holzmann, D., Speich, R., Kaufmann, T., Laube, I., Russi, E.W., Simmen, D., Weder, W., Boehler, A., 2004. Effects of sinus surgery in patients with cystic fibrosis after lung transplantation: a 10-year experience. Transplantation 77, 134–136.

Horz, H.-P., Scheer, S., Vianna, M.E., Conrads, G., 2010. New methods for selective isolation of bacterial DNA from human clinical specimens. Anaerobe 16, 47–53.

Huse, H.K., Kwon, T., Zlosnik, J.E.A., Speert, D.P., Marcotte, E.M., Whiteley, M., 2010. Parallel evolution in Pseudomonas aeruginosa over 39,000 generations in vivo. MBio 1, pii: e00199–10.

Høiby, N., 1982. Microbiology of lung infections in cystic fibrosis patients. Acta Pædiatrica 71, 33–54.

Høiby, N., Frederiksen, B., 2000. Microbiology of cystic fibrosis, in: Hodson, M.E., Geddes, D.M. (Eds.), Cystic Fibrosis. Hodder Arnold Publishers, pp. 83–107.

Høiby, N., Frederiksen, B., Pressler, T., 2005. Eradication of early Pseudomonas aeruginosa infection. J Cyst Fibros. 4, 49–54.

Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., Iwasaki, A., 2011. Microbiota Regulates Immune Defense Against Respiratory Tract Influenza A Virus Infection. PNAS 108, 5354–5359.

Jacob, D., Sauer, U., Housley, R., Washington, C., Sannes-Lowery, K., Ecker, D.J., Sampath, R., Grunow, R., 2012. Rapid and High-Throughput Detection of Highly Pathogenic Bacteria by Ibis PLEX-ID Technology. PLoS ONE 7, e39928.

Jensen, P.Ø., Moser, C., 2010. Innate Immune Responce to Infectious Biofims, in: Bjarnsholt, T., Jensen, P.Ø., Moser, C., Høiby, N. (Eds.), Biofilm Infections. Springer, pp. 185–199.

Jing, H., Ning, B., Hao, H., Zheng, Y., Chang, D., Jiang, W., Jiang, Y., 2006. Epidemiological analysis of group A streptococci recovered from patients in China. J Med Microbiol 55, 1101–1107.

Johansen, H.K., Kovesi, T.A., Koch, C., Corey, M., Høiby, N., Levison, H., 1998. Pseudomonas aeruginosa and Burkholderia cepacia infection in cystic fibrosis patients treated in Toronto and Copenhagen. Pediatric Pulmonology 26, 89–96.

Johansen, H.K., Aanaes, K., Pressler, T., Nielsen, K.G., Fisker, J., Skov, M., Høiby, N., von Buchwald, C., 2012. Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response. J Cyst Fibros *Epub*.

Jorgensen, J.H., Ferraro, M.J., 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. Clin Infect Dis 49, 1749–1755.

Kalcioglu, M.T., Durmaz, B., Aktas, E., Ozturan, O., Durmaz, R., 2003. Bacteriology of chronic maxillary sinusitis and normal maxillary sinuses: using culture and multiplex polymerase chain reaction. Am J Rhinol 17, 143–147.

Kaul, M., McGeer, Ms., Low, M., Green, R., Schwartz, M., Simor, M., 1997. Population-Based Surveillance for Group A Streptococcal Necrotizing Fasciitis: Clinical Features, Prognostic Indicators, and Microbiologic Analysis of Seventy-Seven Cases. The American Journal of Medicine 103, 18–24.

Keech, D.R., Ramadan, H., Mathers, P., 2000. Analysis of Aerobic Bacterial Strains Found in Chronic Rhinosinusitis Using the Polymerase Chain Reaction. Otolaryngol Head Neck Surg 123, 363–367.

Kerstens, H.M., Poddighe, P.J., Hanselaar, A.G., 1995. A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. J Histochem Cytochem 43, 347–352.

Kidd, T.J., Ramsay, K.A., Hu, H., Bye, P.T.P., Elkins, M.R., Grimwood, K., Harbour, C., Marks, G.B., Nissen, M.D., Robinson, P.J., Rose, B.R., Sloots, T.P., Wainwright, C.E., Bell, S.C., 2009. Low rates of Pseudomonas aeruginosa misidentification in isolates from cystic fibrosis patients. J Clin Microbiol 47, 1503–1509.

Kim, S.-H., Roh, K.H., Yoon, Y.K., Kang, D.O., Lee, D.W., Kim, M.J., Sohn, J.W., 2012. Necrotizing fasciitis involving the chest and abdominal wall caused by Raoultella planticola. BMC Infect Dis 12, 59.

Kimbrell, D.A., Beutler, B., 2001. The evolution and genetics of innate immunity. Nat Rev Genet 2, 256–267.

Kirketerp-Moller, K., Jensen, P.O., Fazli, M., Madsen, K.G., Pedersen, J., Moser, C., Tolker-Nielsen, T., Hoiby, N., Givskov, M., Bjarnsholt, T., 2008. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. J Clin Microbiol 46, 2717–2722.

Kittang, B.R., Langeland, N., Skrede, S., Mylvaganam, H., 2010. Two Unusual Cases of Severe Soft Tissue Infection Caused by Streptococcus dysgalactiae subsp. equisimilis. J Clin Microbiol 48, 1484–1487.

Klepac-Ceraj, V., Lemon, K.P., Martin, T.R., Allgaier, M., Kembel, S.W., Knapp, A.A., Lory, S., Brodie, E.L., Lynch, S.V., Bohannan, B.J.M., Green, J.L., Maurer, B.A., Kolter, R., 2010. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and Pseudomonas aeruginosa. Environ Microbiol 12, 1293–1303.

Kliem, M., Sauer, S., 2012. The essence on mass spectrometry based microbial diagnostics. Curr Opin Microbiol 15, 397–402.

Kolak, M., Karpati, F., Monstein, H.-J., Jonasson, J., 2003. Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. Int J Med Microbiol 293, 309–317.

Kommedal, Ø., Karlsen, B., Sæbø, Ø., 2008. Analysis of Mixed Sequencing Chromatograms and Its Application in Direct 16S rRNA Gene Sequencing of Polymicrobial Samples. J Clin Microbiol 46, 3766–3771.

Kommedal, Ø., Kvello, K., Skjåstad, R., Langeland, N., Wiker, H.G., 2009. Direct 16S rRNA Gene Sequencing from Clinical Specimens, with Special Focus on Polybacterial Samples and Interpretation of Mixed DNA Chromatograms. J Clin Microbiol 47, 3562–3568.

Korman, T.M., Boers, A., Gooding, T.M., Curtis, N., Visvanathan, K., 2004. Fatal Case of Toxic Shock-Like Syndrome Due to Group C Streptococcus Associated with Superantigen Exotoxin. J Clin Microbiol 42, 2866–2869.

Krishnamurthy, T., Ross, P.L., 1996. Rapid Identification of Bacteria by Direct Matrix-assisted Laser Desorption/Ionization Mass Spectrometric Analysis of Whole Cells. Rapid Communications in Mass Spectrometry 10, 1992–1996.

Lam, J., Chan, R., Lam, K., Costerton, J.W., 1980. Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis. Infect Immun 28, 546–556.

Lam, J.Y.W., Wu, A.K.L., Ngai, D.C., Teng, J.L.L., Wong, E.S.Y., Lau, S.K.P., Lee, R.A., Woo, P.C.Y., 2011. Three cases of severe invasive infections caused by Campylobacter rectus and first report of fatal C. rectus infection. J Clin Microbiol 49, 1687–1691.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., *et al.*, 2001. Initial sequencing and analysis of the human genome. Nature 409, 860–921.

Larsen, L.H., Lange, J., Xu, Y., Schønheyder, H.C., 2012. Optimizing Culture Methods for Diagnosis of Prosthetic Joint Infections: A Summary of Modifications and Improvements Reported Since 1995. J Med Microbiol 61, 309–316.

Lee, Y.-L., Shih, S.-D., Weng, Y.-J., Chen, C., Liu, C.-E., 2010. Fatal spontaneous bacterial peritonitis and necrotizing fasciitis with bacteraemia caused by Bacillus cereus in a patient with cirrhosis. J Med Microbiol 59, 242–244.

Leung, M.-K., Rachakonda, L., Weill, D., Hwang, P.H., 2008. Effects of sinus surgery on lung transplantation outcomes in cystic fibrosis. Am J Rhinol 22, 192–196.

Lieberman, T.D., Michel, J.-B., Aingaran, M., Potter-Bynoe, G., Roux, D., Jr, M.R.D., Skurnik, D., Leiby, N., LiPuma, J.J., Goldberg, J.B., McAdam, A.J., Priebe, G.P., Kishony, R., 2011. Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. Nature Genetics 43, 1275–1280.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., Law, M., 2012. Comparison of Next-Generation Sequencing Systems. J Biomed Biotechnol *Epub*.

Louie, L., Simor, A.E., Louie, M., McGeer, A., Low, D.E., 1998. Diagnosis of Group A Streptococcal Necrotizing Fasciitis by Using PCR To Amplify the Streptococcal Pyrogenic Exotoxin B Gene. J Clin Microbiol 36, 1769–1771.

Machen, T.E., 2006. Innate immune response in CF airway epithelia: hyperinflammatory? Am J Physiol Cell Physiol 291, C218–C230.

Madeira, A., Santos, P.M., Coutinho, C.P., Pinto-de-Oliveira, A., Sá-Correia, I., 2011. Quantitative proteomics (2-D DIGE) reveals molecular strategies employed by Burkholderia cenocepacia to adapt to the airways of cystic fibrosis patients under antimicrobial therapy. Proteomics 11, 1313–1328.

Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G., 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 95, 3140–3145.

Mainz, J.G., Naehrlich, L., Schien, M., Käding, M., Schiller, I., Mayr, S., Schneider, G., Wiedemann, B., Wiehlmann, L., Cramer, N., Pfister, W., Kahl, B.C., Beck, J.F., Tümmler, B., 2009. Concordant genotype of upper and lower airways P aeruginosa and S aureus isolates in cystic fibrosis. Thorax 64, 535–540.

Maiwald, R., 2011. Broad-Range PCR for Detection and Identification of Bacteria, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 491–505.

Malic, S., Hill, K.E., Hayes, A., Percival, S.L., Thomas, D.W., Williams, D.W., 2009. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent in situ hybridization (PNA FISH). Microbiology 155, 2603–2611.

Mardis, E.R., 2008. The impact of next-generation sequencing technology on genetics. Trends Genet 24, 133–141.

Marko, D.C., Saffert, R.T., Cunningham, S.A., Hyman, J., Walsh, J., Arbefeville, S., Howard, W., Pruessner, J., Safwat, N., Cockerill, F.R., Bossler, A.D., Patel, R., Richter, S.S., 2012. Evaluation of the Bruker Biotyper and Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of nonfermenting gram-negative bacilli isolated from cultures from cystic fibrosis patients. J Clin Microbiol 50, 2034–2039.

Martin, J.M., Zenilman, J.M., Lazarus, G.S., 2010. Molecular microbiology: new dimensions for cutaneous biology and wound healing. J Invest Dermatol 130, 38–48.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol 10, 1571–1581.

Masoud-Landgraf, L., Badura, A., Eber, E., Feierl, G., Posch, J., Zarfel, G., Zach, M., Marth, E., 2012. Molecular epidemiology of Pseudomonas aeruginosa in cystic fibrosis patients from Southeast Austria. Wien Klin Wochenschr 124, 262–265.

McAdam, P.R., Holmes, A., Templeton, K.E., Fitzgerald, J.R., 2011. Adaptive Evolution of Staphylococcus aureus during Chronic Endobronchial Infection of a Cystic Fibrosis Patient. PLoS One 6, *Epub*.

Meisal, R., Høiby, E.A., Aaberge, I.S., Caugant, D.A., 2008. Sequence Type and emm Type Diversity in Streptococcus pyogenes Isolates Causing Invasive Disease in Norway between 1988 and 2003. J Clin Microbiol 46, 2102–2105.

Menuet, M., Bittar, F., Stremler, N., Dubus, J.-C., Sarles, J., Raoult, D., Rolain, J.-M., 2008. First isolation of two colistin-resistant emerging pathogens, Brevundimonas diminuta and Ochrobactrum anthropi, in a woman with cystic fibrosis: a case report. J Med Case Rep 2, 373.

Miller, L.G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A.S., Tang, A.W., Phung, T.O., Spellberg, B., 2005. Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. N Engl J Med 352, 1445.

Miller, M.B., Tang, Y.-W., 2009. Basic Concepts of Microarrays and Potential Applications in Clinical Microbiology. Clin Microbiol Rev 22, 611–633.

Miller, R., 2011. Solid and liquid phase array technologies, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 275–297.

Monack, D.M., Mueller, A., Falkow, S., 2004. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol 2, 747–765.

Monchy, S., Sanciu, G., Jobard, M., Rasconi, S., Gerphagnon, M., Chabé, M., Cian, A., Meloni, D., Niquil, N., Christaki, U., Viscogliosi, E., Sime-Ngando, T., 2011. Exploring and quantifying fungal diversity in freshwater lake ecosystems using rDNA cloning/sequencing and SSU tag pyrosequencing. Environ Microbiol 13, 1433–1453.

Morgan, W.R., Caldwell, M.D., Brady, J.M., Stemper, M.E., Reed, K.D., Shukla, S.K., 2007. Necrotizing Fasciitis Due to a Methicillin-Sensitive Staphylococcus aureus Isolate Harboring an Enterotoxin Gene Cluster. J Clin Microbiol 45, 668–671.

Mortensen, K.L., Jensen, R.H., Johansen, H.K., Skov, M., Pressler, T., Howard, S.J., Leatherbarrow, H., Mellado, E., Arendrup, M.C., 2011. Aspergillus Species and Other Molds in Respiratory Samples from Patients with Cystic Fibrosis: a Laboratory-Based Study with Focus on Aspergillus fumigatus Azole Resistance. J Clin Microbiol 49, 2243–2251.

Moser, C., Jensen, P.Ø., 2010. Adaptive Immune Responses and Biofilm Infections, in: Bjarnsholt, T., Jensen, P.Ø., Moser, C., Høiby, N. (Eds.), Biofilm Infections. Springer, pp. 201–214.

Moter, A., Göbel, U.B., 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J Microbiol Methods 41, 85–112.

Muhlebach, M.S., Miller, M.B., Moore, C., Wedd, J.P., Drake, A.F., Leigh, M.W., 2006. Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis? Pediatr Pulmonol 41, 445–451.

Muldrew, K.L., Miller, R.R., Kressin, M., Tang, Y.-W., Stratton, C., 2007. Necrotizing Fasciitis from Vibrio vulnificus in a Patient with Undiagnosed Hepatitis and Cirrhosis. J Clin Microbiol 45, 1058–1062.

Muldrew, K.L., Simpson, J.F., Stratton, C.W., Tang, Y.-W., 2005. Molecular Diagnosis of Necrotizing Fasciitis by 16S rRNA Gene Sequencing and Superantigen Gene Detection. J Mol Diagn 7, 641–645.

Myung, D.S., Jung, Y.-S., Kang, S.-J., Song, Y.A., Park, K.-H., Jung, S.-I., Kim, S.H., Shin, J.-H., 2009. Primary Shewanella algae Bacteremia Mimicking Vibrio Septicemia. J Korean Med Sci 24, 1192–1194.

Nei, T., Akutsu, K., Shima, A., Tsuboi, I., Suzuki, H., Yamamoto, T., Tanaka, K., Shinoyama, A., Kojima, Y., Washio, Y., Okawa, S., Sonobe, K., Norose, Y., Saito, R., 2012. A case of streptococcal toxic shock syndrome due to Group G streptococci identified as Streptococcus dysgalactiae subsp. equisimilis. J Infect Chemother *Epub*.

Nelson, A., Soyza, A.D., Perry, J.D., Sutcliffe, I.C., Cummings, S.P., 2012. Polymicrobial challenges to Koch's postulates: Ecological lessons from the bacterial vaginosis and cystic fibrosis microbiomes. Innate Immunity *Epub*.

Nicoletti, M., Iacobino, A., Prosseda, G., Fiscarelli, E., Zarrilli, R., De Carolis, E., Petrucca, A., Nencioni, L., Colonna, B., Casalino, M., 2011. Stenotrophomonas maltophilia strains from cystic fibrosis patients: Genomic variability and molecular characterization of some virulence determinants. Int J Med Microbiol 301, 34–43.

Nielsen, 1999. Applications of peptide nucleic acids. Current Opinion in Biotechnology 10, 71–75.

Nielsen, P.H., Daims, H., Lemmer, H. (Eds.), 2009. FISH Handbook for Biological Wastewater Treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. Intl Water Assn.

Nogva, H.K., Drømtorp, S.M., Nissen, H., Rudi, K., 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. BioTechniques 34, 804–808, 810, 812–813.

Nolte, F.S., Caliendo, A.M., 2011. Molecular Microbiology, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 27–59.

Norrby-Teglund, A., Basma, H., Andersson, J., McGeer, A., Low, D.E., Kotb, M., 1998. Varying Titers of Neutralizing Antibodies to Streptococcal Superantigens in Different Preparations of Normal Polyspecific Immunoglobulin G: Implications for Therapeutic Efficacy. Clin Infect Dis. 26, 631–638.

Oishi, H., Kagawa, Y., Mitsumizo, S., Tashiro, Y., Kobayashi, G., Udo, K., Aoki, S., Takayanagi, M., Nagasawa, Z., Araki, K., Ohza, N., Eguchi, Y., Nakashima, M., 2008. A fatal case of necrotizing fasciitis due to bacterial translocation of Klebsiella oxytoca. J Infect Chemother 14, 62–65.

Olsen, R.J., Musser, J.M., 2010. Molecular pathogenesis of necrotizing fasciitis. Annu Rev Pathol 5, 1–31.

Osborn, A.J., Leung, R., Ratjen, F., James, A.L., 2011. Effect of endoscopic sinus surgery on pulmonary function and microbial pathogens in a pediatric population with cystic fibrosis. Arch Otolaryngol. Head Neck Surg. 137, 542–547.

Paju, S., Bernstein, J.M., Haase, E.M., Scannapieco, F.A., 2003. Molecular analysis of bacterial flora associated with chronically inflamed maxillary sinuses. J. Med. Microbiol. 52, 591–597.

Parsek, M.R., Singh, P.K., 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 57, 677–701.

Patel, J.B., Tenover, F.C., Turnidge, J.D., Jorgensen, J.H., 2011. Susceptibility Test Methods: Dilution and Disk Diffusion Methods, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 1122–1143.

Pedersen, K.M., Kolmos, H.J., 2007. Costs attributed to hospital-acquired infections. Ugeskr Laeger 169, 4135–4138.

Perloff, J.R., Palmer, J.N., 2004. Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis. Am J Rhinol 18, 377–380.

Petti, C.A., Weinstein, M.P., Carroll, K.C., 2011. Systems for Detection and Identification of Bacteria and Yeasts, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 15–26.

Pihl, M., Chávez de Paz, L.E., Schmidtchen, A., Svensäter, G., Davies, J.R., 2010. Effects of clinical isolates of Pseudomonas aeruginosa on Staphylococcus epidermidis biofilm formation. FEMS Immunol Med Microbiol 59, 504–512.

Pimentel, J.D., MacLeod, C., 2008. Misidentification of Pandoraea sputorum Isolated from Sputum of a Patient with Cystic Fibrosis and Review of Pandoraea Species Infections in Transplant Patients. J Clin Microbiol 46, 3165–3168.

Pinheiro, P., Mathers, C.D., Krämer, A., 2010. The Global Burden of Infectious Diseases, in: Krämer, A., Kretzschmar, M., Krickeberg, K. (Eds.), Modern Infectious Disease Epidemiology: Concepts, Methods, Mathematical Models, and Public Health. Springer, pp. 3–21.

Pompilio, A., Pomponio, S., Crocetta, V., Gherardi, G., Verginelli, F., Fiscarelli, E., Dicuonzo, G., Savini, V., D'Antonio, D., Di Bonaventura, G., 2011. Phenotypic and genotypic characterization of Stenotrophomonas maltophilia isolates from patients with cystic fibrosis: Genome diversity, biofilm formation, and virulence. BMC Microbiol 11, 159.

Potera, C., 1999. Forging a Link Between Biofilms and Disease. Science 283, 1837.

Price, L.B., Liu, C.M., Frankel, Y.M., Melendez, J.H., Aziz, M., Buchhagen, J., Contente-Cuomo, T., Engelthaler, D.M., Keim, P.S., Ravel, J., Lazarus, G.S., Zenilman, J.M., 2011. Macro-Scale Spatial Variation in Chronic Wound Microbiota: A Cross-Sectional Study. Wound Repair Regen 19, 80–88.

Price, L.B., Liu, C.M., Melendez, J.H., Frankel, Y.M., Engelthaler, D., Aziz, M., Bowers, J., Rattray, R., Ravel, J., Kingsley, C., Keim, P.S., Lazarus, G.S., Zenilman, J.M., 2009. Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. PLoS ONE 4, e6462.

Prod'hom, G., Bizzini, A., Durussel, C., Bille, J., Greub, G., 2010. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Direct Bacterial Identification from Positive Blood Culture Pellets. J Clin Microbiol 48, 1481–1483.

Qin, X., Emerson, J., Stapp, J., Stapp, L., Abe, P., Burns, J.L., 2003. Use of real-time PCR with multiple targets to identify Pseudomonas aeruginosa and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. J Clin Microbiol 41, 4312–4317.

Quinton, P.M., 1999. Physiological Basis of Cystic Fibrosis: A Historical Perspective. Physiol Rev 79, S3–S22.

Ramadan, H.H., Mathers, P.H., Schwartzbauer, H., 2002. Role of Anaerobes in Chronic Sinusitis: Will Polymerase Chain Reaction Solve the Debate. Otolaryngol Head Neck Surg 127, 384–386.

Ramadan, H.H., Sanclement, J.A., Thomas, J.G., 2005. Chronic rhinosinusitis and biofilms. Otolaryngol Head Neck Surg 132, 414–417.

Rasmussen, J., Aanaes, K., Norling, R., Nielsen, K.G., Johansen, H.K., von Buchwald, C., 2012. CT of the paranasal sinuses is not a valid indicator for sinus surgery in CF patients. J Cyst Fibros 11, 93–99.

Rau, M.H., Hansen, S.K., Johansen, H.K., Thomsen, L.E., Workman, C.T., Nielsen, K.F., Jelsbak, L., Høiby, N., Yang, L., Molin, S., 2010. Early adaptive developments of Pseudomonas aeruginosa after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. Environ Microbiol 12, 1643–1658.

Rau, M.H., Marvig, R.L., Ehrlich, G.D., Molin, S., Jelsbak, L., 2012. Deletion and acquisition of genomic content during early stage adaptation of Pseudomonas aeruginosa to a human host environment. Environ Microbiol 14, 2200–2211.

Roby, B.B., McNamara, J., Finkelstein, M., Sidman, J., 2008. Sinus surgery in Cystic Fibrosis patients: Comparison of sinus and lower airway cultures. Int J Pediatr Otorhinolaryngol 72, 1365–1369.

Rogers, G.B., Carroll, M.P., Serisier, D.J., Hockey, P.M., Jones, G., Bruce, K.D., 2004. Characterization of Bacterial Community Diversity in Cystic Fibrosis Lung Infections by Use

of 16S Ribosomal DNA Terminal Restriction Fragment Length Polymorphism Profiling. J Clin Microbiol 42, 5176–5183.

Rogers, G.B., Carroll, M.P., Serisier, D.J., Hockey, P.M., Jones, G., Kehagia, V., Connett, G.J., Bruce, K.D., 2006. Use of 16S rRNA Gene Profiling by Terminal Restriction Fragment Length Polymorphism Analysis To Compare Bacterial Communities in Sputum and Mouthwash Samples from Patients with Cystic Fibrosis. J Clin Microbiol 44, 2601–2604.

Rogers, G.B., Carroll, M.P., Serisier, D.J., Hockey, P.M., Kehagia, V., Jones, G.R., Bruce, K.D., 2005. Bacterial activity in cystic fibrosis lung infections. Respir Res 6, 49.

Rogers, G.B., Daniels, T.W., Tuck, A., Carroll, M.P., Connett, G.J., David, G.J., Bruce, K.D., 2009. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. BMC Pulm Med 9, 14.

Rogers, G.B., Hart, C.A., Mason, J.R., Hughes, M., Walshaw, M.J., Bruce, K.D., 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol 41, 3548–3558.

Rogers, G.B., Marsh, P., Stressmann, A.F., Allen, C.E., Daniels, T.V.W., Carroll, M.P., Bruce, K.D., 2010. The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. Clin Microbiol Infect 16, 1656–1658.

Rogers, G.B., Skelton, S., Serisier, D.J., Gast, C.J. van der, Bruce, K.D., 2010. Determining Cystic Fibrosis-Affected Lung Microbiology: Comparison of Spontaneous and Serially Induced Sputum Samples by Use of Terminal Restriction Fragment Length Polymorphism Profiling. J Clin Microbiol 48, 78–86.

Rogers, G.B., Stressmann, F.A., Koller, G., Daniels, T., Carroll, M.P., Bruce, K.D., 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagn Microbiol Infect Dis 62, 133–141.

Rudi, K., Moen, B., Drømtorp, S.M., Holck, A.L., 2005. Use of Ethidium Monoazide and PCR in Combination for Quantification of Viable and Dead Cells in Complex Samples. Appl Environ Microbiol 71, 1018–1024.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487–491.

Sanclement, J.A., Webster, P., Thomas, J., Ramadan, H.H., 2005. Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis. Laryngoscope 115, 578–582.

Sanderson, A.R., Leid, J.G., Hunsaker, D., 2006. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope 116, 1121–1126.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. PNAS 74, 5463–5467.

Sauer, S., Kliem, M., 2010. Mass spectrometry tools for the classification and identification of bacteria. Nat Rev Microbiol 8, 74–82.

Savelkoul, P.H.M., Peters, R.P.H., 2011. Molecular Approaches to the Diagnosis of Sepsis, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 751–765.

Schadt, E.E., Turner, S., Kasarskis, A., 2010. A window into third-generation sequencing. Hum Mol Genet 19, R227–R240.

Segonds, C., Clavel-Batut, P., Thouverez, M., Grenet, D., Coustumier, A.L., Plésiat, P., Chabanon, G., 2009. Microbiological and Epidemiological Features of Clinical Respiratory Isolates of Burkholderia gladioli. J Clin Microbiol 47, 1510–1516.

Sen, C.K., Gordillo, G.M., Roy, S., Kirsner, R., Lambert, L., Hunt, T.K., Gottrup, F., Gurtner, G.C., Longaker, M.T., 2009. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen 17, 763–771.

Seng, P., Drancourt, M., Gouriet, F., Scola, B.L., Fournier, P.-E., Rolain, J.M., Raoult, D., 2009. Ongoing Revolution in Bacteriology: Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. Clin Infect Dis 49, 543–551.

Shapiro, E.D., Milmoe, G.J., Wald, E.R., Rodnan, J.B., Bowen, A., 1982. Bacteriology of the maxillary sinuses in patients with cystic fibrosis. J Infect Dis 146, 589 –593.

Shendure, J., Ji, H., 2008. Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135–1145.

Sibley, C.D., Grinwis, M.E., Field, T.R., Eshaghurshan, C.S., Faria, M.M., Dowd, S.E., Parkins, M.D., Rabin, H.R., Surette, M.G., 2011. Culture Enriched Molecular Profiling of the Cystic Fibrosis Airway Microbiome. PLoS One 6.

Sibley, C.D., Parkins, M.D., Rabin, H.R., Duan, K., Norgaard, J.C., Surette, M.G., 2008. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. PNAS 105, 15070–15075.

Sing, A., Trebesius, K., Heesemann, J., 2001. Diagnosis of Streptococcus dysgalactiae subspecies equisimilis (Group C streptococci) associated with deep soft tissue infections using fluorescent in situ hybridization. Eur J Clin Microbiol Infect Dis 20, 146–149.

Slack, R.C.B., 2007. Diagnostic procedures, in: Greenwood, D., Slack, R.C.B., Peutherer, J.F., Barer, M.R. (Eds.), Medical Microbiology: A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control. Churchill Livingstone, pp. 667–673.

Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'Argenio, D.A., Miller, S.I., Ramsey, B.W., Speert, D.P., Moskowitz, S.M., Burns, J.L., Kaul, R., Olson, M.V., 2006. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. PNAS 103, 8487–8492.

Southern, E.M., 2001. DNA microarrays. History and overview. Methods Mol. Biol. 170, 1–15.

Spasenovski, T., Carroll, M.P., Payne, M.S., Bruce, K.D., 2009. Molecular analysis of diversity within the genus Pseudomonas in the lungs of cystic fibrosis patients. Diagn Microbiol Infect Dis 63, 261–267.

Spilker, T., Uluer, A.Z., Marty, F.M., Yeh, W.W., Levison, J.H., Vandamme, P., LiPuma, J.J., 2008. Recovery of Herbaspirillum Species from Persons with Cystic Fibrosis. J Clin Microbiol 46, 2774–2777.

Stephens, P., Wall, I. b., Wilson, M. j., Hill, K. e., Davies, C. e., Hill, C. m., Harding, K. g., Thomas, D. w., 2003. Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro. Br J Dermatol 148, 456–466.

Stephenson, M.-F., Mfuna, L., Dowd, S.E., Wolcott, R.D., Barbeau, J., Poisson, M., James, G., Desrosiers, M., 2010. Molecular characterization of the polymicrobial flora in chronic rhinosinusitis. J Otolaryngol Head Neck Surg 39, 182–187.

Stevens, D.L., Bisno, A.L., Chambers, H.F., Everett, E.D., Dellinger, P., Goldstein, E.J.C., Gorbach, S.L., Hirschmann, J.V., Kaplan, E.L., Montoya, J.G., Wade, J.C., 2005. Practice guidelines for the diagnosis and management of skin and soft-tissue infections. Clin Infect Dis 41, 1373–1406.

Stressmann, F.A., Rogers, G.B., Klem, E.R., Lilley, A.K., Donaldson, S.H., Daniels, T.W., Carroll, M.P., Patel, N., Forbes, B., Boucher, R.C., Wolfgang, M.C., Bruce, K.D., 2011. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. J Clin Microbiol 49, 281–291.

Stressmann, F.A., Rogers, G.B., van der Gast, C.J., Marsh, P., Vermeer, L.S., Carroll, M.P., Hoffman, L., Daniels, T.W.V., Patel, N., Forbes, B., Bruce, K.D., 2012. Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. Thorax *Epub*.

Thomsen, T.R., Xu, Y., Lorenzen, J., Nielsen, P.H., Schønheyder, H.C., 2012. Improved diagnosis of biofilm infections using various molecular methods, in: DeMeo, P.J., Costerton, J.W., Ehrlich, G.D., Winkler, H. (Eds.), Culture Negative Orthopedic Biofilm Infections. Springer. *In press.*

Thuong, T.C., Dac Tho, N., Thi Hoa, N., Minh Phuong, N.T., Van Tuan, L., Song Diep, T., Lindsay, J., The Dung, N., Van Cam, B., Quoc Thinh, L., Thanh Hai, L., Dieu Linh, L., Campbell, J., Kim Tien, N.T., Vinh Chau, N.V., Cockfield, J., Truong Giang, L., Van Nghiem, P., Hoang Son, L., Tan Son, H., Van Phung, L., Counahan, M., Bentsi-Enchill, A., Brown, R., Simmerman, J., Tran Chinh, N., Tinh Hien, T., Farrar, J., Schultsz, C., 2007. An Outbreak of Severe Infections with Community-Acquired MRSA Carrying the Panton-Valentine Leukocidin Following Vaccination. PLoS ONE 2.

Trebesius, K., Leitritz, L., Adler, K., Schubert, S., Autenrieth, I.B., Heesemann, J., 2000. Culture independent and rapid identification of bacterial pathogens in necrotising fasciitis and streptococcal toxic shock syndrome by fluorescence in situ hybridisation. Med Microbiol Immunol 188, 169–175.

Tunney, M.M., Field, T.R., Moriarty, T.F., Patrick, S., Doering, G., Muhlebach, M.S., Wolfgang, M.C., Boucher, R., Gilpin, D.F., McDowell, A., Elborn, J.S., 2008. Detection of Anaerobic Bacteria in High Numbers in Sputum from Patients with Cystic Fibrosis. Am J Respir Crit Care Med 177, 995–1001.

Turnidge, J.D., Ferraro, M.J., Jorgensen, J.H., 2011. Susceptibility Test Methods: General Considerations, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 1115–1121.

Tuttle, M.S., Mostow, E., Mukherjee, P., Hu, F.Z., Melton-Kreft, R., Ehrlich, G.D., Dowd, S.E., Ghannoum, M.A., 2011. Characterization of Bacterial Communities in Venous Insufficiency Wounds by Use of Conventional Culture and Molecular Diagnostic Methods. J Clin Microbiol 49, 3812–3819.

Ulrich, M., Worlitzsch, D., Viglio, S., Siegmann, N., Iadarola, P., Shute, J.K., Geiser, M., Pier, G.B., Friedel, G., Barr, M.L., Schuster, A., Meyer, K.C., Ratjen, F., Bjarnsholt, T., Gulbins, E., Döring, G., 2010. Alveolar inflammation in cystic fibrosis. J Cyst Fibros. 9, 217–227.

van Belkum, A., 1994. DNA fingerprinting of medically important microorganisms by use of PCR. Clin Microbiol Rev 7, 174–184.

van Belkum, A., Renders, N.H.M., Smith, S., Overbeek, S.E., Verbrugh, H.A., 2000. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients1. FEMS Immunol Med Microbiol 27, 51–57.

van der Gast, C.J., Walker, A.W., Stressmann, F.A., Rogers, G.B., Scott, P., Daniels, T.W., Carroll, M.P., Parkhill, J., Bruce, K.D., 2011. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. ISME J 5, 780–791.

van Veen, S.Q., Claas, E.C.J., Kuijper, E.J., 2010. High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. J Clin Microbiol 48, 900–907.

Vandamme, P., 2011. Taxonomy and Classification of Bacteria, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 213–227.

Vartoukian, S.R., Palmer, R.M., Wade, W.G., 2010. Strategies for culture of "unculturable" bacteria. FEMS Microbiol Lett 309, 1–7.

Vital, D., Hofer, M., Boehler, A., Holzmann, D., 2012. Posttransplant sinus surgery in lung transplant recipients with cystic fibrosis: a single institutional experience. Eur Arch Otorhinolaryngol In press.

Wall, I.B., Davies, C.E., Hill, K.E., Wilson, M.J., Stephens, P., Harding, K.G., Thomas, D.W., 2002. Potential role of anaerobic cocci in impaired human wound healing. Wound Repair Regen 10, 346–353.

Walter, S., Gudowius, P., Bosshammer, J., Römling, U., Weissbrodt, H., Schürmann, W., von der Hardt, H., Tümmler, B., 1997. Epidemiology of chronic Pseudomonas aeruginosa infections in the airways of lung transplant recipients with cystic fibrosis. Thorax 52, 318–321.

Wang, D., Coscoy, L., Zylberberg, M., Avila, P.C., Boushey, H.A., Ganem, D., DeRisi, J.L., 2002. Microarray-based detection and genotyping of viral pathogens. Proc Natl Acad Sci U S A 99, 15687–15692.

Warren, A.E., Boulianne-Larsen, C.M., Chandler, C.B., Chiotti, K., Kroll, E., Miller, S.R., Taddei, F., Sermet-Gaudelus, I., Ferroni, A., McInnerney, K., Franklin, M.J., Rosenzweig, F., 2011. Genotypic and phenotypic variation in Pseudomonas aeruginosa reveals signatures of secondary infection and mutator activity in certain cystic fibrosis patients with chronic lung infections. Infect Immun 79, 4802–4818.

Waters, V., Zlosnik, J.E.A., Yau, Y.C.W., Speert, D.P., Aaron, S.D., Guttman, D.S., 2012. Comparison of three typing methods for Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Eur J Clin Microbiol Infect Dis *Epub*.

Wellinghausen, N., Köthe, J., Wirths, B., Sigge, A., Poppert, S., 2005. Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical Pseudomonas aeruginosa, from patients with cystic fibrosis. J Clin Microbiol 43, 4070–4075.

Wellinghausen, N., Wirths, B., Poppert, S., 2006. Fluorescence In Situ Hybridization for Rapid Identification of Achromobacter xylosoxidans and Alcaligenes faecalis Recovered from Cystic Fibrosis Patients. J Clin Microbiol 44, 3415–3417.

Wheeler, D.A., Srinivasan, M., Egholm, M., Shen, Y., Chen, L., McGuire, A., He, W., Chen, Y.-J., Makhijani, V., Roth, G.T., Gomes, X., Tartaro, K., Niazi, F., Turcotte, C.L., Irzyk, G.P., Lupski, J.R., Chinault, C., Song, X., Liu, Y., Yuan, Y., Nazareth, L., Qin, X., Muzny, D.M., Margulies, M., Weinstock, G.M., Gibbs, R.A., Rothberg, J.M., 2008. The complete genome of an individual by massively parallel DNA sequencing. Nature 452, 872–876.

Wiedbrauk, D.L., 2011. Microscopy, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 5–14.

Willner, D., Haynes, M.R., Furlan, M., Schmieder, R., Lim, Y.W., Rainey, P.B., Rohwer, F., Conrad, D., 2012. Spatial distribution of microbial communities in the cystic fibrosis lung. ISME J 6, 471–474.

Wilson, W.J., Strout, C.L., DeSantis, T.Z., Stilwell, J.L., Carrano, A.V., Andersen, G.L., 2002. Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. Mol Cell Probes 16, 119–127.

Wittwer, C.T., Kusukawa, N., 2011. Real-Time-PCR and Melting Analysis, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 63–82.

Wolcott, R., Costerton, J.W., Raoult, D., Cutler, S.J., 2012. The polymicrobial nature of biofilm infection. Clin Microbiol Infect *Epub*.

Wolcott, R., Dowd, S., 2011. The role of biofilms: are we hitting the right target? Plast Reconstr Surg 127 Suppl 1, 28S–35S.

Wolcott, R.D., Gontcharova, V., Sun, Y., Dowd, S.E., 2009. Evaluation of the bacterial diversity among and within individual venous leg ulcers using bacterial tag-encoded FLX and Titanium amplicon pyrosequencing and metagenomic approaches. BMC Microbiol 9, 226.

Wolff, T.Y., Bjarnsholt, T., Kirketerp-Møller, K., Thomsen, T.R., 2011. Influence of microorganisms on the healing of skin grafts from chronic venous leg wounds, in: Spear, M. (Ed.), Skin Grafts - Indications, Applications and Current Research. InTech, Available from: http://www.intechopen.com/books/skin–grafts–indications–applications–and–current–research/influence–of–microorganisms–on–the–healing–of–skin–grafts–from–chronic–venous–leg–wounds.

Wolk, D.M., Hayden, R.T., 2011. Quantitative Molecular Methods, in: Persing, D.H., Tang, Y.-W., Nolte, F.S. (Eds.), Molecular Microbiology: Diagnostic Principles and Practice. ASM Press, pp. 83–105.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K.C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J.R., Randell, S., Boucher, R.C., Döring, G., 2002. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest 109, 317–325.

Yang, L., Haagensen, J.A.J., Jelsbak, L., Johansen, H.K., Sternberg, C., Høiby, N., Molin, S., 2008. In Situ Growth Rates and Biofilm Development of Pseudomonas aeruginosa Populations in Chronic Lung Infections. J. Bacteriol. 190, 2767–2776.

Yang, L., Jelsbak, L., Marvig, R.L., Damkiær, S., Workman, C.T., Rau, M.H., Hansen, S.K., Folkesson, A., Johansen, H.K., Ciofu, O., Høiby, N., Sommer, M.O.A., Molin, S., 2011. Evolutionary dynamics of bacteria in a human host environment. Proc Natl Acad Sci U S A 108, 7481–7486.

Yang, L., Rau, M.H., Yang, L., Høiby, N., Molin, S., Jelsbak, L., 2011. Bacterial adaptation during chronic infection revealed by independent component analysis of transcriptomic data. BMC Microbiol 11, 184.

Zemanick, E.T., Sagel, S.D., Harris, J.K., 2011. The airway microbiome in cystic fibrosis and implications for treatment. Current Opinion in Pediatrics 23, 319–324.

Zhao, J., Schloss, P.D., Kalikin, L.M., Carmody, L.A., Foster, B.K., Petrosino, J.F., Cavalcoli, J.D., VanDevanter, D.R., Murray, S., Li, J.Z., Young, V.B., LiPuma, J.J., 2012. Decade-long bacterial community dynamics in cystic fibrosis airways. PNAS 109, 5809–5814.

Aanaes, K., Rickelt, L.F., Johansen, H.K., von Buchwald, C., Pressler, T., Høiby, N., Jensen, P.Ø., 2011. Decreased mucosal oxygen tension in the maxillary sinuses in patients with cystic fibrosis. J Cyst Fibros 10, 114–120.

Research paper 1

Rudkjøbing, V.B., Thomsen, T.R., Melton-Kreft, R., Ahmed, A., Eickhardt-Sørensen, S.R., Bjarnsholt, T., Nielsen, P.H., Ehrlich, G.D., Moser, C. (in prep). Comparing culture and molecular methods for the identification of microorganisms involved in necrotizing soft tissue infections.

.

Comparing culture and molecular methods for the identification of microorganisms involved in necrotizing soft tissue infections

V.B. Rudkjøbing[1], T.R. Thomsen[1, 2], R. Melton-Kreft[3], A. Ahmed[3], S.R. Eickhardt-Sørensen[4], T. Bjarnsholt[4, 5], P.H. Nielsen[1], G.D. Ehrlich[3,7], C. Moser[5]

[1] Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Denmark

[2] Life Science Division, The Danish Technological Institute, Denmark

[3] Center for Genomic Sciences, Allegheny-Singer Research Institute, Pittsburgh, United States of America

[4] Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark.

[5] Department of Clinical Microbiology, Rigshospitalet, Denmark

[6] Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark.

[7] Department of Microbiology and Immunology, Drexel University College of Medicine, Allegheny Campus, Pittsburgh, United States of America.

Key words: Necrotizing soft tissue infections, microorganisms, 16S rRNA, cloning, direct Sanger sequencing, Ibis T5000 Biosensor, 454 pyrosequencing, qPCR

Short running title: Microorganisms in necrotizing soft tissue infections

Abstract

Necrotizing soft tissue infections (NSTIs) is a group of infections of all soft tissues involving necrosis of the afflicted tissue. NSTI is potentially life threatening due to major and rapid destruction of tissue and potential association of septic shock and organ effect or failure. The gold standard for identification of involved pathogens is culture; however molecular methods for identification of microorganisms may provide a more rapid result and be able to identify additional microorganisms that are not detected by culture.

In this study, tissue samples (n=20) obtained after debridement of 10 NSTI patients were analyzed by standard culture, peptide nucleic acid (PNA) fluorescence in situ hybridization (FISH) and different molecular methods. The molecular methods included analysis of microbial diversity by 1) direct rRNA gene Sanger sequencing 2) construction of near full-length 16S rRNA gene clone libraries (or deep 454-based pyrosequencing) and 3) the Ibis T5000 biosensor. Furthermore, quantitative PCR (qPCR) was used to verify and determine the relative abundance of *Streptococcus pyogenes* in samples.

For approximately half the samples it was possible to identify microorganisms by culture, although some samples did not result in growth presumable due to administration of antimicrobial therapy. The various molecular methods identified microorganisms in all samples, and frequently detected additional microorganisms compared to culture. Although the various molecular methods generally gave concordant results, our results seem to indicate that direct Sanger sequencing may misidentify or overlook microorganisms that can be detected by other molecular methods.

Half of the patients were found to harbor *S. pyogenes*, but several atypical findings were also made including a monomicrobial infection by *Acinetobacter baumannii* and *Streptococcus pneumoniae*, and a polymicrobial infection by fungi, mycoplasma and *Fusobacterium necrophorum*. The faster turnaround time that is offered by some molecular methods makes their use attractive for identification of microorganisms, especially for fulminant life-threatening infections such as NSTI.

Introduction

The spectrum of diseases referred to as soft tissue infections is diverse. Their common characteristic is that they involve infection of the skin, subcutaneous tissue, fascia or muscle (Bowler et al., 2001). The infections range from common superficial epidermal infections to potentially life threatening but rare cases of necrotizing soft tissue infections (NSTI) (Stevens et al., 2005). The incidence of NSTI has been estimated to be 4 cases per 100,000 person-years in the USA (Ellis Simonsen et al., 2006); thus, an average practitioner will only see one or two cases during their career (Anaya and Dellinger, 2007; Sarani et al., 2009), and may therefore not be sufficiently familiar with the disease to ensure a rapid diagnosis and appropriate treatment (Sarani et al., 2009). Treatment of NSTI involves immediate aggressive surgical debridement and administration of intravenous broad-spectrum antibiotics. Some centers also use systemic administration of non-specific immunoglobulin as well as hyperbaric oxygen treatment. Establishing the diagnosis can be a challenge in managing NSTI, because the early signs are non-specific and include local erythema and swelling with warmth and pain out of proportion to physical findings (Wong et al., 2003; Sarani et al., 2009). As the disease progress bullae filled with serous fluid are formed, and eventually large hemorrhagic bullae, skin necrosis, fluctuance, crepitus and sensory and motor deficits become apparent (Wong et al., 2003; Stevens et al., 2005). Despite many advances in the understanding of NSTI and great improvements in medical care, the mortality associated with NSTI remains high (Stevens et al., 2005; Sarani et al., 2009). Different mortality rates have been reported, but are generally in the range of 16-24% (Wong et al., 2003; Anaya and Dellinger, 2007; Golger et al., 2007).

The etiology of necrotizing fasciitis is variable and not fully understood. In some cases an antecedent penetrating injury is present (such as skin trauma, varicella, and burns) (Cunningham, 2000; Wong et al., 2003; Olsen and Musser, 2010). The skin trauma may be caused by surgery or may even be caused by a trivial event such as an insect bite, scratch, or abrasion (Singh et al., 2002; Hasham et al., 2005). In many cases however, no identifiable cause can be found (Cunningham, 2000; Singh et al., 2002; Wong et al., 2003; Johansson et al., 2010; Olsen and Musser, 2010). In these cases it is hypothesized that necrotizing fasciitis may result from hematogeneous seeding from a reservoir in the oropharynx or other anatomic site (Kihiczak et al., 2006; Olsen and Musser, 2010). Most patients who develop necrotizing fasciitis have pre-existing conditions that render them susceptible to infection, including diabetes mellitus, advanced age, immune suppression, peripheral vascular disease, obesity, smoking, drug and alcohol misuse (Wong et al., 2003; Hasham et al., 2005; Anaya and Dellinger, 2007; Angoules et al., 2007; Sarani et al., 2009). The necrotizing changes associated with NSTI lead to devitalization of the infected tissue, which provides a suitable environment for further microbial growth, setting the stage for major and rapid destruction of tissue (Bowler et al., 2001; Stevens et al., 2005). Infection can spread as fast as 1 inch per hour with little overlying skin change (Sarani et al., 2009). It is hypothesized that rapid tissue destruction and severe pain associated with NSTI is caused by the interaction of microorganisms and their toxins with the human coagulation system, leading to hypercoagulation, vascular occlusion and microvascular thrombosis (Bryant, 2003). The resulting poor tissue

perfusion also has implications on the treatment strategy, since the antibiotic concentration at the infection site may be insufficient (Johansson et al., 2010).

Historically NSTI has been classified into specific types based on anatomic location or microbial findings. Based on bacteriology, two types of infections have been defined. Type-I infections are polymicrobial synergistic infections (usually caused by non-group-A streptococci, aerobic organisms and anaerobic organisms) and usually occur in immunocompromised hosts. Type-II infections are usually caused by *Streptococcus pyogenes* alone or with staphylocci and tend to occur in patients with no underlying comorbidities. This type of infection might be associated with toxic shock syndrome (Sarani et al., 2009). A third type has been suggested, although it has not been universally agreed on, caused by marine vibrios, and is usually associated with exposure to seawater or marine animals (Wong et al., 2003; Sarani et al., 2009). However, it has been suggested that such classifications lead to undue complication of the issue. It is argued that the most important information to be established is presence of a necrotizing component, distinguishing NSTI from a milder condition such as cellulitis that should respond to antibiotics alone (Elliott et al., 1996; Singh et al., 2002; Hasham et al., 2005; Stevens et al., 2005; Anaya and Dellinger, 2007). On the other hand, the correct identification of the microorganisms involved has important implications on the antibiotic treatment since *S. pyogenes* or *Clostridium perfringens* need different treatment modalities (Kaul et al., 1997; Cawley et al., 1999; Stevens et al., 2005) than for example methicillin-resistant *Staphylococcus aureus* (Miller et al., 2005), or *Streptococcus pneumoniae* (Kwak et al., 2002).

The microbial communities involved in NSTI have previously only been investigated by culture-dependent methods. It is, however, possible that additional microorganisms, which may not be detectable by standard cultural methods, are involved in the infections as recent studies of numerous infectious conditions using molecular diagnostics have revealed that many of what were once thought to be monomicrobial infections are in fact polymicrobial (Melton-Kreft et al., In Press; Hall-Stoodley et al., 2006; Ehrlich et al., 2010; Costerton et al., 2011; Stoodley, Ehrlich, et al., 2011). Presently, various molecular methods are available that may be able to identify additional microorganisms and offer a more rapid identification of microorganisms than routine cultural methods. Since the various NSTI-causing microorganisms may require different antibiotics, the initial empiric antimicrobial treatment may need to be subsequently modified to target the infecting microorganisms. Because of the rapid progression of the disease it is of paramount importance that the etiologic pathogens can be rapidly identified. In this study, we investigated several molecular methods for identification of microorganisms, including the Ibis T5000 biosensor, quantitative polymerase chain reaction (qPCR) and 16S rRNA gene analysis by direct sequencing, near full length 16S rRNA clone libraries and 454 pyrosequencing. These findings were then compared to those of routine cultural methods.

Methods

Patients and samples

Samples in this study were obtained from NSTI patients by debridement of the infected area, performed at Rigshospitalet (Copenhagen, Denmark). A total of 20 samples from 10 patients were included (Table 1). The debrided tissue were immediately transported to the Department of Clinical Microbiology, Rigshospitalet (Copenhagen, Denmark), where each sample were divided into three subsamples for standard culture experiments, molecular analysis and PNA FISH experiments. The samples for molecular analysis and FISH experiments were transferred to tubes containing glycerol or ethanol respectively, and kept frozen until analysis.

Culture

All culture experiments were performed at the Department of Clinical Microbiology at Rigshospitalet. All biopsies were analyzed by Gram-staining, and culture. Both aerobic and anaerobic conditions were used. Biopsies were plated on brain heart infusion agar (BHIA, SSI, Copenhagen Denmark), coagulated agar, and 5% horse blood agar (SSI) for culture in a 5% CO_2 atmosphere. Aerobic conditions included plating on modified Conradi-Drigalski ("Blue plates", SSI), in serum buillon, in thioglycollate media, on tellurite agar (SSI) in a normal atmosphere. 37 Colonies were further identified by use of Matrix-assisted laser desorption-ionization time of flight mass specroscopy (MALDI-TOF MS), Bruker, Bremen, Germany). Resistance patterns were analyzed by disc diffusion test on blood agar (SSI) using Neosensitabs (Rosco Diagnostica, Taastrup, Denmark).

DNA extraction

DNA was extracted from samples as described previously (Stoodley, Conti, et al., 2011). Briefly, the tissue samples were cut into smaller pieces under sterile conditions. Approximately 1 mm^3 of tissue was transferred to a microcentrifuge tube containing tissue lysis buffer (ATL, Qiagen) and 20 mg/mL proteinase K (Qiagen). The sample was incubated at 55 °C until visual inspection indicated that lysis was achieved. 100µL Zirconia/Silica Beads mixture (50 µL of 0.1 mm diameter, Biospec, PN: 11079101z and 50 µL of 0.7 mm diameter, Biospec, PN:11079107zx) were added to the microcentrifuge tube and the sample was homogenized for 10 min at 25 Hz using a Qiagen Tissuelyser (Model MM300, cat# 85210). DNA from the lysed samples was extracted using the Qiagen DNeasy Tissue kit, according to the manufacturer's protocol. The DNA was eluded in 200 µL AE buffer (10 mM Tris·Cl; 0.5 mM EDTA, pH 9.0).

Direct rRNA gene Sanger sequencing

PCR was performed with primers that targeted the first 500 bases of the bacterial 16S rRNA gene or the D2 region of the fungal 28S rRNA gene. The resulting PCR products were sequenced using the MicroSeq® 500 kit (Applied Biosystems, Carlsbad, California) according to the manufacturer's guidelines. The resulting DNA sequences were compared to the sequence library included in the MicroSeq® ID analysis software. In cases where sequencing resulted in mixed chromatograms due to 16S rRNA gene products from multiple species, these chromatograms were analyzed using RipSeq Mixed at www.ripseq.com.

Construction and analysis of clone libraries

Clone libraries were constructed for near full length 16S rRNA genes (*E. coli* position 26-1390) and sequences were obtained as described previously (Rudkjøbing et al., Unpublished; Xu et al., 2012). Briefly, PCR amplicons of near full-length 16S rRNA genes were cloned using the TOPO TA Cloning ® kit (Invitrogen) according to the manufacturer's instructions. Plasmid purification and sequencing was performed by Macrogen Inc. (Korea) using M13F primer (and M13R primer in some cases). Manual refinement of sequences and construction of consensus sequences were done in CLC Main Workbench (CLC bio, Aarhus, Denmark). Sequences were checked for chimeras using the Mallard software package (Ashelford et al., 2006), aligned using SINA Web Aligner (Pruesse et al., 2007) and imported into the ARB software package (Ludwig et al., 2004) for taxonomic lineage assignment, using the non-redundant (NR) SSU Ref database from SILVA Release 106 as reference database. Operational taxonomic units (OTUs) were constructed across all patient samples for clones having a sequence similarity of more than 97% since these sequences are typically assigned to the same species. One clone from each OTU was sequenced with both M13F and M13R primers. The resulting consensus sequences and their closest relatives in the database were selected to construct phylogenetic trees using neighbor joining, maximum parsimony and maximum likelihood methods. The non-redundant, near full-length 16S rRNA gene sequences, representing each OTU obtained in this study, were deposited in GenBank under the accession numbers (*not yet submitted*).

454-pyrosequencing

454-based pyrosequencing was performed largely as described previously (Mussmann et al., 2007). Briefly, bar-coded FLX-titanium amplicon pyrosequencing of the V2–V3 region of the 16S rRNA gene was performed using the 27F and 338R primers. The DNA fragments were amplified using Platinum Hi-Fi taq polymerase (Invitrogen) with 800 µM dNTP, 2 mM $MgCl_2$ and 400 nM of each primer. To each reaction 5 µL of template DNA was added, and the volume was adjusted to 50 µL. The PCR incubation conditions were 94°C for 2 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 60 s, with a final extension at 72 °C for 7 min. Tag-encoded FLX amplicon pyrosequencing analyses utilized the Roche 454 FLX instrument with titanium reagents and titanium procedures (Roche).

Analysis of 16S rRNA gene amplicon sequences was performed using Quantitative Insights Into Microbial Ecology (QIIME v.1.3.0) pipeline (Caporaso et al., 2010). The sequencing data was processed initially with AmpliconNoise (Quince et al., 2011) to remove noise. Then the QIIME pipeline separates the sequences into individual specimen communities based on the unique 5' barcode sequence and utilizes a suite of external programs to make taxonomic assignments and estimate phylogenetic diversity. These data were used to generate taxonomic summaries. The default settings in QIIME were employed for analysis, except that the sequences were grouped into operational taxonomic units using 99% sequence similarity for clustering; taxonomic assignments were done using Greengenes taxonomy (DeSantis et al., 2006).

Ibis T5000 assay

An aliquot of each DNA extract was loaded into each of 16 wells of an Ibis 96-well BAC (bacteria, antibiotic resistance genes, candida) detection plates (Abbott Molecular) and processed as described previously (Tuttle et al., 2011). Briefly, PCR amplifications were carried out, and the resulting PCR products where then desalted in a 96-well format and sequentially electrosprayed into the TOF MS as described by the manufacturer. The spectral signals were processed to determine the mass of each strand of the PCR products, which in turn were used to derive the base compositions that were then compared to the Ibis database to obtain species level determinations for all microorganisms (Ecker et al., 2008).

Quantitative PCR

Quantification of *S. pyogenes* (Dawson et al., 2009) and 16S rRNA genes (Suzuki et al., 2000) was performed using hydrolysis probe chemistry. The *S. pyogenes* assay is commercially available from Biosearch Technologies (Novato, CA). For each sample duplicate 25 µL reactions were run, each containing: 12.5 µL Brilliant® qPCR Master mix (Agilent Technologies, Santa Clara, California), 25 µg BSA (Sigma-Aldrich, Brøndby, Denmark), appropriate concentration of primers and TaqMan® probes (*S. pyogenes*: 400 nM primers and 100 nM probe, 16S rRNA: 900 nM primers and 200 nM probe), 0.75 µM ROX reference dye (Agilent Technologies) and 2 µL of template DNA. Measurements were obtained by absolute quantification using genomic DNA isolated from *S. pyogenes* (DSM 20565) and *P. aeruginosa* (DSM 1253) for total bacteria quantification. The number of isolated genomes was calculated based on DNA concentration (Quant-iT™ dsDNA Assay Kit (Invitrogen)) and genome size estimated to be 1.8Mbp for *S. pyogene*s and 6.5 Mbp for *P. aeruginosa* (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Dilution series of the genomic DNA covered a range of 10^6-10^0 genome copies. Reactions were run on an Mx3005P (Agilent Technologies) with the following program: 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 60 °C.

Analysis of quantitative data

The number of gene copies measured by qPCR was converted to number of CFU per gram sample using $CFU/g = {C_{measured}/C_{genome} * (V_{total}/V_{used})} \Big/ m_{sample}$. Here $C_{measured}$ is the number of copies measured and C_{genome} is the number of gene copies in the genome of one CFU. The standard deviation of all measurements above the detection limit of the assays was calculated. For samples where more than one bacterium could be quantified, a two tailed T-test was used to provide a hypothesis test of the difference between population means. A statistical value of ≤ 0.05 was considered significant.

Visualization of samples

The samples were prepared for visualization by imbedding in paraffin, which was sectioned (4 µm) and mounted on microscope slides. Before staining or hybridization, the slides of five randomly selected samples (patient 4A, 6A, 8A, 8C and 9B) were deparaffinized by using 2x 5 min xylene, 2x 3 min 99.9% EtOH, 2x 3min 96% EtOH, and washed 3x 3 min in sterile water. The de-paraffinized NSTI sections were analyzed by FISH using PNA probes (Stender, 2003). A mixture of fluorescein isothiocyanate (FITC)-labeled *S. aureus*-specific PNA probe and Texas Red-labeled universal bacterium PNA probe both in hybridization solution

(AdvanDx, Inc., Woburn, MA) was added to each section and hybridized in a PNA FISH Workstation at 55°C for 90 min covered by a lid (Bjarnsholt et al., 2009).The slides were washed for 30 min. at 55°C in Wash Solution (AdvanDx). 200 µl of DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Life technologies, Paisley, Scotland) was added and incubated in darkness for 15 minutes at RT. The sample was then washed twice with PBS. Once dry, 20 µl Prolong Gold (Life technologies) was added and a cover slip was added. To fix the coverslip, a small amount of clear nail polish was brushed on each side. The entire PNA FISH procedure required approx. 2.5 h. Slides were investigated using a LSM 710 confocal laser scanning microscope (Zeiss, Germany).

Results

Identification of microorganisms by routine culture

The findings by routine culture at the Department of Clinical Microbiology, Rigshospitalet (Copenhagen, Denmark), mostly revealed monomicrobial infections of the surgical samples (in 7 of the 10 patients) (Table 2). These monomicrobial infections were primarily caused by streptococci (71% of the monomicrobial infections), specifically *S. pyogenes*, *S. pneumoniae* and non-hemolytic streptococci. The remaining monomicrobial culture findings were identified as *Acinetobacter baumanii* (patient 3) and fungal infection (patient 7). Furthermore, two patients were found to harbor more than one microorganism (patients 5 and 6), where *Bacteroidees fragilis* with *Clostridium paraputrificum* and *S. pyogenes* with *Escherichia coli* were found. One fourth of the samples investigated by culture did not result in growth of microorganisms. Three of these surgical samples originated from patients where other samples taken from the site of infection resulted in growth of microorganisms. The remaining culture-negative samples originated from a patient, where none of the samples resulted in growth of microorganisms (patient 10).

Identification of microorganisms by molecular methods

Generally, the molecular methods confirmed the cultural findings (Table 2). However, using the multiple molecular methods, microorganisms were found in all samples including those that were culture-negative, and in most culture-positive cases additional microorganisms were identified by the molecular methods (Table 3). Overall, the different molecular methods gave largely concordant results, although the results obtained for patient 10 was difficult to interpret since the various methods gave differing results. 16S rRNA clone libraries were not constructed for six of the samples, instead deep 454-based pyrosequencing of 16S rRNA gene was performed (Figure 1). For four of the samples (patients 4A, 8A, 8B and 8C) the deep sequencing confirmed the findings by the other molecular methods. For the remaining two samples, deep sequencing confirmed the other molecular diagnostics and also revealed additional microorganisms (in patient 6A these were *Peptoniphilus* sp. and *Porphyromonas* sp., in patient 7A *Prevotella* sp. and *Sharpea* sp. were additionally found). In two of the samples some of the findings detected by a single molecular method were not confirmed by the 454 pyrosequencing (in sample 4A this was *Streptococcus didelphis* detected by the Ibis T5000 Biosensor, and in sample 6A it was staphylococci by the Ibis T5000 Biosensor and *Mycoplasma hominis* by direct Sanger sequencing). Of the used molecular methods only the Ibis T5000 biosensor could identify *Candida albicans* in sample 7B and *Cladosporium cladosporioides* in sample 6A, since this was the only method not entirely based on the bacterial 16S rRNA gene.

Verification of findings by qPCR

The findings of *S. pyogenes* by molecular methods could generally be confirmed by qPCR (Figure 2). Based on the measurements of bacterial 16S rRNA genes, *S. pyogenes* was the dominant microorganism in most of the samples, except 6A and 7B.

Visualization of samples

Of the five randomly selected NSTI samples investigated by FISH and DAPI staining, only two were found to have detectable amounts of bacterial cells (Figure 3). The observed bacteria were located in clusters. Generally, the areas of tissue destruction were not found to contain detectable bacterial cells, but large amounts of inflammatory stained with DAPI.

Discussion

NSTI is a serious, potentially lethal condition induced by microorganisms. The gold standard for identification of microorganisms involved in NSTI is culture and a number of studies have described microbiological findings during NSTI. However, newer molecular techniques may provide more rapid results with the added benefit of being able to provide additional information relating to the detection of unculturable organisms. Therefore, the present study was designed to investigate the potential of adding molecular diagnostics to cultural studies in the diagnosis of NSTI. For the majority (15 of 20: 75%) of samples included in this study, pathogens could be identified by culture (Table 2) with monomicrobial infections caused by *S. pyogenes* being the most frequently finding, although one patient (patient 6) was found to harbor additional microorganisms. The incidence of monomicrobial *S. pyogenes* NSTIs is higher in this study than reported elsewhere, with an accordingly decreased incidence of polymicrobial infections (Elliott et al., 2000; Wong et al., 2003; Levine and Manders, 2005; Sarani et al., 2009).

For all samples three different molecular methods were used to identify microorganisms: 1) direct rRNA gene Sanger sequencing; 2) construction of 16S rRNA gene clone libraries and Sanger sequencing (or 454-based 16S rRNA gene pyrosequencing in samples 4A, 6A, 7A, 8A, 8B and 8C); and 3) the Ibis T5000 Biosensor. Although all of the molecular methods generally provided concordant results, there were some cases where a microorganism was only detected by one of the three methods (Table 3). Only microorganisms found by at least two molecular methods were considered to be present in the sample, with the exception of fungal species which were only found by the Ibis T5000 biosensor, since these were not targeted by 16S rRNA gene based methods. For a single patient (patient 10) the various molecular methods gave discrepant or negative results. Since the confidences of the Ibis T5000 biosensor results were generally low (< 0.72) as was the number of genomes/well (data not shown) the results may be contaminants or background, which would correspond with the inability to construct clone libraries due to negative PCR. Discrepancies in results were most often caused by direct Sanger sequencing, which misidentified or missed the microorganisms that could be found by the other two molecular methods. Since the DNA molecules in the direct Sanger sequencing reactions are competing for the same reagents it is possible that microorganisms present in low quantities cannot be identified by this method. Furthermore the Ripseq algorithm used for interpretation of mixed chromatograms has only been validated in samples containing up to three different species (Kommedal et al., 2008), which may explain the less complex communities that are being found by direct Sanger sequencing. Although these considerations might explain some of the discrepant results, it does not explain the cases of misidentifications. The fact that all misidentifications by direct Sanger sequencing is identified as the same species (*S. pyogenes*) indicates that the results are based on contamination, potentially during the PCR setup in order for the DNA sequences from this species to outcompete the DNA from the microorganisms within the sample.

By the molecular methods microorganisms could be identified in all samples (with the exception of samples from patient 10), including those where no growth was observed by culture. Overall, there were a total of 17 samples where culture and molecular methods were in agreement, giving either concordant (13 samples) or partially concordant results (four samples). In the remaining cases the disagreement between culture and

molecular methods were caused by lack of growth, which was most likely caused by antibiotic therapy, since other samples taken from patients earlier in the course of disease did show growth of microorganisms (Table 2). The partial concordance is caused by finding of a greater diversity by molecular methods compared to culture (Table 2 and 3), which is consistent with similar comparative studies of other clinical conditions evaluating microbial detection methods (Jacovides et al., In Press; Yun et al., Submitted for publication; Stoodley et al., 2008; Kathju et al., 2009; Gallo et al., 2011; Stoodley, Ehrlich, et al., 2011).

An interesting aspect of the 454 pyrosequencing results is their reportedly quantitative nature. When the deep sequencing results for sample 6A are compared to the culture results, it is seen that culture only identified pathogens that were found in low abundance by 454 pyrosequencing; *S. pyogenes* and *E. coli* constituted >2% and >1% respectively of the total number of sequences. The Ibis T5000 Biosensor also has a capacity for generating quantitative results due to an internal calibration standard. According to the Ibis T5000 biosensor, *S. pyogenes* and *E. coli* constituted 42% and 30% respectively of the total number of genomes found per well, which is more than expected based on the culture results. By qPCR, the presence of *S. pyogenes* was quantified and related to the total number of bacteria in the sample (estimates of cell numbers based on 16S rRNA gene measurements). Based on these qPCR results *S. pyogenes* appear to be the dominant pathogen in the samples where the pathogen was found by other methods, except samples 6A and 7B, where the total number of bacteria seemed to exceed the number of *S. pyogenes*. In these samples were contained a larger number of different species, which supports the findings by qPCR. The high relative abundance of *S. pyogenes* in sample 6A, seems to be in agreement with the quantitative results by the Ibis T5000 Biosensor, but not the 454 pyrosequencing data. An unexpected finding by qPCR was the presence of *S. pyogenes* in relatively high abundance in sample 7B, since the pathogen was not found in the sample by any other method. The use of qPCR furthermore seems to support our criteria of detection of bacteria by at least two methods, since *S. pyogens* could not be quantified in samples 3A, 5A, 10A and 10B where direct Sanger sequencing had indicated the presence of the pathogen (data not shown).

Using FISH it was possible to detect bacteria in 2 out of 5 samples of debrided tissue from the NSTI patients. Interestingly, the areas of damaged tissue were found to contain large amounts of DAPI stained DNA. Based on the morphology this was likely inflammatory cells, which would be possible to determine in future studies using specific immunostaining. The general the lack of detection of bacterial cells but observation of high amounts of inflammatory cells may indicate that the tissue damage might in large part be mediated by the host immune system. However, it is also possible that lack of bacterial detection in the debrided tissue is due to successful antimicrobial treatment and that the vast majority of causative pathogens have rendered metabolically inactive prior to debridement of tissue. This seems plausible since the majority of patients in this study survived the NSTI. It is also possible that the microorganisms have moved on to other tissue areas after having destroyed the tissue that is investigated here. We cannot, however, preclude that pathogens were present in the tissue but not detected due problems during transport and storage of samples for FISH or because they were present in concentrations below the limit of detection for PNA FISH.

The polymicrobial findings in this study included *Escherichia coli*, streptococci and the anaerobes *Bacteroides fragilis, Fusobacterium* spp. and *Clostridium* spp. These findings correspond with bacteria previously reported to be present in polymicrobial NSTIs (Elliott et al., 2000; Kihiczak et al., 2006; Pandey et al., 2009). Furthermore, fungal NSTI due to *Candida albicans* have also been reported (Elliott et al., 2000; Wong et al., 2003). The detection of Mycoplasma spp. in polymicrobial NSTI (patient 7 by all molecular methods), is to the best of our knowledge unique. However, animal studies have shown that ulcerative dermal necrosis can be induced in mice by *Mycoplasma arthritidis* (Cole et al., 1985). Mycoplasmas are associated with the mucosa and residing primarily in the respiratory tract and rarely penetrating the submucosa except in cases of immunosupression or instrumentation. The lack of cell wall makes the mycoplasmas very sensitive to environmental conditions and isolation of mycoplasmas is complicated due specific nutrient requirements and lack of a single optimal media formulation (Waites and Taylor-Robinson, 2011), which may explain why they have not been isolated in NSTI patients before. Interestingly, the localization of infection in patient 7 where mycoplasmas were detected was the neck, which corresponds with the association of mycoplasmas with the respiratory tract. It is possible that repeated surgical revision of the patient is the cause for the finding of mycoplasmas, and that they may not be playing an etiological role in the NSTI pathogenesis.

Monomicrobial infections by other species such as *A. baumannii* (patient 3) and *S. pneumoniae* (patient 8) are unusual findings. However, *A. baumanii* is an emergent pathogen and has increasingly been recognized as a prevalent and significant nosocomial pathogen associated with sepsis, wound infections, and pneumonia (Charnot-Katsikas et al., 2009). *A. baumannii* and other *Acinetobacter* sp. have been described as participants in polymicrobial NSTIs (Anaya and Dellinger, 2007; Angoules et al., 2007; Guerrero et al., 2010) and several reports have identified *A. baumannii*as the sole agent in NSTIs. (Liu et al., 2005; Charnot-Katsikas et al., 2009; Corradino et al., 2010; Sullivan et al., 2010). The increased prevalence of *A. baumannii* coupled with its increasing resistance to even the most-broad-spectrum antibiotics, means that NSTI infections caused by this pathogen may become an increasing problem (Charnot-Katsikas et al., 2009). *S. pneumoniae* is a common pathogen implicated in community-acquired pneumonia, sinusitis, otitis media and meningitis, which displays enormous heterogenetiy with respect to phenotype and pathogenicity (Forbes et al., 2008; Hall-Stoodley et al., 2008). However, NSTI due to *S. pneumoniae* is rare and has primarily been reported in cases where patients were immunosuppressed or had other underlying conditions (Ballon-Landa et al., 2001; Frick and Cerny, 2001; Kwak et al., 2002; Imhof et al., 2003; Dawar et al., 2008; Park et al., 2011), which does not correspond to the patient history in this case (Table 1).

The realization that many pathogens can cause NSTIs, and that no specific combination of species are found in all cases means that clinicians should be prepared to treat any combination of microbial pathogens (Elliott et al., 2000; Anaya and Dellinger, 2007). Although appropriate antimicrobial treatment cannot cure NSTI, it can help during the acute phase of the infection (Anaya and Dellinger, 2007), which highlights the importance of rapid and comprehensive identification of the pathogens involved.

In conclusion, most of the microorganisms found in this study have previously been described in NSTIs. However, several atypical findings were made with the aid of the molecular diagnostis including monomicrobial infections by *A. baumannii* and *S. pneumoniae* and a polymicrobial infection including mycoplasma. The detection of mycoplasma was consistent across all samples from the affected patient and across the various molecular methods and therefore strongly suggests that the culture results represent false-negatives. The culture reports from the Department of Clinical Microbiology only included microorganisms known as potential human pathogens, which may contribute to the finding of a larger microbial diversity by molecular methods. The fact that all microorganisms identified by culture were also identified by molecular methods indicates that these would be suitable alternatives for rapid identification of NSTI pathogens. The much faster turnaround time for the diagnostic molecular methods (Ibis and qPCR) makes the use of these methods attractive for pathogen identification in diseases that have rapid progression such as NSTI. However the use of molecular methods may increase the risk of identifying colonizers or contaminants to a higher degree. Based on the findings in this study, the Ibis T5000 biosensor appears to be the best of the molecular methods, but it has not yet been FDA approved for routine use in clinical practice. A problem with the molecular methods is that antimicrobial susceptibility testing cannot be performed, however the Ibis T5000 biosensor has the ability to detect some resistance genes, and did in fact detect the *mecA* gene in sample 6A where staphylococci were identified (data not shown). Furthermore, if an organism is not cultured it can never be examined for antibiotic sensitivity, thus speciating molecular diagnostics that incorporate antimicrobial resistance gene determinants such as the Ibis T5000 are an attractive alternative to culture, particularly when a rapid answer is needed as in NSTIs.

Acknowledgements

We are indebted to the technicians at the Department of Clinical Microbiology at Rigshospitalet for performing the culture-dependent experiments. We thank Steen Seir Poulsen for paraffin embedding and slicing of samples and Lene Bay for performing direct Sanger sequencing.

References

Anaya, D.A., Dellinger, E.P., 2007. Necrotizing soft-tissue infection: diagnosis and management. Clin. Infect. Dis. 44, 705–710.

Angoules, A.G., Kontakis, G., Drakoulakis, E., Vrentzos, G., Granick, M.S., Giannoudis, P.V., 2007. Necrotising fasciitis of upper and lower limb: a systematic review. Injury 38 Suppl 5, S19–26.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J., 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. Appl Environ Microbiol 72, 5734–5741.

Ballon-Landa, G.R., Gherardi, G., Beall, B., Krosner, S., Nizet, V., 2001. Necrotizing fasciitis due to penicillin-resistant Streptococcus pneumoniae: case report and review of the literature. J. Infect. 42, 272–277.

Bowler, P.G., Duerden, B.I., Armstrong, D.G., 2001. Wound microbiology and associated approaches to wound management. Clin. Microbiol. Rev 14, 244–269.

Bryant, A.E., 2003. Biology and Pathogenesis of Thrombosis and Procoagulant Activity in Invasive Infections Caused by Group A Streptococci and Clostridium perfringens. Clin Microbiol Rev 16, 451–462.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

Cawley, M.J., Briggs, M., Haith, L.R., Jr, Reilly, K.J., Guilday, R.E., Braxton, G.R., Patton, M.L., 1999. Intravenous immunoglobulin as adjunctive treatment for streptococcal toxic shock syndrome associated with necrotizing fasciitis: case report and review. Pharmacotherapy 19, 1094–1098.

Charnot-Katsikas, A., Dorafshar, A.H., Aycock, J.K., David, M.Z., Weber, S.G., Frank, K.M., 2009. Two cases of necrotizing fasciitis due to Acinetobacter baumannii. J. Clin. Microbiol. 47, 258–263.

Cole, B.C., Piepkorn, M.W., Wright, E.C., 1985. Influence of genes of the major histocompatibility complex on ulcerative dermal necrosis induced in mice by Mycoplasma arthritidis. J. Invest. Dermatol. 85, 357–361.

Corradino, B., Toia, F., di Lorenzo, S., Cordova, A., Moschella, F., 2010. A difficult case of necrotizing fasciitis caused by Acinetobacter baumannii. Int J Low Extrem Wounds 9, 152–154.

Costerton, J.W., Post, J.C., Ehrlich, G.D., Hu, F.Z., Kreft, R., Nistico, L., Kathju, S., Stoodley, P., Hall-Stoodley, L., Maale, G., James, G., Sotereanos, N., DeMeo, P., 2011. New methods for the detection of orthopedic and other biofilm infections. FEMS Immunol. Med. Microbiol. 61, 133–140.

Cunningham, M.W., 2000. Pathogenesis of Group A Streptococcal Infections. Clin. Microbiol. Rev. 13, 470–511.

Dawar, M., Russell, B., McClean, K., Levett, P.N., Tyrrell, G.J., Irvine, J., 2008. A case of necrotizing fasciitis due to Streptococcus pneumoniae serotype 5 in Saskatchewan. Can J Infect Dis Med Microbiol 19, 69–71.

Dawson, E.D., Taylor, A.W., Smagala, J.A., Rowlen, K.L., 2009. Molecular detection of Streptococcus pyogenes and Streptococcus dysgalactiae subsp. equisimilis. Mol. Biotechnol. 42, 117–127.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072.

Ecker, D.J., Sampath, R., Massire, C., Blyn, L.B., Hall, T.A., Eshoo, M.W., Hofstadler, S.A., 2008. Ibis T5000: a universal biosensor approach for microbiology. Nat. Rev. Microbiol. 6, 553–558.

Ehrlich, G.D., Ahmed, A., Earl, J., Hiller, N.L., Costerton, J.W., Stoodley, P., Post, J.C., DeMeo, P., Hu, F.Z., 2010. The distributed genome hypothesis as a rubric for understanding evolution in situ during chronic bacterial biofilm infectious processes. FEMS Immunology & Medical Microbiology no–no.

Elliott, D., Kufera, J.A., Myers, R.A.., 2000. The microbiology of necrotizing soft tissue infections. The American Journal of Surgery 179, 361–366.

Elliott, D.C., Kufera, J.A., Myers, R.A., 1996. Necrotizing soft tissue infections. Risk factors for mortality and strategies for management. Annals of surgery 224, 672.

Ellis Simonsen, S.M., van Orman, E.R., Hatch, B.E., Jones, S.S., Gren, L.H., Hegmann, K.T., Lyon, J.L., 2006. Cellulitis incidence in a defined population. Epidemiol. Infect. 134, 293–299.

Forbes, M.L., Horsey, E., Hiller, N.L., Buchinsky, F.J., Hayes, J.D., Compliment, J.M., Hillman, T., Ezzo, S., Shen, K., Keefe, R., Barbadora, K., Post, J.C., Hu, F.Z., Ehrlich, G.D., 2008. Strain-Specific

Virulence Phenotypes of Streptococcus pneumoniae Assessed Using the Chinchilla laniger Model of Otitis Media. PLoS ONE 3, e1969.

Frick, S., Cerny, A., 2001. Necrotizing Fasciitis Due to Streptococcus pneumoniae after Intramuscular Injection of Nonsteroidal Anti-Inflammatory Drugs: Report of 2 Cases and Review. Clin Infect Dis. 33, 740–744.

Gallo, P.H., Melton-Kreft, R., Nistico, L., Sotereanos, N.G., Sewecke, J.J., Stoodley, P., Ehrlich, G.D., Costerton, J.W., Kathju, S., 2011. Demonstration of Bacillus cereus in Orthopaedic-Implant-Related Infection with Use of a Multi-Primer Polymerase Chain Reaction-Mass Spectrometric AssayReport of Two Cases. J Bone Joint Surg Am 93, e85 1–6.

Golger, A., Ching, S., Goldsmith, C.H., Pennie, R.A., Bain, J.R., 2007. Mortality in patients with necrotizing fasciitis. Plast. Reconstr. Surg. 119, 1803–1807.

Guerrero, D.M., Perez, F., Conger, N.G., Solomkin, J.S., Adams, M.D., Rather, P.N., Bonomo, R.A., 2010. Acinetobacter baumannii-Associated Skin and Soft Tissue Infections: Recognizing a Broadening Spectrum of Disease*. Surg Infect (Larchmt) 11, 49–57.

Hall-Stoodley, L., Hu, F.Z., Gieseke, A., Nistico, L., Nguyen, D., Hayes, J., Forbes, M., Greenberg, D.P., Dice, B., Burrows, A., Wackym, P.A., Stoodley, P., Post, J.C., Ehrlich, G.D., Kerschner, J.E., 2006. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA 296, 202–211.

Hall-Stoodley, L., Nistico, L., Sambanthamoorthy, K., Dice, B., Nguyen, D., Mershon, W.J., Johnson, C., Hu, F.Z., Stoodley, P., Ehrlich, G.D., Post, J.C., 2008. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates. BMC Microbiol. 8, 173.

Hasham, S., Matteucci, P., Stanley, P.R.W., Hart, N.B., 2005. Necrotising fasciitis. BMJ 330, 830–833.

Imhof, A., Maggiorini, M., Zbinden, R., Walter, R.B., 2003. Fatal necrotizing fasciitis due to Streptococcus pneumoniae after renal transplantation. Nephrol. Dial. Transplant. 18, 195–197.

Jacovides, C., Kreft, R., Adeli, B., Hozack, B., Ehrlich, G.D., Parvizi, J., In Press. Successful Identification of Pathogens by ESI-TOF-MS in Culture-Negative Periprosthetic Joint Infections. J Bone Joint Surg Am.

Johansson, L., Thulin, P., Low, D.E., Norrby-Teglund, A., 2010. Getting Under the Skin: The Immunopathogenesis of Streptococcus Pyogenes Deep Tissue Infections. Clin Infect Dis. 51, 58–65.

Kathju, S., Nistico, L., Hall-Stoodley, L., Post, J.C., Ehrlich, G.D., Stoodley, P., 2009. Chronic surgical site infection due to suture-associated polymicrobial biofilm. Surg Infect (Larchmt) 10, 457–461.

Kaul, M., McGeer, Ms., Low, M., Green, R., Schwartz, M., Simor, M., 1997. Population-Based Surveillance for Group A Streptococcal Necrotizing Fasciitis: Clinical Features, Prognostic Indicators, and Microbiologic Analysis of Seventy-Seven Cases. The American Journal of Medicine 103, 18–24.

Kihiczak, G., Schwartz, R., Kapila, R., 2006. Necrotizing fasciitis: a deadly infection. Journal of the European Academy of Dermatology and Venereology 20, 365–369.

Kommedal, Ø., Karlsen, B., Sæbø, Ø., 2008. Analysis of Mixed Sequencing Chromatograms and Its Application in Direct 16S rRNA Gene Sequencing of Polymicrobial Samples. J Clin Microbiol 46, 3766–3771.

Kwak, E.J., McClure, J.-A., McGeer, A., Lee, B.C., 2002. Exploring the Pathogenesis of Necrotizing Fasciitis Due to Streptococcus pneumoniae. Scandinavian Journal of Infectious Diseases 34, 639–644.

Levine, E.G., Manders, S.M., 2005. Life-threatening necrotizing fasciitis. Clinics in dermatology 23, 144–147.

Liu, Y.-M., Chi, C.-Y., Ho, M.-W., Chen, C.-M., Liao, W.-C., Ho, C.-M., Lin, P.-C., Wang, J.-H., 2005. Microbiology and factors affecting mortality in necrotizing fasciitis. J Microbiol Immunol Infect 38, 430–435.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K., 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363 –1371.

Melton-Kreft, R., Costerton, J.W., Ehrlich, G.D., In Press. Eclipsing Culture: How the rising sun of molecular diagnostics will replace the “gold standard”. Microbe Wash DC.

Miller, L.G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A.S., Tang, A.W., Phung, T.O., Spellberg, B., 2005. Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. The New England journal of medicine 352, 1445.

Mussmann, M., Hu, F.Z., Richter, M., de Beer, D., Preisler, A., Jørgensen, B.B., Huntemann, M., Glöckner, F.O., Amann, R., Koopman, W.J.H., Lasken, R.S., Janto, B., Hogg, J., Stoodley, P., Boissy, R., Ehrlich, G.D., 2007. Insights into the genome of large sulfur bacteria revealed by analysis of single filaments. PLoS Biol. 5, e230.

Olsen, R.J., Musser, J.M., 2010. Molecular pathogenesis of necrotizing fasciitis. Annu Rev Pathol 5, 1–31.

Pandey, A., Gangopadhyay, A.N., Sharma, S.P., Kumar, V., Upadhyaya, V.D., 2009. Necrotizing fasciitis in children with special reference to anaerobes. Journal of Pediatric Infectious Diseases 4, 47–52.

Park, S.-Y., Park, S.Y., Moon, S., Son, J.S., Lee, M.S., 2011. Fatal Necrotizing Fasciitis Due to Streptococcus pneumoniae: A Case Report. J Korean Med Sci 26, 131–134.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188 –7196.

Quince, C., Lanzen, A., Davenport, R.J., Turnbaugh, P.J., 2011. Removing noise from pyrosequenced amplicons. BMC Bioinformatics 12, 38.

Rudkjøbing, V.B., Aanaes, K., Wolff, T.Y., von Buchwald, C., Johansen, H.K., Thomsen, T.R., Unpublished. Microorganisms involved in sinus infection of cystic fibrosis patients determined by culture and molecular-based methods. PloS One.

Sarani, B., Strong, M., Pascual, J., Schwab, C.W., 2009. Necrotizing fasciitis: current concepts and review of the literature. J. Am. Coll. Surg. 208, 279–288.

Singh, G., Sinha, S.K., Adhikary, S., Babu, K.S., Ray, P., Khanna, S.K., 2002. Necrotising infections of soft tissues--a clinical profile. Eur J Surg 168, 366–371.

Stevens, D.L., Bisno, A.L., Chambers, H.F., Everett, E.D., Dellinger, P., Goldstein, E.J.C., Gorbach, S.L., Hirschmann, J.V., Kaplan, E.L., Montoya, J.G., Wade, J.C., 2005. Practice guidelines for the diagnosis and management of skin and soft-tissue infections. Clin. Infect. Dis. 41, 1373–1406.

Stoodley, P., Conti, S.F., DeMeo, P.J., Nistico, L., Melton-Kreft, R., Johnson, S., Darabi, A., Ehrlich, G.D., Costerton, J.W., Kathju, S., 2011. Characterization of a mixed MRSA/MRSE biofilm in an explanted total ankle arthroplasty. FEMS Immunology & Medical Microbiology 62, 66–74.

Stoodley, P., Ehrlich, G.D., Sedghizadeh, P.P., Hall-Stoodley, L., Baratz, M.E., Altman, D.T., Sotereanos, N.G., William Costerton, J., DeMeo, P., 2011. Orthopaedic biofilm infections. Current Orthopaedic Practice 22, 558–563.

Stoodley, P., Nistico, L., Johnson, S., Lasko, L.-A., Baratz, M., Gahlot, V., Ehrlich, G.D., Kathju, S., 2008. Direct Demonstration of Viable Staphylococcus aureus Biofilms in an Infected Total Joint Arthroplasty. J Bone Joint Surg Am 90, 1751–1758.

Sullivan, D.R., Shields, J., Netzer, G., 2010. Fatal case of multi-drug resistant Acinetobacter baumannii necrotizing fasciitis. Am Surg 76, 651–653.

Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative Analysis of Small-Subunit rRNA Genes in Mixed Microbial Populations via 5′-Nuclease Assays. Appl Environ Microbiol 66, 4605–4614.

Tuttle, M.S., Mostow, E., Mukherjee, P., Hu, F.Z., Melton-Kreft, R., Ehrlich, G.D., Dowd, S.E., Ghannoum, M.A., 2011. Characterization of bacterial communities in venous insufficiency wounds by use of conventional culture and molecular diagnostic methods. J. Clin. Microbiol. 49, 3812–3819.

Waites, K.B., Taylor-Robinson, E., 2011. Mycoplamsa and Ureaplasma, in: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM Press, pp. 970–985.

Wong, C.-H., Chang, H.-C., Pasupathy, S., Khin, L.-W., Tan, J.-L., Low, C.-O., 2003. Necrotizing fasciitis: clinical presentation, microbiology, and determinants of mortality. J Bone Joint Surg Am 85-A, 1454–1460.

Xu, Y., Rudkjøbing, V.B., Simonsen, O., Pedersen, C., Lorenzen, J., Schønheyder, H.C., Nielsen, P.H., Thomsen, T.R., 2012. Bacterial diversity in suspected prosthetic joint infections:An exploratory study using 16S rRNA gene analysis. FEMS Immunol Med Microbiol 65, 291–304.

Yun, H.C., Kreft, H.E., Castillo, M.A., Ehrlich, G.D., Guymon, C.H., Crouch, H.K., Chung, K.K., Wenke, J.C., Hsu, J.R., Spirk, T., Costerton, J.W., Mende, K., Murray, C.K., Submitted for publication. Comparison of PCR/Electron spray Ionization - Time-of-Flight - Mass Spectrometry versus Traditional Clinical Microbiology for Detection of Organisms Contaminating High-use Surfaces in a Burn Unit, an Orthopaedic Ward and Healthcare Workers. BMC Infect Dis.

Tables

Table 1: Patient information [a]. N indicates the number of surgical samples investigated by molecular methods in this study.

Patient	Anatomical site	Age	Gender	Clinical manifestations	Antibiotics	Outcome
1 (n=4)	Right femur	71	F	NSTI emanating from femur spreading to shoulder and thorax, accompanied by diarrhea and septic shock with organ failure.	MEPM, CPFX and MNZ (supplemented with CLDM after sampling).	Survival.
2 (n=2)	Left crus	28	M	NSTI emanating from left knee, accompanied by organ failure.	MEPM, CPFX and CLDM.	Survival.
3 (n=1)	Left crus	67	M	Paranoid schizophrenia, adiposity and chronic leg ulcer. Femur was amputated. Organ failure including cardiac arrest and resuscitation.	MEPM, CPFX and CLDM. (Cefur and gentamycin before transferral)	Death within 24 hours.
4 (n=2)	Right arm	67	M	NSTI emanating from burn wound on finger. Septic shock with organ failure.	MEPM, CPFX and CLDM.	Survival.
5 (n=1)	Inguina	65	M	Diabetes mellitus. Previous Fourniers gangrene in 2008. Present NSTI emanating from inguinal hernia.	MEPM, CPFX and CLDM (suppl. MNZ after sampling).	Survival.
6 (n=1)	Vulva	63	F	Previous ischemic heart disease. Chronic lymphatic leukemia and polyarteritis nodosa receiving immunosuppressants. NSTI meaning from wound on labia pudenda, accompanied by organ failure and leucopenia.	MEPM, CPFX and CLDM.	Death due to cerebral lymphoma.
7 (n=2)	Neck	74	M	Diabetes mellitus. NSTI emanating from dental focus, accompanied by renal impairment (sepsis). Recurrence of infection after surgery (after 14 days and again after 24 days).	PEN and MNZ. After recurrence: MEPM, MNZ, linesolid and moxifloxacin.	Survival.
8 (n=3)	Right shoulder	66	M	Surgery based on NSTI suspicion. Finding of necrotic bursa.	MEPM, CPFX and CLCM.	Survival.
9 (n=2)	Left shoulder	43	M	Thalidomide limb defects. One week of discomfort and fever. NSTI emanating from neck and truncus, accompanied by septic shock and organ effect.	MEPM, CPFX and CLCM.	Survival.
10 (n=2)	Arm	57	M	Leukemia diagnosed during course of NSTI and is predisposing factor. NSTI emanating from finger bulla, accompanied by septic shock. Received many surgical revisions.	MEPM, CPFX, CLCM, MNZ	Survival.

Table 1 footnote: [a] Antibiotics abbreviations: MEPM, meropenem; CPFX, ciprofloxacin, MNZ, metronidazol; CLDM, clindamycin; PEN, penicillin.

Table 2: Microorganisms detected by routine culture methods in surgical and other (often previous) samples from NSTI patients. In many cases the findings by culture could be confirmed by the molecular methods (✓), or the molecular methods identified additional microorganisms (*).

	Other samples	Surgical samples		
Patient	**Culture**	**Sample**	**Culture**	**Molecular methods**
1	-	A	*Streptococcus pyogenes*	✓
		B	*Streptococcus pyogenes*	✓
		C	*Streptococcus pyogenes*	✓
		D	*Streptococcus pyogenes*	✓
2	*Streptococcus pyogenes* (blood culture)	A	*Streptococcus pyogenes*	✓
		B	*Streptococcus pyogenes*	✓
3	No growth	A	*Acinetobacter baumannii* (Gram positive cocci in chains by light microscopy)	✓*
4	*Streptococcus pyogenes* and CNS (Gram negative rods by light microscopy)	A	Non-hemolytic streptococci	✓
		B	No growth	*
5	-	A	*Bacteroides fragilis, Clostridium paraputrificum*	✓*
6	*Streptococcus pyogenes, Staphylococcus aureus* and Enterobacteriaceae	A	*Streptococcus pyogenes, Escherichia coli*	✓*
7	*Fusobacterium necrophorum*	A	Fungus	✓*
		B	No growth	*
8	*Streptococcus pneumoniae*	A	*Streptococcus pneumoniae*	✓
		B	*Streptococcus pneumoniae*	✓
		C	No growth	*
9	*Streptococcus pyogenes*	A	*Streptococcus pyogenes*	✓
		B	*Streptococcus pyogenes*	✓
10	*Staphylococcus aureus*	A	No growth	✓
		B	No growth	✓

- Indicates that no previous samples were taken for culture.

Table 3: Overview of the surgical samples where findings by molecular methods differed from culture results. Cases where the microorganisms were identified by culture are marked by ✓.

Patient	Sample	Direct Sanger sequencing	Clone libraries and Sanger sequencing	Ibis T5000 Biosensor
3	A	(*Streptococcus pyogenes*)	*Acinetobacter baumannii* ✓	*Acinetobacter baumannii* ✓
4	A	*Streptococcus pyogenes* ✓	454-pyrosequencing (Figure 1)	*Streptococcus pyogenes* ✓ *Streptococcus didelphis* ✓
	B	*Streptococcus pyogenes*	*Streptococcus pyogenes*	*Streptococcus pyogenes*
5	A	(*Streptococcus pyogenes*)	*Clostridium paraputrificum* ✓ Uncultured bacterium	*Clostridium paraputrificum* ✓ *Bacteroides fragilis* ✓ (*Streptococcus agalactiae*)
6	A	*Streptococcus pyogenes* ✓ *Mycoplasma hominis*	454-pyrosequencing (Figure 1)	*Streptococcus pyogenes* ✓ *Escherichia coli* ✓ *Bacteroides fragilis* (*Staphylococcus hominis*) (*Staphylococcus epidermidis*) (*Cladosporium cladosporioides*)
7	A	*Mycoplasma* spp. *Fusobacterium necrophorum*	454-pyrosequencing (Figure 1)	*Mycoplasma* spp. *Fusobacterium necrophorum* *Candida albicans* ✓
	B	*Mycoplasma salivarium*	*Mycoplasma salivarium* *Fusobacterium necrophorum*	*Mycoplasma* spp. *Fusobacterium necrophorum*
8	A	*Streptococcus pneumoniae* ✓	454-pyrosequencing (Figure 1)	*Streptococcus pneumoniae* ✓
	B	*Streptococcus pneumoniae* ✓	454-pyrosequencing (Figure 1)	*Streptococcus pneumoniae* ✓
	C	*Streptococcus pneumoniae*	454-pyrosequencing (Figure 1)	*Streptococcus pneumoniae*
9	A	*Streptococcus pyogenes*	*Streptococcus pyogenes*	*Streptococcus pyogenes*
	B	*Streptococcus pyogenes*	*Streptococcus pyogenes*	*Streptococcus pyogenes*
10	A	(*Streptococcus pyogenes*)	No PCR	(*Streptococcus pneumoniae*) (*Clostridium septicum*)
	B	(*Streptococcus pyogenes*)	No PCR	(*Staphylococcus capitis/caprae*)

Table 3 footnote: () indicates that microorganism could only be found by one molecular method and not by culture.

Figures

Figure 1: Taxa identified by pyrosequencing. The stacked graph illustrates the relative abundance of each taxon identified by pyrosequencing from the six samples (color coded according to the key).

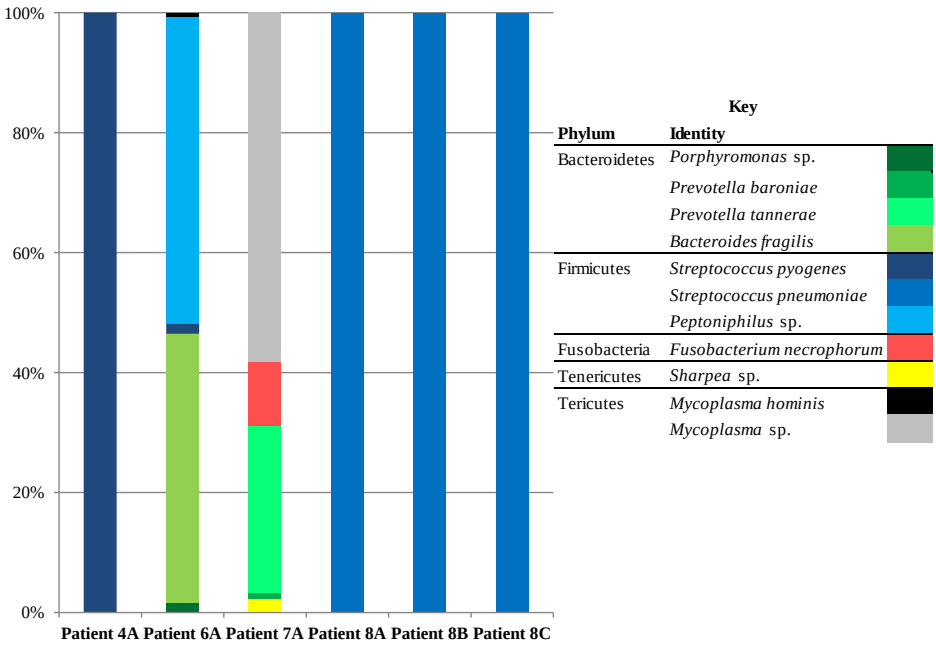


Figure 2: Results by taqman qPCR for *S. pyogenes* (grey) and the 16S rRNA gene of all bacteria (black) given as CFU/mg sample.

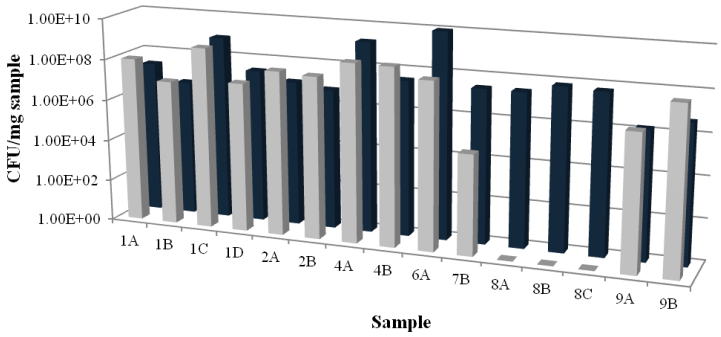
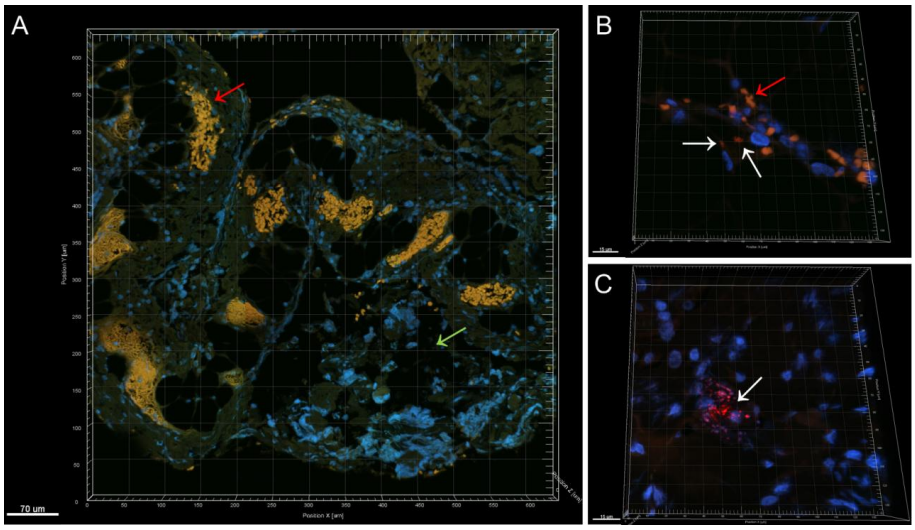


Figure 3: Visualization of NSTI samples where microorganisms could be detected. Frame (A) show an overview of sample 9B, where DAPI stained nucleic acid (blue) and coagulated blood (orange, indicated by red arrow) can be seen in the tissue. The black areas in the tissue is fat cells (defined areas), but also areas of tissue destruction (green arrow). Frame (B) and (C) show 63x magnification of samples where bacterial cells could be detected by the universal bacterium PNA probe (scalebar represents 15 µm). Frame (B) shows sample 9B where bacterial cells could be detected (white arrow) among DAPI stained DNA and coagulated blood. Frame (C) show a large cluster of bacterial cell in sample 8A.



Research paper 2

Rudkjøbing, V.B., Thomsen, T.R., Alhede, M., Kragh, K.N., Nielsen, P.H., Johansen, U.R., Givskov, M., Høiby, N., Bjarnsholt, T. (2011). True Microbiota Involved in Chronic Lung Infection of Cystic Fibrosis Patients Found by Culturing and 16S rRNA Gene Analysis. *Journal of Clinical Microbiology* 49, 4352-4355.

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2011, p. 4352–4355 Vol. 49, No. 12
0095-1137/11/$12.00 doi:10.1128/JCM.06092-11

True Microbiota Involved in Chronic Lung Infection of Cystic Fibrosis Patients Found by Culturing and 16S rRNA Gene Analysis▽

Vibeke B. Rudkjøbing,[1] Trine R. Thomsen,[1,2] Morten Alhede,[3] Kasper N. Kragh,[3] Per H. Nielsen,[1] Ulla R. Johansen,[4] Michael Givskov,[3] Niels Høiby,[3,4] and Thomas Bjarnsholt[3,4]*

Department of Biotechnology, Chemistry, and Environmental Engineering, Faculty of Engineering and Science, Aalborg University, Aalborg, Denmark[1]; The Danish Technological Institute, Life Science Division, Aarhus C, Denmark[2]; Department of International Health, Immunology, and Microbiology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark[3]; and Department of Clinical Microbiology, Rigshospitalet, University Hospital of Copenhagen, Copenhagen, Denmark[4]

Received 13 October 2011/Accepted 13 October 2011

Patients suffering from cystic fibrosis (CF) develop chronic lung infection. In this study, we investigated the microorganisms present in transplanted CF lungs ($n = 5$) by standard culturing and 16S rRNA gene analysis. A correspondence between culturing and the molecular methods was observed. In conclusion, standard culturing seems reliable for the identification of the dominating pathogens.

Cystic fibrosis (CF) is the most common lethal autosomal recessively inherited disorder of Caucasians. Although several organs are affected, the most severe effect is observed in the lungs, which is the major cause of deaths of patients (5). Here, genetic alterations of the chloride channel in epithelial cells lead to dehydration of the airway mucus, increasing its viscosity. This means that the cilia are unable to transport the mucus in which inhaled material and, importantly, bacteria are entrapped, enabling microorganisms to colonize and establish infections within the mucus (9). In the early stages of CF, intermittent colonizations occur, which can be treated with antibiotics (10). Establishment of chronic infection occurs over time and is characterized by the formation and establishment of bacterial aggregates (the so-called biofilms) (1, 5). Formation of biofilm is problematic since not only does this afford protection against the different components of the host defense in the lungs but the bacteria also become extremely tolerant to antibiotics (1, 4, 5). Most pathogenic bacteria are easily diagnosed by standard culture-based techniques; however, many less well recognized bacteria can be difficult to culture due to their growth requirements or being very slow growing or not growing at all if the patient has been treated with antibiotics. In these cases, the standard culture techniques may fail to detect these bacteria and detect only the more readily culturable bacteria (14). In the CF centers in Denmark, an intensive antibiotic treatment strategy has been shown to prolong the life expectancy of the CF patients (10). In recent studies, the chronically infected lungs of CF patients have been observed to harbor multiple species (19, 21). However, the strict antibiotic strategy employed in Denmark has led to only a small variety of microorganisms being found in the lungs of CF patients, compared to what is found in other studies (8, 18, 23). In a previous study, we applied fluorescence *in situ* hybridization (FISH) using peptide nucleic acid (PNA) probes to investigate the spatial distribution of *Pseudomonas aeruginosa* in the lungs of end-stage Danish CF patients by using both general and specific probes and found *P. aeruginosa* to be present alone (1). The end stage is defined as the time when the lungs are destroyed and the lung function is reduced to an extent where lung transplantation is required for the patient to survive (3).

In the present study, we investigated the true microbiota of the end-stage CF lung by investigating fresh samples directly from explanted lungs of Danish CF patients undergoing double lung transplantations. This was to avoid possible contamination by the patient's oral and pharyngeal flora during expectoration of sputum, which is the typical type of sample investigated in CF studies.

We included 34 lung tissue and mucopurulent pus/sputum samples excised directly and sterilely from the lungs of five Danish end-stage CF patients undergoing double lung transplantation at Rigshospitalet (Copenhagen, Denmark). The lungs were collected with the consent of the patients and in accordance with the biomedical project protocol (KF-01278432) approved by the Danish Council of Ethics. To investigate the microorganisms of the true microbiota present within the lungs of the patients, both standard culturing and 16S rRNA gene analysis were performed. All culture experiments were performed at the Department of Clinical Microbiology, Rigshospitalet (Copenhagen University Hospital, Denmark), according to standard protocols (2). All samples were incubated both aerobically and anaerobically. Aerobic culturing was performed on blood agar, chocolate agar, and eosin-methylene blue (EMB) agar with an incubation time of up to 1 week. Anaerobic culturing was performed on blood agar and chocolate agar, using an atmosphere of 7% CO_2 and 7% H_2 in N_2 for up to 2 weeks.

Before extraction of DNA for 16S rRNA gene analysis, samples were lysed by proteinase K (40 μl) and ATL buffer (360 μl) from the DNeasy blood and tissue kit (Qiagen, Copenhagen, Denmark) for each 500 mg of tissue and incubated overnight at 56°C. The samples were then centrifuged at 13,000

* Corresponding author. Mailing address: Department of International Health, Immunology and Microbiology, University of Copenhagen, DK-2100 Copenhagen, Denmark. Phone: 4535457774. Fax: 4535327853. E-mail: tbjarnsholt@sund.ku.dk.

▽ Published ahead of print on 19 October 2011.

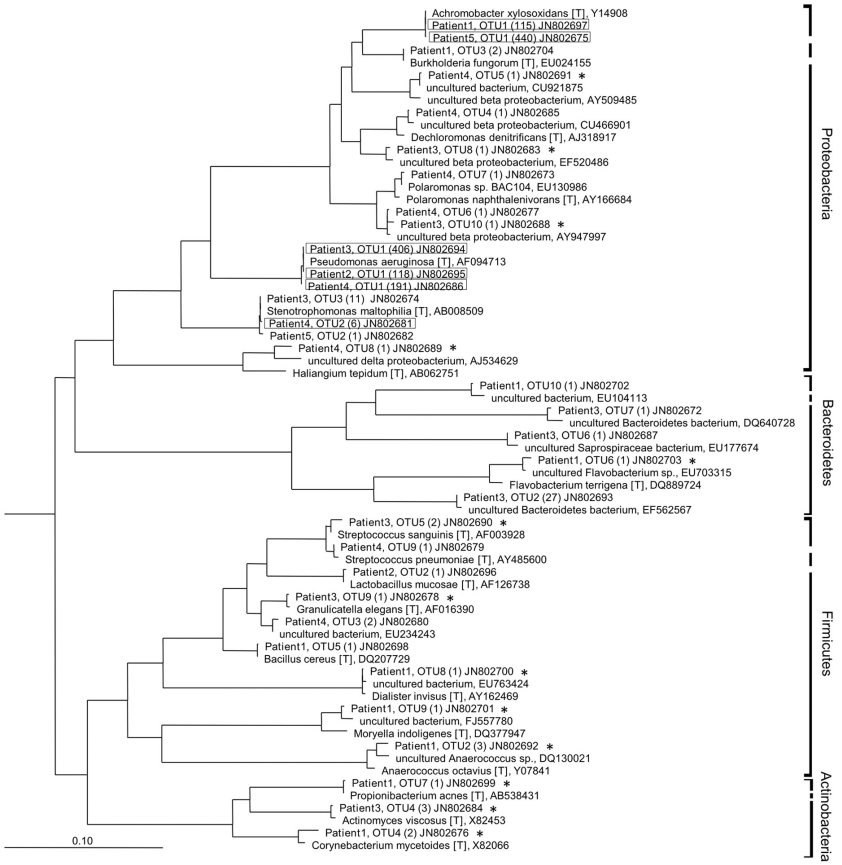


FIG. 1. Maximum likelihood tree of the sequences in the clone libraries with their closest relatives. The OTUs from the clone libraries from the five patients are given with the numbers of sequences in parentheses. The out-group (consisting of 24 sequences of the *Chloroflexi* phylum) was set as the root, not shown in the figure. The scale bar represents a 10% deviation of sequence. Asterisks indicate sequences where identification by BLAST search gave different results. The identities of microorganisms found by culturing are highlighted by a box; these are also the clones most often identified in the respective clone libraries.

rpm for 1 min, and DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's protocol (revision 6560-200-07DEC); starting from step 6, DNA was eluted with 60 μl diethyl pyrocarbonate (DEPC)-treated water. Nearly full-length 16S rRNA genes were amplified as described in the literature (22), using two different combinations of universal bacterial primers: 26F (5′-AGAGTTTGATCCTGGCTCAG-3′) with either 1390R (5′-GACGGGCGGTGTCTACAA-3′) or 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (15). The resulting 16S rRNA gene fragments were pooled and purified using Nucleospin Extract II columns (Macherey-Nagel, Düren, Germany). The PCR products were cloned into a pCR4-TOPO vector, transformed into One Shot Top 10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA), and incubated overnight at 37°C on LB agar plates containing 50 μg/ml kanamycin and 50 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Either plasmids were purified using the Illustra TempliPhi DNA amplification kit (GE Healthcare, Brøndby, Denmark) and sequenced commercially by Macrogen (South Korea), or plasmid purification was performed by Macrogen before sequencing. Sequences were obtained using

TABLE 1. Overview of bacteria found in the explanted lung samples by culturing and 16S rRNA gene analysis

Patient	Culturing	16S rRNA gene analysis		
		Species (BLAST)[a]	OTU	No.[b]
Patient 1	*Achromobacter xylosoxidans*	*Achromobacter xylosoxidans*	1	115
		Uncultured bacterium	2*[c]	3
		Burkholderia fungorum	3	2
		Uncultured bacterium	4*	2
		Bacillus cereus	5	1
		Uncultured *Flavobacterium*	6*	1
		Uncultured bacterium	7*	1
		Uncultured bacterium	8*	1
		Uncultured bacterium	9*	1
		Uncultured bacterium	10*	1
Patient 2	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	1	118
		Lactobacillus mucosae	2	1
Patient 3	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	1	406
		Uncultured *Bacteroidetes* bacterium	2	27
		Stenotrophomonas maltophilia	3	11
		Uncultured bacterium	4*	3
		Uncultured bacterium	5*	2
		Uncultured *Saprospiraceae* bacterium	6	1
		Uncultured *Bacteroidetes* bacterium	7	1
		Uncultured bacterium	8*	1
		Uncultured bacterium	9*	1
		Uncultured bacterium	10*	1
Patient 4	*Pseudomonas aeruginosa* *Stenotrophomonas maltophilia*	*Pseudomonas aeruginosa*	1	191
		Stenotrophomonas maltophilia	2	6
		Uncultured bacterium	3	2
		Uncultured betaproteobacterium	4	1
		Uncultured betaproteobacterium	5*	1
		Uncultured betaproteobacterium	6	1
		Polaromonas sp.	7	1
		Uncultured bacterium	8*	1
		Uncultured bacterium	9	1
Patient 5	*Achromobacter xylosoxidans*	*Achromobacter xylosoxidans*	1	440
		Stenotrophomonas maltophilia	2	1

[a] The species found by 16S rRNA gene analysis is given by the closest relatives of the bacterial OTUs in clone libraries for the patients.
[b] The number of sequences that make up the OTUs.
[c] Asterisks indicate clones where the identification by BLAST differed from the identification made by phylogenetic analysis.

the M13F primer (5′-GTAAAACGACGGCCAGT-3′) and checked for chimeric sequences with the program Bellerophon (12), using the Huber-Hugenholtz correction and a window size of 300 nucleotides. The BlastN function in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for initial identification of closest relatives with standard parameter settings, except that the database was set to the nucleotide collection (nr/nt).

Alignment of the sequences was performed using the SILVA web aligner (17) with default settings and refined manually in ARB (16). The sequences from the 34 clone libraries were compiled into overall libraries for each of the 5 patients; within these, the sequences were grouped into operational taxonomy units (OTUs) if they had a sequence similarity of more than 97% (13). Representative clones for all OTUs were also sequenced using the M13R primer (5′-GCGGATAACAATTT CACACAGG-3′) in order to obtain consensus sequences covering the entire length of the fragments. Consensus sequences representing the different OTUs and their closest relatives in the nonredundant SSU Ref database from SILVA release 104 were used for calculation of trees by distance matrix, parsimony, and maximum likelihood approaches using default settings in the ARB software but omitting hypervariable regions of the gene. Twenty-four out-group sequences from the phylum *Chloroflexi* were added to the tree calculations.

Culture analysis showed the presence of monospecies infection in the lungs of four patients, and two bacterial species were found for the last patient (patient 4). No growth of anaerobic bacteria was observed. The isolated bacteria were *P. aeruginosa*, *Stenotrophomonas maltophilia*, or *Achromobacter xylosoxidans* (Table 1), and the same result was found on all types of media used. The 5 patients expectorated sputum just prior to their lung transplantation. The culture analysis of this sputum revealed the exact same bacteria as those found by the culture analysis from the explanted lungs (not shown). The initial identification of clone library sequences (as determined by BLAST search) showed that the organisms found by culture analysis were present in high numbers in the clone libraries (Table 1). The phylogenetic trees (neighbor joining, maximum parsimony, and maximum likelihood) were constructed to vi-

sualize the phylogenetic relationship of the microorganisms and showed congruent topology (the maximum likelihood tree is shown in Fig. 1). The locations of the sequences in the tree confirmed the result of the BLAST search and in several cases gave identification of sequences that had been determined to be uncultured bacteria by BLAST search, as indicated by asterisks in Table 1 and Fig. 1. This is due to the fact that, unlike the BLAST tool at NCBI, only quality-checked sequences were used in the ARB database used. Another factor is that, in ARB, the secondary structure of the 16S rRNA gene was taken into account. Some of the bacteria identified in the clone libraries have previously been associated with cystic fibrosis, such as *Stenotrophomonas maltophilia* (6, 7, 20), *Burkholderia fungorum* (6, 19), and *Streptococcus* sp. (13), but the clinical relevance of these bacteria and others found in small amounts in the samples is unknown (2, 11). Compared to the results obtained by culture analysis, the 16S rRNA gene analysis showed a greater diversity of bacteria, with sequences distributed into 4 phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. As the bacteria found by culturing were also represented by the highest numbers of sequences in the clone libraries, it is very likely that these bacteria were dominant in the lung. We are currently investigating this thoroughly by FISH and quantitative PCR.

The results presented here correlate with results that we have previously published (1) that the end-stage CF lung harbors relatively few bacterial species that could be identified by culturing. However, this might not represent the other levels of chronic infection in the CF lungs. In fact, many of the non-end-stage CF patients at the Copenhagen CF Clinic harbor several species in their lungs, which should also be investigated further.

Nucleotide sequence accession numbers. The nonredundant, nearly full-length 16S rRNA gene sequences representing each OTU obtained in this study were deposited in GenBank under the accession numbers JN802672 to JN802704.

REFERENCES

1. **Bjarnsholt, T., et al.** 2009. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr. Pulmonol. **44:**547–558.
2. **Bjarnsholt, T., X. C. Nielsen, U. Johansen, L. Nørgaard, and N. Høiby.** 2011. Methods to classify bacterial pathogens in cystic fibrosis, p. 143–171. *In* M. D. Amaral and K. Kunzelmann (ed.), Cystic fibrosis: diagnosis and protocols, vol. II. Methods and resources to understand cystic fibrosis. Humana Press, Totowa, NJ.
3. **Braun, A. T., and C. A. Merlo.** 2011. Cystic fibrosis lung transplantation. Curr. Opin. Pulm. Med. **17:**467–472.
4. **Conese, M., E. Copreni, S. D. Gioia, P. D. Rinaldis, and R. Fumarulo.** 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. J. Cyst. Fibros. **2:**129–135.
5. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
6. **Demko, C. A., R. C. Stern, and C. F. Doershuk.** 1998. *Stenotrophomonas maltophilia* in cystic fibrosis: incidence and prevalence. Pediatr. Pulmonol. **25:**304–308.
7. **Ferroni, A., et al.** 2002. Use of 16S rRNA gene sequencing for identification of nonfermenting gram-negative bacilli recovered from patients attending a single cystic fibrosis center. J. Clin. Microbiol. **40:**3793–3797.
8. **Harrison, F.** 2007. Microbial ecology of the cystic fibrosis lung. Microbiology **153:**917–923.
9. **Heijerman, H.** 2005. Infection and inflammation in cystic fibrosis: a short review. J. Cyst. Fibros. **4:**3–5.
10. **Høiby, N., B. Frederiksen, and T. Pressler.** 2005. Eradication of early *Pseudomonas aeruginosa* infection. J. Cyst. Fibros. **4**(Suppl. 2)**:**49–54.
11. **Høiby, N., and T. Pressler.** 2006. Emerging pathogens in cystic fibrosis. ERM **35:**66–79.
12. **Hugenholtz, P., and T. Huber.** 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int. J. Med. Microbiol. **53:**289–293.
13. **Juretschko, S., A. Loy, A. Lehner, and M. Wagner.** 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst. Appl. Microbiol. **25:**84–99.
14. **Kolak, M., F. Karpati, H.-J. Monstein, and J. Jonasson.** 2003. Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. Int. J. Med. Microbiol. **293:**309–317.
15. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics, 1st ed. John Wiley & Sons, London, United Kingdom.
16. **Ludwig, W., et al.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
17. **Pruesse, E., et al.** 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. **35:**7188–7196.
18. **Rogers, G. B., et al.** 2004. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16S ribosomal DNA terminal restriction fragment length polymorphism profiling. J. Clin. Microbiol. **42:**5176–5183.
19. **Rogers, G. B., F. A. Stressmann, A. W. Walker, M. P. Carroll, and K. D. Bruce.** 2010. Lung infections in cystic fibrosis: deriving clinical insight from microbial complexity. Expert Rev. Mol. Diagn. **10:**187–196.
20. **Saiman, L.** 2004. Microbiology of early CF lung disease. Paediatr. Respir. Rev. **5:**S367–S369.
21. **Stressmann, F. A., et al.** 2011. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. J. Clin. Microbiol. **49:**281–291.
22. **Thomsen, T. R., K. Finster, and N. B. Ramsing.** 2001. Biogeochemical and molecular signatures of anaerobic methane oxidation in a marine sediment. Appl. Environ. Microbiol. **67:**1646–1656.
23. **Worlitzsch, D., et al.** 2009. Antibiotic resistant obligate anaerobes during exacerbations of cystic fibrosis patients. Clin. Microbiol. Infect. **15:**454–460.

Research paper 3

Rudkjøbing, V.B., Thomsen, T.R., Alhede, M., Kragh, K.N., Nielsen, P.H., Johansen, U.R., Givskov, M., Høiby, N., Bjarnsholt, T. (2012). The microorganisms in chronically infected end-stage and non-end-stage cystic fibrosis patients. *FEMS Immunology & Medical Microbiology* 65, 236–244.

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

The microorganisms in chronically infected end-stage and non-end-stage cystic fibrosis patients

Vibeke B. Rudkjøbing[1], Trine R. Thomsen[1,2], Morten Alhede[3], Kasper N. Kragh[3], Per H. Nielsen[1], Ulla R. Johansen[4], Michael Givskov[3], Niels Høiby[3,4] & Thomas Bjarnsholt[3,4]

[1]Department of Biotechnology, Chemistry, and Environmental Engineering, Faculty of Engineering and Science, Aalborg University, Aalborg, Denmark; [2]Life Science Division, The Danish Technological Institute, Aarhus C, Denmark; [3]Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark; and [4]Department of Clinical Microbiology, Rigshospitalet, University Hospital of Copenhagen, Copenhagen, Denmark

Correspondence: Thomas Bjarnsholt, Department of International Health, Immunology and Microbiology, University of Copenhagen, DK-2100 Copenhagen, Denmark. Tel.: +4535457774; fax: +4535327853; e-mail: tbjarnsholt@sund.ku.dk

Received 2 November 2011; revised 12 December 2011; accepted 13 December 2011.
Final version published online 23 January 2012.

DOI: 10.1111/j.1574-695X.2011.00925.x

Editor: Ake Forsberg

Keywords
cystic fibrosis; chronic infection; molecular diagnostic; 16S rRNA gene analysis; *Pseudomonas aeruginosa*; *Stenotrophomonas maltophilia*; *Achromobacter xylosoxidans*; biofilms.

Abstract

Patients suffering from cystic fibrosis (CF) develop chronic lung infections because of highly viscous mucus, where bacteria can form biofilms. In this study, we investigated the microorganisms present in the lungs of end-stage and non-end-stage patients using standard culturing techniques and molecular methods. Tissue and sputum samples (n = 34) from explanted lungs of five end-stage patients were examined along with routine expectorates (n = 15) from 13 patients with non-end-stage CF, representing earlier stages of chronic lung infections. Previously, using peptide nucleic acid (PNA) fluorescence *in situ* hybridization (FISH), we have shown that *Pseudomonas aeruginosa* was the sole pathogen in end-stage CF lungs (*Pediatr Pulmonol* 2009, 44: 547). In this study, this tendency was supported by the results of real-time PCR, confirming previous results obtained by standard culturing and 16S rRNA gene analysis (*J Clin Microbiol* 2011, 49: 4352). Conversely, the non-end-stage patients were found to harbor several species by culturing. PNA FISH confirmed heterogeneous microbiota and showed that the bacteria were located in monospecies aggregates with no apparent physical interaction between the different microcolonies. In conclusion, standard culturing identifies the dominating pathogens, which seem to reside in monospecies microcolonies. The possibility of signaling between the distinct microcolonies still has to be verified and elucidated.

Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessively inherited disorder of Caucasians. The genetic defect leads to a decrease in epithelial chloride secretion and an increase in sodium absorption and as such affect several organs. The most severe effect is on the lungs, where the airway mucus becomes dehydrated, increasing its viscosity. Normally, the mucus entraps bacteria and other foreign material, which is then transported by cilia for mechanical clearing, for example, by coughing. In patients with CF, the cilia are unable to transport the highly viscous mucus, making it a reservoir for inhaled bacteria (Boucher, 2004). The airway microbiology of patients with CF changes over time; early in life, intermittent acute infections of many different bacteria occur, the most common being *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, the *Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia*. Of these pathogens, *H. influenza*, *S. aureus*, and *P. aeruginosa* are predominant (Gilligan, 1991; Saiman, 2004). The viscous mucus enables microorganisms to establish chronic infections characterized by the formation and establishment of bacterial aggregates (the so-called biofilm) (Costerton *et al.*, 1999; Bjarnsholt *et al.*, 2009). Up to 80% of young CF adults are chronically infected with *P. aeruginosa* (Gillian, 1991; Bjarnsholt *et al.*, 2009), but other species, like *Achromobacter xylosoxidans*, are

increasingly detected as a cause of chronic infections (Rønne Hansen *et al.*, 2006). The mucus of a chronically infected patient contains an abundance of microcolonies, alginate, and some planktonic bacteria, and a continuous recruitment of polymorphonuclear neutrophils (PMNs) occurs. Despite the abundance of PMNs, the bacteria persist for decades, and the constant neutrophil-dominated inflammation leads to lung tissue destruction and reduced respiratory function in the patient (Bjarnsholt, 2009; Moser *et al.*, 2009). At some point the lung function is reduced to an extent where lung transplantation is required in order for the patient to survive, this is called the end-stage of the disease. Without transplantation, the disease leads to premature deaths of the patients (Braun & Merlo, 2011).

Formation of bacterial biofilms in the CF lung is problematic as the bacteria are protected from the host immune system and become extremely tolerant to antibiotics, which explains why the chronic infections cannot be eradicated (Costerton *et al.*, 1999; Conese *et al.*, 2003; Bjarnsholt *et al.*, 2009). The intermittent colonizations, however, can be treated successfully with antibiotics. In Denmark, an aggressive antibiotic treatment strategy has been employed, and the onset of the chronic infection has been postponed for decades, prolonging the life expectancy of the patients (Høiby *et al.*, 2005).

We have previously studied spatial distribution of *P. aeruginosa* in the lungs of Danish patients with end-stage CF using both general and specific peptide nucleic acid (PNA) probes for fluorescence *in situ* hybridization (FISH) (Bjarnsholt *et al.*, 2009). Recently, we investigated the true microbiota in the lungs of patients with end-stage CF by examining fresh samples from explanted lungs using culturing and 16S rRNA gene analysis, and the results indicated that culturing could identify the organisms that were dominant in the 16S rRNA gene libraries (Rudkjøbing *et al.*, 2011). In this study, we investigated whether *P. aeruginosa* in fact dominates in the lungs of these patients by quantifying these by real-time PCR. As in our previous PNA FISH studies, we also investigated sputum samples from chronically infected non-end-stage patients using culturing and PNA FISH to compare different stages of chronic lung infection.

Materials and methods

Patients and samples

Samples included in this study were obtained from a group of Danish patients with end-stage and non-end-stage CF. Samples from five patients with end-stage CF (34 lung tissue and mucupurulent pus/sputum samples excised from explanted lungs), as described elsewhere (Rudkjøbing *et al.*, 2011), were investigated by real-time PCR. Samples from 13 Danish patients with non-end-stage CF (15 sputum samples) were obtained during bacteriology examinations as part of the routine diagnostics. These patients ranged from nonchronic infected to chronic infected for up to 34 years with *P. aeruginosa*. The samples were investigated using standard culturing and also prepared for PNA FISH visualization by fixation in PBS with 4% paraformaldehyde and imbedding in paraffin, which was sectioned (4 μm) and mounted on glass slides. The end-stage lungs were collected with the acceptance of the patients and in accordance with the biomedical project protocol (KF-01278432) approved by the Danish scientific ethical board.

Culture experiments

All culture experiments were performed at the Department of Clinical Microbiology, Rigshospitalet (Copenhagen, Denmark) according to standard protocols. All samples were investigated using our routine CF media and culture conditions, as described by (Bjarnsholt *et al.*, 2011). Sputum samples were not subjected to anaerobic culture because of heavy growth of the normal anaerobic flora from the mouth (Bjarnsholt *et al.*, 2011).

DNA extraction efficiency – spiking experiment

DNA was extracted from the samples of patients with end-stage CF, as described previously (Rudkjøbing *et al.*, 2011). The efficiency of this DNA extraction method was evaluated by spiking known amounts of *S. aureus* (DSM 6148) cells to a lung tissue sample. *Staphylococcus aureus* was chosen as model organism, as Gram-positive cells often pose the biggest challenge to DNA extraction. The tissue was digested by proteinase K solution (40 μL proteinase K and 360 μL ATL buffer from DNeasy® Blood & Tissue kit; Qiagen), followed by homogenization. Degraded tissue was divided into five fractions, to which five different amounts of *S. aureus* cells grown in LB media were added (25 000, 7500, 1500, 300 cells, and 0). Cell concentration was determined using a 4′,6-diamidino-2-phenylindole (DAPI) solution (1 mg mL^{-1}) for staining cells filtrated onto a polycarbonate filter and inspected under a Carl Zeiss Axioskop 2 Plus epifluorescence microscope using a 100 times oil immersion lens. After addition of cells, 20 μL proteinase K and 200 μL ATL buffer were added to each sample and incubated for 2 h. The samples were homogenized and divided into three equally sized subsamples (only two subsamples for the samples with highest and lowest concentration of added cells), from which DNA was extracted according to (Rudkjøbing *et al.*, 2011). The quantity of *S. aureus* cells was

measured using real-time PCR, targeting the *nuc* gene of *S. aureus* according to (Hein *et al.*, 2001). Measurements were taken in triplicate, and comparison of subsamples with equal amounts of added cells was made (data not shown).

Real-time PCR

The concentration of *P. aeruginosa* was determined by real-time PCR, targeting *oprL* gene (Jaffe *et al.*, 2001), and hydrolysis probe-based real-time PCR, targeting the *ecfX* gene (Anuj *et al.*, 2009). Also, the total content of bacteria was quantified using bacterial primer 341F and universal primer 907R (Skovhus *et al.*, 2004). Measurements were obtained by absolute quantification using genomic DNA isolated from *P. aeruginosa* (DSM 1253) and *Escherichia coli* (DSM 30083) for total bacteria quantification. The number of isolated genomes was calculated based on DNA concentration (Quant-iT™ dsDNA Assay kit; Invitrogen) and genome size estimated to be 6.5 Mbp (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Dilution series of the genomic *P. aeruginosa* DNA covered a range of 10^{6}–10^{-2} genome copies and 10^{7}–10^{0} genome copies for *E. coli*.

Triplicate 25 μL SYBR-based real-time PCR reactions were run containing: 12.5 μL Brilliant® II SYBR® Green REAL-TIME PCR Master mix (Stratagene), 1 μg μL^{-1} BSA (New England Biolabs), 400 nM of both forward and reverse primer, 38 nM ROX reference dye (Stratagene), and 5 μL of sample, standard, or control. Reactions were run on Mx3005P (Stratagene) with 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min at 60 °C, 1 min at 72 °C, and 15 s at 82 °C (data capture), and finally melting profile analysis (57–95 °C). For the general bacteria assay, the program was 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min at 56 °C, 30 s at 72 °C, and 20 s at 80 °C (data capture), and finally melting profile analysis. For the hydrolysis probe-based real-time PCR triplicate 25 μL, reactions were run containing: 12.5 μL Brilliant® II QPCR Master mix (Stratagene), 1 μg μL^{-1} BSA (New England Biolabs), 1 μM of both primers, 200 nM hydrolysis probe, 38 nM ROX reference dye (Stratagene), and 5 μL of sample, standard, or control. The reactions were run with 10 min at 95 °C, 50 cycles of 30 s at 95 °C, and 1 min at 60 °C (data capture). The gene measurements were converted into a measure of DNA content, assuming nucleotide base pair weight of 660 Da, that all bacteria in the samples have the same genome size as *P. aeruginosa* and contain the same number of 16S rRNA gene copies in the genome (total of four).

The products of a selected number of experiments were verified on a 2% agarose gel by gel electrophoresis. The specificity of the assays targeting *P. aeruginosa* was confirmed by cloning amplicons into pCR®4–TOPO® plasmid, sequencing, and BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA quantification

DNA content of the lung samples from patients with end-stage CF was quantified using the Picogreen assay (Invitrogen). Briefly, standards of Lambda DNA (Invitrogen), ranging from 0 to 1000 pg μL^{-1}, were prepared, and 50 μL of the standards and of the diluted DNA samples were added to a black, solid 96-well microtiter plate (Cayman). Fifty microlitres of Picogreen diluted according to the manufacturer's instructions was added to the plate. Fluorescence was measured using Infinite® M1000 plate reader (Tecan) with excitation of 480 nm, emission of 524 nm, and a gain of 159.

Statistical analysis

The gene copy numbers measured by real-time PCR were normalized to two factors, in order to estimate whether the mean reported is the true mean, the relative expression was calculated along with the standard error of the relative expression (Jacobs & Dinman, 2004). To compare the number of genes quantified by the two real-time PCR assays targeting *P. aeruginosa*, the paired samples *t*-test was used on the mean values of triplicate measurements, both normalized to DNA content and bacterial content in PASW Statistics program (formerly SPSS Statistics). This provided a hypothesis test of the difference between population means for pairs of samples, and a statistical value of ≤ 0.05 was considered significant.

PNA FISH

Before staining or hybridization, the paraffin was removed from the samples using 2× 5 min xylene, 2× 3 min 99.9% EtOH, 2× 3 min 96% EtOH and washed 3× 3 min in sterile water. The de-paraffinized sputum sections were analyzed by FISH using PNA probes (Stender, 2003). A mixture of Texas Red-labeled *P. aeruginosa*-specific PNA probe and fluorescein isothiocyanate (FITC)-labeled universal bacterium PNA probe or FITC-labeled *S. aureus*-specific PNA probe and Texas Red-labeled universal bacterium PNA probe both in hybridization solution (AdvanDx, Inc., Woburn, MA) was added to each section and hybridized in a PNA FISH Workstation at 55 °C for 90 min covered by a lid (Bjarnsholt *et al.*, 2009). The slides were washed for 30 min at 55 °C in Wash Solution (AdvanDx). Vectashield mounting media with DAPI (Vector laboratories, Burlingame, CA) were applied, and a cover slip was added to each slide. The entire PNA FISH procedure required approx. 2.5 h. Slides

were investigated using a LSM 710 confocal laser scanning microscope (Zeiss, Germany).

Results

Patients with end-stage CF

From previous investigations of the five end-stage patients, it was found that *P. aeruginosa* was hardly detectable in clone libraries constructed for two patients (patients 1 and 5), whereas they were dominant in three patients (patients 2–4). Generally, it was found that 1–2 bacteria dominated the clone libraries, but the presence of other bacteria from up to four different phyla could be identified (Rudkjøbing *et al.*, 2011). To determine whether the dominance in the clone library was caused by dominance of the pathogen in the sample or by an artifact in the construction of the libraries, real-time PCR experiments were performed. Only the results from samples with *P. aeruginosa* genes above the limit of detection are shown. The samples from the patients varied greatly in size, and therefore the gene measurements were normalized before comparison. Measurements were normalized both to the total bacterial content, which was quantified by targeting their 16S rRNA genes (Table 1) and to the total concentration of the extracted DNA (Table 2). The two *P. aeruginosa* assays generally gave differing results, with the assay targeting the *ecfX* gene always measuring the highest number (as indicated by the means given in Tables 1 and 2). A paired samples *t*-test revealed that the difference in measurements by the two assays was statistically significant. Normalization of *P. aeruginosa* to the number of bacteria in samples generally showed that the number of *P. aeruginosa* cells (based on measurements of both *ecfX* and *oprL* genes) was higher than the number of bacterial cells (based on 16S rRNA gene measurements) (Table 1). Although this is not realistic (see Discussion), it indicates that *P. aeruginosa* is highly abundant in the samples. The normalization of *P. aeruginosa* and bacteria to the total DNA concentration showed that these constituted only a small percentage of the extracted DNA (Table 2). The abundance of *P. aeruginosa* was found to vary considerably between the patients and also between the different samples from a single patient, which is to be expected based on our previous findings of a heterogeneous distribution of microcolonies. Samples from patients 1 and 5 generally did not contain quantifiable amounts of *P. aeruginosa* (not shown), which corresponded to our previous results using 16S rRNA gene analysis and culturing (Rudkjøbing *et al.*, 2011). Only a single sample from patient 1 contained *P. aeruginosa* genes above the limit of detection, and the normalized results show that *P. aeruginosa* constituted a very small percentage of all bacteria present (Table 1).

Table 1. Real-time PCR measurements of the *oprL* and *ecfX* genes of *Pseudomonas aeruginosa* in samples from patients with end-stage CF. Measurements are normalized to the total number of bacteria, with the standard error of the relative expression (as percentage of the standard error values)

	oprL ng per ng DNA (‰)	*ecfX* ng per ng DNA (‰)
End-stage patient 1		
3 tissue	0.029 ± 18	0.608 ± 4
End-stage patient 2		
1 tissue	256 ± 6	9300 ± 7
2 tissue	380 ± 8	–
3 tissue	130 ± 17	5940 ± 13
End-stage patient 3		
1 sputum	–	–
2 sputum	–	–
3 sputum	1150 ± 7	3930 ± 8
4 sputum	2640 ± 11	11 000 ± 12
5 sputum	787 ± 7	2910 ± 9
1 tissue	1040 ± 6	2000 ± 7
2 tissue	3760 ± 10	9620 ± 9
3 tissue	–	–
4 tissue	504 ± 7	525 ± 15
5 tissue	174 ± 16	394 ± 8
End-stage patient 4		
1 tissue	1160 ± 7	3050 ± 5
2 tissue	38.1 ± 30	58.0 ± 10
3 tissue	705 ± 4	617 ± 9
4 tissue	42.3 ± 6	113 ± 6
4 sputum	–	–
Mean	651	2600
Significance value*		0.011

'–' Indicates that data could not be given. For sample 2 tissue from end-stage patient 2, this was caused by lack of DNA extract. For the rest, it was not possible to normalize the measured *P. aeruginosa* genes because the 16S rRNA assay could not detect the target gene.
*The significance value calculated by paired samples *t*-test is below 0.05, indicating that the difference between mean value observed for the two assays targeting *P. aeruginosa* genes is statistical significant.

Non-end-stage patients

Culture analysis

The culture analysis of the non-end-stage sputum samples showed a variety of bacteria, ranging from 1 to 3 different CF-related microorganisms identified in each sample. The organisms identified were as follows: Mucoid and nonmucoid *P. aeruginosa*, *H. influenzae*, *S. aureus*, *S. maltophilia*, *B. cepacia*, Candida species, and *Aspergillus* species. Compared to culture analysis of end-stage patients, this is a relatively large diversity (Table 3).

Table 2. Real-time PCR measurements of the *oprL* and *ecfX* genes of *Pseudomonas aeruginosa* and the 16S rRNA gene of *Bacteria* in samples from patients with end-stage CF. Measurements are normalized to the total mass of DNA extracted from samples, with the standard error of the relative expression (as percentage of the standard error values)

	16S rRNA ng per ng DNA (%)	*oprL* ng per ng DNA (%)	*ecfX* ng per ng DNA (%)
End-stage patient 1			
3 tissue	0.200 ± 4	0.00006 ± 17	0.001 ± 2
End-stage patient 2			
1 tissue	0.006 ± 4	0.016 ± 4	0.580 ± 7
2 tissue	0.001 ± 4	0.002 ± 8	–
3 tissue	0.012 ± 7	0.016 ± 16	0.734 ± 11
End-stage patient 3			
1 sputum	ND	0.041 ± 11	0.090 ± 8
2 sputum	ND	0.129 ± 9	1.171 ± 6
3 sputum	0.035 ± 7	0.406 ± 8	1.391 ± 9
4 sputum	0.024 ± 11	0.554 ± 3	2.306 ± 6
5 sputum	0.077 ± 7	0.422 ± 1	1.562 ± 5
1 tissue	0.001 ± 4	0.011 ± 6	0.021 ± 7
2 tissue	0.003 ± 9	0.101 ± 5	0.259 ± 4
3 tissue	ND	0.005 ± 14	0.011 ± 15
4 tissue	0.060 ± 7	0.305 ± 5	0.317 ± 14
5 tissue	0.010 ± 7	0.018 ± 14	0.040 ± 4
End-stage patient 4			
1 tissue	0.012 ± 5	0.137 ± 5	0.360 ± 2
2 tissue	0.018 ± 11	0.007 ± 29	0.010 ± 3
3 tissue	0.038 ± 4	0.266 ± 3	0.233 ± 9
4 tissue	0.027 ± 6	0.011 ± 4	0.030 ± 4
4 sputum	ND	0.067 ± 46	0.368 ± 4
Mean		0.109	0.412
Significance value*		0.007	

'–' Indicates that data could not be given because of lack of DNA extract; ND indicates that the genes were not detected, because the quantity was below the level of detection for the assay.

*The significance value calculated by paired samples *t*-test is below 0.05, indicating that the difference between mean value observed for the two assays targeting *P. aeruginosa* genes is statistical significant.

FISH

To investigate the spatial distribution of species in patients with CF chronically infected with more than one species, we applied general and specific PNA FISH visualization to sputum samples of patients with non-end-stage CF (representative selection shown in Fig. 1). Examining these spatial observations, we were not able to observe any direct interaction between microcolonies of different species. No mixing took place at least between *P. aeruginosa* (red) and other bacterial species (green) (Fig. 1a, c, and d) or *S. aureus* (red) and other bacteria (green) (Fig. 1b). We have previously described this segregation of species for chronic infections (Burmølle *et al.*, 2010). Even though the lung infection status of the patients ranged from nonchronic to 34 years of chronic, we could not detect any difference in the spatial distribution of biofilms and species.

Discussion

In the present study, we investigated the true microbiota involved in different stages of chronic infection of CF lungs. The normal type of sample investigated in CF studies is sputum that has been expectorated by the patients, and this is also the sample used in routine diagnostics where standard culturing is employed. It is, however, possible that these samples contain oral or oropharyngeal contamination (Bjarnsholt *et al.*, 2011), which may be particularly problematic in respect to culturing as it becomes difficult to distinguish pathogens from contaminating organisms. Some authors claim that the potential contamination is not significant (Rogers *et al.*, 2006) or that contaminating saliva can be removed from the sputum sample by a series of washing steps (Rogers *et al.*, 2006; Guss *et al.*, 2011). To avoid this disputed point, the samples included in this study were either from explanted lungs (end-stage patients), which were investigated by culture analysis and PCR-based approaches, or expectorated sputum samples (non-end-stage patients), which were investigated by culture analysis and FISH. The visualization by FISH makes it possible to determine whether the bacteria are integrated in the sample, which minimizes the risk of observing contaminating microorganisms. At present, only few publications are available where direct sampling of lung tissue is used, although this allows for a more specific characterization of microbiota than other sampling methods (Rudkjøbing *et al.*, 2011; Willner *et al.*, 2011).

The use of molecular methods for the identification of bacteria has revealed that the complexity and microbial diversity of many samples is greater than that found by culturing. This is partly due to the fact that culturing requires assumptions to be made regarding which species will be present so that appropriate media can be used (Rogers *et al.*, 2006; Guss *et al.*, 2011). In a previous study, we compared culturing and 16S rRNA gene analysis on samples from patients with end-stage CF. In all cases, culture analysis identified the organism that was the most frequently represented clone in the clone libraries, but other bacteria in low abundance with unknown clinical relevance were also found (Rudkjøbing *et al.*, 2011). The real-time PCR measurements confirmed that *P. aeruginosa* (the clone most frequently found in clone libraries from the three patients) was in fact the dominant bacteria in the lungs and not due to an artifact in the construction of the clone libraries (Table 1). The finding of a few dominating bacteria (1 or 2) in the CF

Table 3. Identification of microorganisms found in patients with end-stage and non-end-stage CF by culturing

	Identified microorganisms		
End-stage patient 1*	*Achromobacter xylosoxidans*		
End-stage patient 2*	*Pseudomonas aeruginosa*		
End-stage patient 3*	*P. aeruginosa*		
End-stage patient 4*	*P. aeruginosa*	*Stenotrophomonas maltophilia*	
End-stage patient 5*	*A. xylosoxidans*		
Non-end-stage patient 1	*P. aeruginosa*	Yeast	
Non-end-stage patient 2	*Staphylococcus aureus*	*P. aeruginosa*	
Non-end-stage patient 3	*P. aeruginosa*	*Aspergillus* sp.	Yeast
Non-end-stage patient 4	*P. aeruginosa*		
Non-end-stage patient 5	*P. aeruginosa*		
Non-end-stage patient 6	*S. aureus*	*Burkholderia cepacia*	Yeast
Non-end-stage patient 7	*P. aeruginosa*	Yeast	
Non-end-stage patient 8	*P. aeruginosa*	Yeast	
Non-end-stage patient 9	*S. aureus*	*Haemophilus influenza*	*Aspergillus* sp.
Non-end-stage patient 10	*P. aeruginosa*		
Non-end-stage patient 11	*P. aeruginosa*	Yeast	
Non-end-stage patient 12	*S. aureus*	*Haemophilus influenza*	Yeast
Non-end-stage patient 13	*S. aureus*	*S. maltophilia*	*Aspergillus* sp.
Non-end-stage patient 14	*P. aeruginosa*	Yeast	
Non-end-stage patient 15	*P. aeruginosa*	*S. aureus*	Yeast

*Data from Rudkjøbing *et al.* (2011).

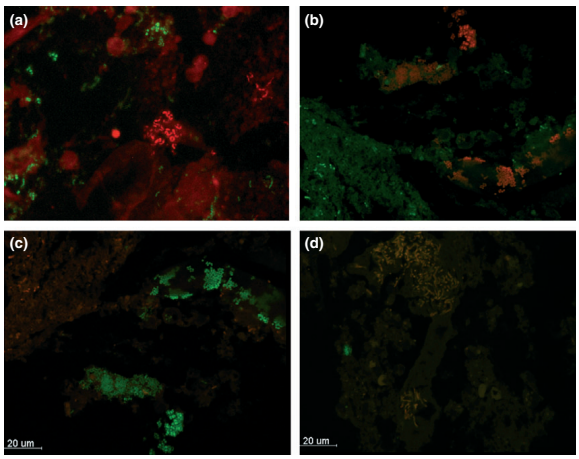


Fig. 1. PNA FISH visualization of bacterial aggregates/biofilms in expectorated sputum of patients with non-end-stage CF. Frame (a), (c), and (d) show *Pseudomonas aeruginosa* (red), and other bacterial species (green) and frame (b) show *Staphylococcus aureus* (red) and other bacteria (green). As seen from the pictures, the biofilms of different species seem to be segregated from each other.

lung, which can be identified by culture analysis, correlates with results we have previously published, where the sole presence of *P. aeruginosa* in end-stage CF lungs was found (Bjarnsholt *et al.*, 2009). This is in contrast to most other studies of diversity in CF, either performed on samples from ex-planted lungs (Willner *et al.*, 2011) or expectorated sputum samples (Harrison, 2007; Worlitzsch *et al.*, 2009; Rogers *et al.*, 2010; Stressmann *et al.*, 2011). The reason for this is probably the aggressive antibiotic treatment administered in Denmark.

The measurements of the two *P. aeruginosa*-specific genes gave significantly different results, with the assay targeting *ecfX* giving the highest numbers (Tables 1 and 2). This could be caused by the use of different fluorescent reporters in the assays or by the length of the amplicons (*ecfX* amplicon was 64 bp, compared to 504 bp for *oprL*); a smaller amplicon is preferable to ensure reliable amplification. Further problems with the *oprL* assay have been discussed elsewhere (Anuj *et al.*, 2009). The measurements were normalized, both to the total number of bacterial cells (based on number of 16S rRNA genes) and to the total DNA content of the samples. It was found that *P. aeruginosa* constituted more than 100% of all bacteria in all cases except for two subsamples of patient 4 (Table 1). It is not realistic, the reason probably being the general nature of the 16S rRNA gene assay, because nucleotide mismatches are allowed during primer design to target as many bacteria as possible. The 907R primer has one mismatch in the 16S rRNA gene of *P. aeruginosa*, so although the target sequence is amplified, the efficiency may be insufficient to provide correct quantification of *P. aeruginosa* 16S rRNA genes. Also, it was not possible to quantify the 16S rRNA genes in four samples (patient 3 subsamples sputum 1, sputum 2, and tissue 3, and patient 4 subsample sputum 4) (Table 2), although both *P. aeruginosa*-specific assays showed that the number of *P. aeruginosa* was above the limit of detection for the 16S rRNA gene assay. This indicates that this 16S rRNA assay is not suitable for the task and emphasizes the caution one should exercise when examining these types of results. Therefore, the real-time PCR measurements were normalized to the total DNA content to give an impression of the heterogeneous distribution of *P. aeruginosa* in the samples (Table 2). The low percentage obtained by this normalization is because of the large amount of DNA originating from host cells.

Molecular methods are strongly influenced by the DNA extraction protocol. Despite this fact, generally, few publications exist where the extraction method is evaluated before molecular methods are applied to CF-related samples. For CF sputum, one study exists, which is conducted by Deschaght *et al.* (2009), where different protocols were used to extract DNA from *P. aeruginosa* and compared to culturing sensitivity. The extraction protocol giving the best sensitivity included a proteinase K pretreatment step with a sensitivity equal to that of culturing (Deschaght *et al.*, 2009). The extraction protocol used in our study included proteinase K pretreatment. We tested the efficiency of the selected extraction procedure and found it to be appropriate for lung tissue samples when the target Gram-positive organism was present in sufficient numbers (over 500 cells). However, the efficiency of our protocol should be investigated with regard to Gram-positive and Gram-negative bacteria and also compared to other available DNA extraction protocols before it can be recommended as a universal protocol for lung tissue samples.

The low diversity found in Danish end-stage patients does not represent the other levels of chronic infection in the CF lungs. In fact, many of the patients with CF at the Copenhagen CF Clinic harbor several species in their lungs. The reports of the multispecies microbiota of the CF lung (Rogers *et al.*, 2010; Guss *et al.*, 2011; Stressmann *et al.*, 2011) and other investigations of chronic infections such as chronic wounds (Dowd *et al.*, 2008) speculate that an interaction occur between species, increasing the pathogenesis. In order for different species to interact, they must reside within close proximity to each other. As seen from the culturing (Table 3) and PNA FISH data from the non-end-stage samples (Fig. 1), earlier in the chronic infection, more species are often present. Depicted from the PNA FISH observations, a direct interaction likely does not take place, as we have previously discussed (Burmølle *et al.*, 2010). Whether a synergy exists between the different species is difficult to determine at the present time. The segregation of species is the same as has been observed in chronic wounds (Bjarnsholt, 2008; Kirketerp-Moller *et al.*, 2008; Fazli *et al.*, 2009), but very different from the intestines and oral flora (Burmølle *et al.*, 2010). We believe that this is a general phenomenon of the chronic infections we have studied and caused by the lack of competition between the species. It is possible that the bacteria signal to each other between the distinct microcolonies, a possibility we are currently testing *in vitro*. On the other hand, the Danish patients with CF do not seem to experience improvements in lung function going from multispecies to a more monospecies infection. Factors mediating an improvement or decline in lung function are either eradication or shift of the dominating bacteria, respectively, because of, at least in part, the intensive antibiotic treatment. A decline in lung function is observed when the patient with CF experiences a downshift in diversity, for instance when dominated by *B. cepacia* complex (Hansen *et al.*, 2010) or *Pandoraea apista* (Jørgensen *et al.*, 2003).

As the techniques for the identification of bacteria are improved, our knowledge of the microorganisms present during various diseases is expanded, and the challenge becomes to identify whether the dominant bacterium alone causes the pathology or the less abundant bacteria add to this as well. To determine the role of these microorganisms in a diverse community, we need to study these in the habitat of interest (Koch, 1884; Bjarnsholt *et al.*, 2011).

In conclusion, our results show that the microbial diversity in Danish patients with CF is minimized by the time of lung transplantation, compared to earlier stages of chronic infection. Furthermore, even when multiple

species were identified in CF sputum, no physical interaction could be observed between the different species. The results indicate that Danish patients with CF harbor more homogenous microbiota than patients in other countries because of administration of aggressive antibiotic treatment and the fact that standard culturing identifies the dominating pathogens.

Acknowledgements

The authors would like to thank Lise Schorling Strange og Heidi Maria Paulsen for laboratory assistance, AdvanDx Inc., Woburn, MA, for supplying the PNA probes for the FISH experiments, and grants to M.G. from strategically research council, Svend Andersen foundation, and Villum Foundation.

References

Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, Nissen MD & Sloots TP (2009) Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. *Diagn Microbiol Infect Dis* **63**: 127–131.

Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, Høiby N & Givskov M (2008) Why chronic wounds will not heal: a novel hypothesis. *Wound Repair Regen* **16**: 2–10.

Bjarnsholt T, Jensen P, Fiandaca M, Pedersen J, Hansen C, Andersen C, Pressler T, Givskov M & Høiby N (2009) *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol* **44**: 547–558.

Bjarnsholt T, Nielsen X, Johansen U, Nørgaard L & Høiby N (2011) Methods to classify bacterial pathogens in cystic fibrosis. *Cystic Fibrosis: Diagnosis and Protocols, Vol. II: Methods and Resources to Understand Cystic Fibrosis*, vol. **742** (Amaral M & Kunzelmann K, eds), pp. 143–171. Humana Press, Totowa, NJ.

Boucher RC (2004) New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur. Respir. J.* **23**: 146–158.

Braun AT & Merlo CA (2011) Cystic fibrosis lung transplantation. *Curr Opin Pulm Med*. **17**: 467–472.

Burmølle M, Thomsen TR, Fazli M *et al.* (2010) Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol* **59**: 324–336.

Conese M, Copreni E, Gioia SD, Rinaldis PD & Fumarulo R (2003) Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *J Cyst Fibros* **2**: 129–135.

Costerton JW, Stewart PS & Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**: 1318–1322.

Deschaght P, De Baere T, Van Simaey L, Van daele S, De Baets F, De Vos D, Pirnay J-P & Vaneechoutte M (2009) Comparison of the sensitivity of culture, PCR and quantitative real-time PCR for the detection of *Pseudomonas aeruginosa* in sputum of cystic fibrosis patients. *BMC Microbiol* **9**: 244.

Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA & Wolcott RD (2008) Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol* **8**: 43.

Fazli M, Bjarnsholt T, Kirketerp-Moller K, Jorgensen B, Andersen AS, Krogfelt KA, Givskov M & Tolker-Nielsen T (2009) Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *J Clin Microbiol* **47**: 4084–4089.

Gilligan PH (1991) Microbiology of airway disease in patients with cystic fibrosis. *Clin Microbiol Rev* **4**: 35–51.

Guss AM, Roeselers G, Newton ILG, Young CR, Klepac-Ceraj V, Lory S & Cavanaugh CM (2011) Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. *ISME J* **5**: 20–29.

Hansen CR, Pressler T, Nielsen KG, Jensen PØ, Bjarnsholt T & Høiby N (2010) Inflammation in *Achromobacter xylosoxidans* infected cystic fibrosis patients. *J Cyst Fibros* **9**: 51–58.

Harrison F (2007) Microbial ecology of the cystic fibrosis lung. *Microbiology* **153**: 917–923.

Hein I, Lehner A, Rieck P, Klein K, Brandl E & Wagner M (2001) Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. *Appl Environ Microbiol* **67**: 3122–3126.

Høiby N, Frederiksen B & Pressler T (2005) Eradication of early *Pseudomonas aeruginosa* infection. *J Cyst Fibros* **4**: 49–54.

Jacobs JL & Dinman JD (2004) Systematic analysis of bicistronic reporter assay data. *Nucleic Acids Res* **32**: e160.

Jaffe RI, Lane JD & Bates CW (2001) Real-time identification of *Pseudomonas aeruginosa* direct from clinical samples using a rapid extraction method and polymerase chain reaction (PCR). *J Clin Lab Anal* **15**: 131–137.

Jørgensen IM, Johansen HK, Frederiksen B, Pressler T, Hansen A, Vandamme P, Høiby N & Koch C (2003) Epidemic spread of *Pandoraea apista*, a new pathogen causing severe lung disease in cystic fibrosis patients. *Pediatr Pulmonol* **36**: 439–446.

Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M & Bjarnsholt T (2008) Distribution, organization, and ecology of bacteria in chronic wounds. *J Clin Microbiol* **46**: 2717–2722.

Koch R (1884) Die Aetiologie der Tuberkulose. *Berliner Klinische Wochenschrift* **2**: 1–88.

Moser C *et al.* (2009) Novel experimental *Pseudomonas aeruginosa* lung infection model mimicking long-term host–pathogen interactions in cystic fibrosis. *APMIS* **117**: 95–107.

Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Kehagia V, Connett GJ & Bruce KD (2006) Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. *J Clin Microbiol* **44**: 2601–2604.

Rogers GB, Stressmann FA, Walker AW, Carroll MP & Bruce KD (2010) Lung infections in cystic fibrosis: deriving clinical insight from microbial complexity. *Expert Rev Mol Diagn* **10**: 187–196.

Rønne Hansen C, Pressler T, Høiby N & Gormsen M (2006) Chronic infection with *Achromobacter xylosoxidans* in cystic fibrosis patients; a retrospective case control study. *J Cyst Fibros* **5**: 245–251.

Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Givskov M, Høiby N & Bjarnsholt T (2011) True microbiota involved in chronic lung infection of cystic fibrosis patients found by culturing and 16S rRNA gene analysis. *J Clin Microbiol* **49**: 4352–4355.

Saiman L (2004) Microbiology of early CF lung disease. *Paediatr Respir Rev* **5**: S367–S369.

Skovhus TL, Ramsing NB, Holmstrom C, Kjelleberg S & Dahllof I (2004) Real-time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. *Appl Environ Microbiol* **70**: 2373–2382.

Stender H (2003) PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases. *Expert Rev Mol Diagn* **3**: 649–655.

Stressmann FA *et al.* (2011) Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. *J Clin Microbiol* **49**: 281–291.

Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, Rohwer F & Conrad D (2011) Spatial distribution of microbial communities in the cystic fibrosis lung. *ISME J*. doi:10.1038/ismej.2011.104

Worlitzsch D, Rintelen C, Böhm K, Wollschläger B, Merkel N, Borneff-Lipp M & Döring G (2009) Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. *Clin Microbiol Infect* **15**: 454–460.

Research paper 4

Rudkjøbing, V.B., Aanaes, K., Wolff, T., von Buchwald, C., Johansen, H.K., Thomsen, T.R. (submitted). Microorganisms involved in sinus infection of cystic fibrosis patients determined by culture and molecular-based methods. *PLoS ONE*.

Microorganisms involved in sinus infection of cystic fibrosis patients determined by culture and molecular-based methods

Vibeke Børsholt Rudkjøbing[1], Kasper Aanaes[2], Tine Yding Wolff[3], Christian von Buchwald[2], Helle Krogh Johansen[4], Trine Rolighed Thomsen[1, 3]#

[1] Department of Biotechnology, Chemistry, and Environmental Engineering, Aalborg University, Sohngaardholmsvej 49, DK-9000 Aalborg, Denmark.

[2] Department of Otorhinolaryngology, Head and Neck Surgery and Audiology, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.

[3] Life Science Division, Danish Technological Institute, Kongsvang Allé 29, DK-8000 Aarhus C, Denmark.

[4] Department of Clinical Microbiology, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.

Corresponding author footnote:

Trine Rolighed Thomsen

Life Science Division

Danish Technological Institute

DK-8000 Aarhus C, Denmark

E-mail: trt@teknologisk.dk

Telephone: +45 7220 1828

Running title: Microorganisms in sinuses of cystic fibrosis patients

Abstract

Cystic fibrosis (CF) is a genetic disease characterized by intermittent and chronic lung infections. Nearly all CF patients have bacteria in their sinuses, which may serve as reservoirs for lung colonizations. Culture often detects *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae,* coagulase-negative staphylococci, *Aspergillus* sp. and other known CF-related microorganisms in the sinuses, but surprisingly few anaerobic bacteria are cultured.

In this study, sinus samples from 19 CF patients were examined using routine culture methods and molecular methods performed on microbial DNA of intact cells including broad-range 16S rRNA gene clone libraries and species-specific quantitative polymerase chain reaction (qPCR). The objective was to study the bacterial diversity, and determine if anaerobes were present in the sinuses.

In 63% of the cases, polymicrobial infections were found by culture. The molecular methods found a greater bacterial diversity and 84% of cases were found to be polymicrobial. Some of the microorganisms found only by molecular methods were anaerobic and facultative anaerobic bacteria. The greater diversity found by molecular methods may be due to growth of microorganisms in biofilms, which represents a challenge for identification of microorganisms by culture. However, contamination by the nasal flora during sample acquisition cannot be ruled out and may influence the additional bacteria detected by the molecular methods. Using qPCR it was found that *P. aeruginosa* and *S. aureus* were generally more abundant than the anaerobe *Propionibacterium acnes*. Tests based on RNA indicated that the selective DNA extraction protocol using Molzym's MolYsis™ pretreatment had worked and that DNA originated from intact bacterial cells. Though CF pathogens were extraordinary found by molecular methods, the clinical relevance of the diverse microbial flora found by these methods needs to be further examined.

Introduction

Cystic fibrosis (CF) is an autosomal recessively inherited disease characterized by abnormal transport of sodium and chloride across epithelial cells, leading to increased mucus viscosity and decreased mucociliary clearance. This makes the mucus more susceptible to infections [1,2]. The disease affects several organs, and the primary cause of the high mortality in CF is chronic lung infections [3,4].

CF patients have thickened mucosa in the paranasal sinuses, decreased oxygen tension [5], and most of them are thought to have bacteria in their paranasal sinuses [6]. Culture-based methods have shown that sinus infections in CF patients most often are caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, [3,7,8] with some reports of *Haemophilus influenzae* [3,8,9], α-hemolytic streptococci [7,8] and few reports of anaerobes (including *Peptostreptococcus* sp., *Bacteroides oralis* and *Propionibacterium acnes*) [8]. Cross infections between sinuses and lower airways are likely to occur in CF patients since a high correlation between sinus and sputum cultures has been found [2–4,10,11]. Recent studies using DNA-based techniques have shown that the sinuses serve as a reservoir for lung colonization and infection of the lower airways [2,12]. Since the sinuses have some physiological similarities with the lungs, such as similar mucous lining and defective ion channel [5,12], the bacteria in the sinuses can become pre-adapted to the conditions of the lungs [12,13]. This may account for the recurrent lung infections after antimicrobial eradication and is a concern for lung transplant patients, since it is possible that the lung become re-colonized with pathogens from the sinus reservoir [2].

Eradication of pathogenic microorganisms in CF sinuses may delay chronic lung infections, thus improving morbidity and prolonging lifespan [4,14,15]. We have investigated the microbial community in sinus samples obtained during extended image guided functional endoscopic sinus surgery (FESS) [6]. The experience at the Copenhagen CF center has been that samples from such surgeries do not result in growth of anaerobic microorganisms. No anaerobic species have been cultured from the sinuses during a two-year sampling period, and anaerobic culture has been stopped for this sample type due to cost considerations, which has also been suggested by other authors [16]. This is in contrast to the findings by others, where anaerobes could be found in up to 15 % of CF sinus cultures [8,9]. The lack of anaerobic microorganisms in the sinuses of CF patients at the Copenhagen CF center, raises the question whether the used culture methods identifies all the microorganisms present in the sinuses. Since microorganisms may reside in biofilms within the sinuses, culture methods may not be able to identify them. In this study, we compared the findings of routine culture methods to molecular methods including construction of broad-range 16S rRNA gene clone libraries and species-specific quantitative polymerase chain reaction (qPCR).

Methods

Patients and treatment

All patients included in this study have been followed at the Copenhagen CF Centre in Denmark by monthly visits to the outpatient clinic. A total of 19 patients undergoing sinus surgery at the Department of Otorhinolaryngology, Head and Neck Surgery and Audiology (Rigshospitalet, Copenhagen, Denmark) were included in the study, 10 females, 9 males (age 6–45 years, median 22 years).

Patients were selected for surgery based on the following criteria: 1) Intermittent lung colonization with prolonged declining lung function, despite intensive antibiotic-chemotherapy and/or increasing antibodies against pathogenic Gram-negative bacteria. 2) Patients who had undergone lung transplantation within one year. 3) Patients with severe symptoms of chronic rhinosinusitis according to European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) guidelines [17].

Ethics

The study was approved by the local ethics committee (H-A-2008-141). All patients gave informed consent; for patients <18 years of age a consent was also obtained from their parents.

Surgical procedure

Surgery was performed as an extended FESS, comprising at least an anterior ethmoidectomy and a medial antrostomy enlarging the natural maxillary ostium, so the sinuses could be explored and cultured during and post surgery, as described previously [6]. Briefly, visible intramucosal abscess looking structures were resected along with swollen and inflamed tissue when accessible. The purpose of surgery was to create ventilation and drainage for the sinuses, allowing postoperative medical irrigations. No local disinfectants were used in the nose. Samples for culture and molecular investigations were obtained in parallel. Multiple samples were prioritized for culture containing nasal secretions, pus, mucosal tissue and polyps (if present). Samples from each anatomic location where mixed and one sample containing tissue and secretions was taken for molecular investigations. Samples for culture were divided into subsamples and were investigated immediately, while samples for molecular methods were stored in glycerol for subsequent DNA extraction. For four patients (patient 14, 15, 16 and 19), an additional subsample was stored in RNA*later*® solution (Invitrogen, Carlsbad, California) for subsequent RNA extraction. Stored samples were kept at -80 °C. All molecular investigations were performed at the Life Science Division (Danish Technological Institute, Aarhus, Denmark) and the Department of Biotechnology, Chemistry, and Environmental Engineering (Aalborg University, Aalborg, Denmark).

Culture experiments

Samples for culture were investigated at the Department of Clinical Microbiology (Rigshospitalet, Copenhagen, Denmark). Investigations included Gram-stained smears along with aerobic cultures on a range of media: Sabouraud plate, 7% NaCl plate, *B. cepacia* plate containing Colistimethate sodium and Gentamicin, "blue plate" (modified Conradi Drigalski's medium) selective for Gram-negative bacteria and non-selective media including 5% Danish blood agar and chocolate agar (State Serum Institute, Copenhagen,

Denmark). Isolated bacteria were identified as previously described [18]**.** Direct plating of tissue samples and pus on a 14 cm blood agar plate with discs containing anti-pseudomonas antibiotics was used for primary susceptibility testing (Rosco® Neosensitabs, Taastrup, Denmark)**.** The culture reports included only microorganisms known as potential human pathogens.

DNA extraction

Genomic DNA (gDNA) from intact bacterial cells was extracted from all 19 samples using MolYsis Basic (Molzym, Bremen, Germany) followed by the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturers' protocols. The gDNA was eluted in 75 µL DEPC-treated water.

RNA extraction and cDNA synthesis

RNA was extracted from samples from four patients (patient 14, 15, 16 and 19) using the RNeasy PLUS mini kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. The optional DNase treatment step was included to minimize the amount of gDNA co-eluting with the RNA. To secure a cleaner product, the RNA extraction was repeated for each extract. The RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware), and complementary DNA (cDNA) was synthesized from 100 ng RNA using the SuperScriptTM III First Strand Synthesis System for RT-PCR (Invitrogen) with random hexamers according to the manufacturer's guidelines. Controls without reverse transcriptase were included to check for the presence of gDNA.

Construction and analysis of clone libraries

Clone libraries were constructed for the extracted gDNA and analyzed as described previosly [19]. Briefly, PCR amplicons of near full-length 16S rRNA genes were cloned using TOPO TA Cloning ® kit (Invitrogen) and DNA templates were prepared by rolling circle amplification with Illustra^{TM} TempliPhi Kit (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Sequencing was performed by Macrogen Inc. (Korea) using M13F primer (and M13R primer in some cases). Manual refinement of sequences and construction of consensus sequences were done in CLC Main Workbench (CLC bio, Aarhus, Denmark). Sequences were checked for chimeras using the Mallard software package [20], aligned using SINA Web Aligner [21] and imported into the ARB software package [22] for taxonomic lineage assignment, using the non-redundant (NR) SSU Ref database from SILVA Release 106 as reference database. Operational taxonomic units (OTUs) were constructed across all patient samples for clones having a sequence similarity of more than 97% since these sequences are typically assigned to the same species [23]. One clone from each OTU was sequenced with both M13F and M13R primers. The resulting consensus sequences and their closest relatives in the database were selected to calculate phylogenetic trees using neighbor joining, maximum parsimony and maximum likelihood methods. The non-redundant, near full-length 16S rRNA gene sequences, representing each OTU obtained in this study, were deposited in GenBank under the accession numbers JQ794610-JQ794658. The proportion of the population represented in the clone library was estimated by the coverage ratio $C = (1 - n_1/N) \cdot 100\%$. Here n_1 is the number of OTUs containing only one sequence and N is the total number of sequences in the clone library [24].

Quantitative PCR

Based on the findings in the clone libraries and by culture, a variety of qPCR assays were applied to the gDNA and cDNA obtained from the patient samples. The assays used hydrolysis probe chemistry and targeted *P. aeruginosa*, *P. acnes* and *S. aureus* (Table 1). For each sample triplicate 25 µL reactions were run, each containing: 12.5 µL Brilliant® qPCR Master mix (Agilent Technologies, Santa Clara, California), 25 µg BSA (Sigma-Aldrich, Brøndby, Denmark), appropriate concentration of primers and TaqMan® probes (Table 1), 0.75 µM ROX reference dye (Agilent Technologies, Santa Clara, CA) and 2 µL of template DNA. The DNA standard was synthesized plasmids containing the respective target gene sequences (GenScript, Piscataway, New Jersey). The standard curve was prepared from serial dilution of plasmid (10^0 to 10^7 copies μL^{-1}). Reactions were run on an Mx3005P (Agilent Technologies) with the following program: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 sec at 60°C and 60 s at 72°C.

Analysis of quantitative data

The number of gene copies measured by qPCR on gDNA was converted to number of CFU per gram sample using $CFU/g = {C_{measured}/C_{genome} * (V_{total}/V_{used})} \Big/ m_{sample}$. Here $C_{measured}$ is the number of copies measured and C_{genome} is the number of gene copies in the genome of one CFU. The standard deviation of all measurements above the detection limit of the assays was calculated. For samples where more than one bacterium could be quantified, a two tailed T-test was used to provide a hypothesis test of the difference between population means. A statistical value of ≤ 0.05 was considered significant.

Results

Molecular methods

The 16S rRNA gene clone libraries constructed for all 19 patients showed a great diversity of microorganisms with clones grouped into 49 OTUs. All clone libraries had a coverage ratio above 90%, except for patient 5 (86% coverage ratio), indicating that the majority of microorganisms in the samples was detected. Identity of the clones was determined based on their closest relatives in the SILVA NR SSU Ref database, release 106. 20 of the found OTUs consisted of only 1-2 sequences found in the clone library of a single patient. The identity of the clones were either opportunistic human pathogens (*Brevundimonas vesicularis*, *Brevibacterium frigoritolerans*, *Alcaligenes faecalis*, *Aerococcus viridians*, *Dermabacter hominis* and *Massilia* sp.), environmental bacteria (*Mesorhizobium plurifarium*, *Telmatospirillum siberiense*, *Alkalibacterium pelagium*, *Shimazuella kribbensis*, *Burkholderia tuberum*, *Burkholderia soli*, *Defluviicoccus* sp., and *Myxococcales* sp.) or bacteria where the source could not be determined (6 different OTUs identified as uncultured bacteria). For clarity reasons these 20 OTUs were not included in the following analyses. The phylogenetic trees constructed from near full-length consensus sequences, representing each OTU, were considered to be robust, as congruent phylogenetic relationships were obtained by neighbor joining, maximum parsimony and maximum likelihood methods. Sequences were distributed into 6 phyla: Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Cyanobacteria and Acidobacteria. The last three phyla contained only one OTU each (OTU-26, OTU-13 and OTU-23 respectively). Maximum likelihood trees of the three major phyla are available as supporting information (Figure S1).

The most frequently detected sequences across all clone libraries were identified as *Cyanobacterium* sp. (95 clones in 9 patients), *P. acnes* (65 clones in 9 patients), *Ralstonia* sp. (57 clones in 9 patients), *S. aureus* (166 clones in 6 patients), *A. xylosoxidans* (125 clones in 6 patients), *P. aeruginosa* (47 clones in 3 patients), *Serratia marcescens* (28 clones in 2 patients) and *Streptococcus salivarius* (35 clones in 2 patients) (Table 2). These 8 OTUs constituted 82% of all sequences. QPCR was performed to confirm presence of *P. aeruginosa*, *P. acnes* and *S. aureus* and determine the relative abundance of these pathogens in the samples (Table 3). For *P. aeruginosa* and *S. aureus* (except for patient 17), qPCR confirmed the findings in the clone libraries. For *P. acnes*, qPCR confirmed the clone libraries in only one of nine cases (patient 11). The quantitative measurements indicated that *P. aeruginosa* and *Staphylococcus* sp. were generally present in high numbers, whereas *P. acnes* was less abundant than other species in the same sample. For patient 2, 8 and 11 it was possible to quantify more than one bacterium, and the measurements obtained by the different assays were statistically significant (P value 0.01, 0.0004 and 0.01 respectively). Surprisingly, in three cases (patient 8, 11 and 18) it was possible to detect *P. aeruginosa* by qPCR, although this species was not found in the clone libraries. To confirm that the molecular findings resulted from intact living bacteria and not from naked DNA residing in the samples (which was the rationale for using the MolYsis technology for DNA extraction) RNA was extracted from four samples (patient 14, 15, 16 and 19) and cDNA synthesized before qPCR. The results of qPCR on cDNA were in accordance with the measurements made on gDNA (data not shown), indicating that the applied extraction protocol, targeting only intact bacterial cells, was effective.

Comparison of culture and molecular methods

The bacterial diversity revealed by culture was generally smaller than what was found by molecular methods: overall, eight different microorganisms were identified by culture methods (Table 2). Of all microorganisms identified in this study, only a small part could be identified by both culture and molecular-based methods, as illustrated in Figure 1 A and B. These microorganisms were *P. aeruginosa*, *S. aureus*, *S. marcescens*, *A. xylosoxidans* and coagulase-negative staphylococci (CNS) (Table 2 and 3). Although the methods had the ability to identify these microorganisms, culture results could not be confirmed by molecular methods in all cases or vice versa (Table 3); importantly, the CF pathogen *A. xylosoxidans* was not found by culture in two patients (patient 12 and 13), where molecular methods indicated the pathogen was present. Furthermore, for five patients, none of the microorganisms identified by culture was found by molecular methods, as illustrated in Figure 1 C and D. The molecular methods identified eight different anaerobic and facultative anaerobic bacteria (Table 2), which could not be found by culture due to lack of anaerobic testing.

Discussion

The realization that cross-infections between sinuses and lower airways take place in CF patients has led to increased focus on eradication of pathogenic bacteria from the sinuses. FESS enables irrigation of the sinuses with antibiotics, which may hamper microbial colonization of the sinuses and thereby possibly reduce the incidence of lower airways infection. Correct identification of the involved microorganisms is important to target the microorganisms present in the sinuses. The literature regarding sinus flora in CF patients has primarily been based on culture methods. The microorganisms found by culture in this study (Table 2) generally corresponded to findings by other authors; *P. aeruginosa* and *S. aureus* [2,3,7–9], *Aspergillus* sp. [2,7], *S. marcescens*, *Enterobacter aerogenes* and CNS [2]. *A. xylosoxidans* is a recently recognized pathogen in CF and may as such be found in the sinuses [9]. The remaining cultured microorganism is *Bordetella bronchiseptica*, which is a respiratory tract pathogen of mammals [25]. However, this bacterium has a high phenotypic affinity to *A. xylosoxidans* [26] and has probably been misidentified based on the findings in the clone library (Table 2). By culture methods, a total of eight different microorganisms were found in 19 patients, with a median value of two different microorganisms per patient. The incidence of polymicrobial infections was 63%, which is higher than reported elsewhere [3,8,9].

The flora found in the sinuses by molecular methods was very diverse compared with that found by culture (Table 2). The clone libraries revealed that anaerobes and facultative anaerobes (OTU1-8) were present in the sinuses of Danish CF patients followed at the Copenhagen CF center, although lack of anaerobic growth over a two-year period had led to discontinuation of anaerobic culture of sinus samples. A reason for the lack of anaerobic detection may be that organisms such as *P. acnes* (which were identified in the sinuses of nine patients) may require >10 days' incubation [27], or that species such as *P. acnes* are not reported since they are considered a part of the normal skin flora. Overall, the clones were grouped into 49 OTUs, with a median value of 5 OTUs per patient. The incidence of polymicrobial infections found by molecular methods was 84%. Of the 49 OTUs identified in the clone libraries, only five of the bacteria could be found by the used culture methods (Figure 1 A and B), these bacteria were: *A. xylosoxidans*, *P. aeruginosa*, *S. aureus*, *S. marcescens* and CNS (since some clinical strains of *S. aureus* may be atypical and have no production of coagulase [28], CNS were considered found in the clone libraries if *S. aureus* was identified). However, the two methods did not agree in all cases where these five bacteria were identified (Table 2). *A. xylosoxidans* was found in the clone libraries for two patients and *S. aureus* in one clone library, where culture could not confirm the findings. Both bacteria are known to be CF pathogens, and therefore the lack of detection by culture is serious. Conversely, there were several cases where the microorganisms detected by culture could not be confirmed in the clone libraries or by qPCR; for CNS, a total of eight cases, four cases of *P. aeruginosa* and two cases of *A. xylosoxidans*. For *P. aeruginosa* there were 3 additional cases where the bacterium could not be found in the clone library but was quantified by qPCR (Table 3). It is possible that the universal primers that were used during construction of the clone libraries have a low affinity to the target region in the *P. aeruginosa* genome or that the high G+C content in the genome (approximately 66% [29]) influenced the efficiency of

amplification. Generally, the so-called universal 16S rRNA primers are not truly universal since no primers of sufficient length can be designed to match all bacteria [30].

The clinical relevance of the diverse microbial flora detected in the clone libraries is unknown, and evaluation of the findings is a recurring issue for studies using similar molecular methods. It has been suggested that the mere presence of a bacterium does not necessarily mean that it contributes to the pathogenesis of an infection and, thus, may not merit treatment [31]. However, it has also been argued that high bacterial diversity promotes persistence of chronic infections [32]. Besides the bacteria described above, most of the OTUs consisting of many clones have previously been identified in the sinuses of CF patients. These included *P. acnes* [8], corynebacteria (in this study *C. appendicis* and *C. simulans*) [2,7], *Ralstonia* sp. [2], α-haemolytic streptococci (in this study *Streptococcus salivarius*) [2,8] and *Stenotrophomonas maltophilia* [2,3]. Furthermore, many of the microorganisms detected in this study are known CF pathogens (indicated by * in Table 2). Some of the identified microorganisms may be contaminants from the skin, which are liberated on squamous epithelial cells and inhaled through the nose which will indiscriminately be detected by molecular methods. There seemed to be no clear correlation between frequency of detection in clone libraries, number of bacteria (based on qPCR measurements) and identification by culture. By performing qPCR, the presence of *P. aeruginosa*, *S. aureus* and *P. acnes* was confirmed in some but not all cases, and their relative abundance in the samples could be determined. Although only one sample (patient 11) contained sufficient amounts of *P. acnes* genes for reliable quantification, a low level amplification just below the quantification limits was observed in samples where bacterium was found in the clone libraries. The results indicated that *P. aeruginosa* and *S. aureus* were more abundant than the anaerobic *P. acnes*.

The finding of a larger microbial diversity by molecular methods compared to the findings by culture is common for this type of comparison. An explanation of this discrepancy is often that molecular methods cannot distinguish living and dead microorganisms, whereas culture only identifies living and dividing microorganisms. This should not be the case in this study, since the DNA extraction protocol was targeted DNA of intact microbial cells by inclusion of a MolYsis (Molzym, Bremen, Germany) pretreatment step that degrades human DNA as well as any naked bacterial DNA residing in the sample. Since qPCR on gDNA and cDNA gave comparable results for the four samples where RNA had been extracted, our results suggest that the DNA extraction protocol yielded DNA from active microorganisms.

It has been proposed that chronic sinus infections are due to formation of biofilm [33]. The bacteria in the sinuses can be locally faced with nutrient limitations i.a. reduced airflow, lower oxygen tension and anoxic conditions [5], which may facilitate mucoid phenotypes and biofilm formation abilities [12]. Bacterial biofilm represents a challenge for culture methods, which are primarily suitable for planktonic microorganisms. It is now recognized that this phenotype represents only a minor aspect of the life cycle of microorganisms involved in chronic diseases. It is therefore highly probable that culture methods underestimate bacteria present in disease [31,32]. Biofilms have now been implicated in many infectious diseases and it has been well established that these exhibit a heterogeneous distribution of microorganisms, with a trend of monospecies

aggregates even though the infection overall is multispecies [31]. This may account for the discrepancy between findings by the different methods in this study, since it is possible that the one sample used for molecular methods from each patient does not represent the entire microbial community. Also, since the results from culture were based on several samples it is possible that these samples contain microorganisms that were not present in the one sample used for molecular methods.

The clinical implication of the microbial flora found in the sinuses of CF patients is difficult to ascertain. Studies have been conducted where the isolates of normal sinus flora were identified [34], but to our knowledge no studies have compared the normal and CF sinus flora as determined by molecular methods. Such a comparison will be of interest to perform in future studies of the sinus flora in CF patients.

Study limitations

When obtaining samples from the sinuses, we cannot exclude that the samples were contaminated with flora from the air and anterior part of the nasal cavity, since the focus during the FESS operation was to avoid upper pharynx flora. This may account for presence of environmentally related microorganisms found using sensitive molecular methods. Further these microorganisms may not be included in the culture reports from the Department of Clinical Microbiology where only microorganisms known as potential human pathogens were reported.

Furthermore, we cannot be certain that the samples for culture and the sample for molecular methods contained exactly the same bacterial species, since the sample volume for culture was larger than for molecular methods. A future study should include investigations by molecular methods on multiple samples in order to enable investigation of a possible heterogeneous distribution of the microorganisms. Also, different sample types (nasal secretion, mucosal tissue, polyps etc.) should be segregated in order to access which sample types has the highest yield of microbial diversity and quantity of different species.

Conclusion

Of potentially great clinical importance is the detection of anaerobic bacteria, which were not cultured due to a discontinuation of anaerobic cultures for CF sinus samples. The anaerobic and facultative anaerobic bacteria may be relevant human pathogens, and calls into question the used diagnosis methods. Anaerobic and other microorganisms may reside in biofilms in the sinus, which could explain the lack of detection by culture. Another important finding involves *A. xylosoxidans*, a CF-related pathogen of great clinical relevance due to its ability to rapidly develop multi-drug resistance, spread from patient to patient and cause lung morbidity. Detection of the pathogen by culture and molecular methods gave discrepant findings and in two cases culture was not able to detect the bacterium (either due to lack of growth or misidentification). Such lacks of identification can have major implications for the patients e.g. delayed antibiotic therapy, decreased lung function and patient to patient spread. The findings in this study indicate that a combination of culture and molecular methods may improve diagnosis of sinus infections, since both methods have advantages and limitations. If bacteria are cultured, their antibiotic susceptibility can be easily investigated, but the bacterial flora may be underestimated. For molecular methods, the problem is that extraction of DNA/RNA may be insufficient. The ability of performing sensitive molecular-based detection of microorganisms has posed a great challenge of our understanding of pathogenesis of infection and the definitions of positive culture reports, which needs to be addressed before the full potential of the methods can be exploited.

Acknowledgements

We thank Susanne Bielidt, Masumeh Chavoshi, Katja Bloksted, Maria Kristin Bjørnsdottir and Pia Poss for their valuable technical assistance.

References

1. Gysin C, Alothman GA, Papsin BC (2000) Sinonasal disease in cystic fibrosis: Clinical characteristics, diagnosis, and management. Pediatr Pulmonol 30: 481–489.
2. Mainz JG, Naehrlich L, Schien M, Käding M, Schiller I, et al. (2009) Concordant genotype of upper and lower airways P aeruginosa and S aureus isolates in cystic fibrosis. Thorax 64: 535–540.
3. Muhlebach MS, Miller MB, Moore C, Wedd JP, Drake AF, et al. (2006) Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis? Pediatr Pulmonol 41: 445–451.
4. Roby BB, McNamara J, Finkelstein M, Sidman J (2008) Sinus surgery in Cystic Fibrosis patients: Comparison of sinus and lower airway cultures. Int J Pediatr Otorhinolaryngol 72: 1365–1369.
5. Aanaes K, Rickelt LF, Johansen HK, von Buchwald C, Pressler T, et al. (2011) Decreased mucosal oxygen tension in the maxillary sinuses in patients with cystic fibrosis. J Cyst Fibros 10: 114–120.
6. Rasmussen J, Aanaes K, Norling R, Nielsen KG, Johansen HK, et al. (2012) CT of the paranasal sinuses is not a valid indicator for sinus surgery in CF patients. J Cyst Fibros 11: 93–99.
7. Godoy JM, Godoy AN, Ribalta G, Largo I (2011) Bacterial pattern in chronic sinusitis and cystic fibrosis. Otolaryngol Head Neck Surg 145: 673 –676.
8. Shapiro ED, Milmoe GJ, Wald ER, Rodnan JB, Bowen A (1982) Bacteriology of the maxillary sinuses in patients with cystic fibrosis. J Infect Dis 146: 589 –593.
9. Robertson JM, Friedman EM, Rubin BK (2008) Nasal and sinus disease in cystic fibrosis. Paediatr Respir Rev 9: 213–219.
10. Dosanjh A, Lakhani S, Elashoff D, Chin C, Hsu V, et al. (2000) A comparison of microbiologic flora of the sinuses and airway among cystic fibrosis patients with maxillary antrostomies. Pediatr Transplant 4: 182–185.
11. Taylor L, Corey M, Matlow A, Sweezey NB, Ratjen F (2006) Comparison of throat swabs and nasopharyngeal suction specimens in non-sputum-producing patients with cystic fibrosis. Pediatr Pulmonol 41: 839–843
12. Hansen SK, Rau MH, Johansen HK, Ciofu O, Jelsbak L, et al. (2012) Evolution and diversification of Pseudomonas aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. ISME J 6: 31–45.
13. Johansen HK, Aanaes K, Pressler T, Nielsen KG, Fisker J, et al. (2012) Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response. J Cyst Fibros: In press.
14. Holzmann D, Speich R, Kaufmann T, Laube I, Russi EW, et al. (2004) Effects of sinus surgery in patients with cystic fibrosis after lung transplantation: a 10-year experience. Transplantation 77: 134–136.
15. Vital D, Hofer M, Boehler A, Holzmann D (2012) Posttransplant sinus surgery in lung transplant recipients with cystic fibrosis: a single institutional experience. Eur Arch Otorhinolaryngol: In press.
16. Ghegan MD, Wise SK, White DR, Flume PA, Bowman CM, et al. (2009) Cost-effective airway cultures in the cystic fibrosis patient. Am J Otolaryngol 30: 150–152.

17. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, et al. (2012) EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinology 50: 1–12.
18. Høiby N, Frederiksen B (2000) Microbiology of cystic fibrosis. In: Hodson ME, Geddes DM, editors. Cystic Fibrosis. Hodder Arnold Publishers. pp. 83–107.
19. Xu Y, Rudkjøbing VB, Simonsen O, Pedersen C, Lorenzen J, et al. (2012) Bacterial diversity in suspected prosthetic joint infections:An exploratory study using 16S rRNA gene analysis. FEMS Immunol Med Microbiol 65: 291–304.
20. Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. Appl Environ Microbiol 72: 5734–5741.
21. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, et al. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35: 7188 –7196.
22. Ludwig W, Strunk O, Westram R, Richter L, Meier H, et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32: 1363 –1371.
23. Stackebrandt E, Goebel BM (1994) Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. Int J Syst Bacteriol 44: 846–849.
24. Good IJ (1953) The Population Frequencies of Species and the Estimation of Population Parameters. Biometrika 40: 237–264.
25. Gueirard P, Weber C, Le Coustumier A, Guiso N (1995) Human Bordetella bronchiseptica infection related to contact with infected animals: persistence of bacteria in host. J Clin Microbiol 33: 2002–2006.
26. De Ley J, Segers P, Kersters K, Mannheim W, Lievens A (1986) Intra- and Intergeneric Similarities of the Bordetella Ribosomal Ribonucleic Acid Cistrons: Proposal for a New Family, Alcaligenaceae. Int J Syst Bacteriol 36: 405 –414.
27. Lutz M-F, Berthelot P, Fresard A, Cazorla C, Carricajo A, et al. (2005) Arthroplastic and osteosynthetic infections due to Propionibacterium acnes: a retrospective study of 52 cases, 1995-2002. Eur J Clin Microbiol Infect Dis 24: 739–744.
28. Vandenesch F, Lebeau C, Bes M, McDevitt D, Greenland T, et al. (1994) Coagulase deficiency in clinical isolates of Staphylococcus aureus involves both transcriptional and post-transcriptional defects. J Med Microbiol 40: 344 –349.
29. LaBaer J, Qiu Q, Anumanthan A, Mar W, Zuo D, et al. (2004) The Pseudomonas Aeruginosa PA01 Gene Collection. Genome Res 14: 2190–2200.
30. Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. J Microbiol Methods 55: 541–555.
31. Burmølle M, Thomsen TR, Fazli M, Dige I, Christensen L, et al. (2010) Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol 59: 324–336.

32. Ehrlich GD, Hu FZ, Shen K, Stoodley P, Post JC (2005) Bacterial Plurality as a General Mechanism Driving Persistence in Chronic Infections. Clin Orthop Relat Res 437: 20–24.
33. Leid JG, Cope EJ, Parmenter S, Shirtliff ME, Dowd S, et al. (2010) The Importance of Biofilms in Chronic Rhinosinusitis. In: Bjarnsholt T, Jensen PØ, Moser C, Høiby N, editors. Biofilm Infections. Springer. pp. 139–160.
34. Brook I (2011) Microbiology of Sinusitis. Proc Am Thorac Soc 8: 90–100.
35. Eishi Y, Suga M, Ishige I, Kobayashi D, Yamada T, et al. (2002) Quantitative Analysis of Mycobacterial and Propionibacterial DNA in Lymph Nodes of Japanese and European Patients with Sarcoidosis. J Clin Microbiol 40: 198–204.
36. Qin X, Emerson J, Stapp J, Stapp L, Abe P, et al. (2003) Use of real-time PCR with multiple targets to identify Pseudomonas aeruginosa and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. J Clin Microbiol 41: 4312–4317.
37. Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, et al. (2009) Identification of Pseudomonas aeruginosa by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. Diagn Microbiol Infect Dis 63: 127–131.
38. Francois P, Pittet D, Bento M, Pepey B, Vaudaux P, et al. (2003) Rapid Detection of Methicillin-Resistant Staphylococcus aureus Directly from Sterile or Nonsterile Clinical Samples by a New Molecular Assay. J Clin Microbiol 41: 254–260.

Figures

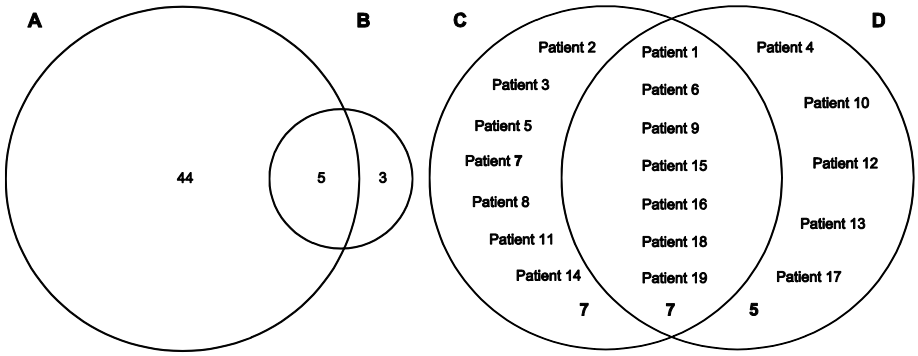


Figure 1: Venn diagrams of microbial findings. A: the number of microorganisms found only by molecular methods and B: the number of microorganisms found only by culture, five microorganisms could be found by both types of methods. C: The patients, where all the cultured microorganisms could be found by molecular methods, and D: patients, where none of the cultured microorganisms could be found by molecular methods. For seven patients some, but not all, of the cultured microorganisms could be confirmed by molecular methods.

Tables

Table 1: Assays used for qPCR in this study.

Target (gene)		**Sequence** (5'→3')	**Conc.** [nM]	**Ref.**
P. acnes (16S rRNA)	F	GCGTGAGTGACGGTAATGGG	500	[35] *
P. acnes (16S rRNA)	R	TTCCGACGCGATCAACCA	500	[35]
P. acnes (16S rRNA)	TM	*FAM*-CGCCCAATAAATCCGGACAACGCT-*BHQ*	200	[35]*
P. aeruginosa (*GyrB*)	F	CCTGACCATCCGTCGCCACAAC	250	[36]
P. aeruginosa (*GyrB*)	R	CGCAGCAGGATGCCGACGCC	250	[36]
P. aeruginosa (*GyrB*)	TM	*FAM*-CCGTGGTGGTAGACCTGTTCCCAGACC-*BHQ*	200	[37]
S. aureus (*FemA*)	F	TGCCTTTACAGATAGCATGCCA	1000	[38]
S. aureus (*FemA*)	R	AGTAAGTAAGCAAGCTGCAATGACC	500	[38]
S. aureus (*FemA*)	TM	*FAM*-TCATTTCACGCAAACTGTTGGCCACTATG-*BHQ*	200	[38]

Table 1 footnote: * the sequence has been modified from that in the reference by removing the last two nucleotides in F primer and using the reverse complimentary sequence for the TM probe.

Table 2: The microorganisms found in the sinuses by culture and 16S rRNA gene clone libraries analysis. For clarity reasons OTUs were only included if the a clone was found in more than one clone library, or if a clone were found more than two times in a single clone library. Identity of the clones was based on their closest relatives in the SILVA non-redundant SSU Ref database, release 106. For each patient the number of clones belonging to an OTU is given, and detection of bacteria by culture is marked by C.

Identity		**Patient**																		
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Anaerobes																				
Actinomyces massiliensis	OTU-1													4						
Propionibacterium acnes	OTU-2	2		1							5	5	3	20			9	3	1	
Propionibacterium granulosum	OTU-3								2										5	
Facultative anaerobes																				
Brochothrix thermosphacta	OTU-4	7																		
Carnobacterium maltaromaticum	OTU-5	15																		
Corynebacterium appendicis	OTU-6	1													2					
Haemophilus influenzae *	OTU-7			11																
Serratia marcescens *	OTU-8	1		27, C																
Aerobes																				
Achromobacter xylosoxidans *	OTU-9						53, C						35	4	4, C	17, C	12, C			C
Aquabacterium citratiphilum	OTU-10											1						1		
Aspergillus sp. *											C									
Bacillus amyloliquefaciens	OTU-11	3																		
Bordetella bronchiseptica *													C							
Bradyrhizobium betae	OTU-12				3															
Corynebacterium simulans	OTU-13								29											
Cyanobacterium sp.	OTU-14				6						10	12	4		12	12	3	17	7	
Dyella sp. *	OTU-15				6															
Enterobacter aerogenes																			C	

Micrococcus luteus	OTU-16					3							3										
Petrobacter succinatimandens	OTU-17																	3					
Pseudomonas aeruginosa *	OTU-18	10, C	2, C			C			35, C	C				C		C				C	C	C	
Ralstonia solanacearum *	OTU-19				1	7							14	5	2		12	3			9		4
Staphylococcus aureus *	OTU-20		32, C				41			9	53, C										4		27
Staphylococcus sp. (coagulase negative)		C	C				C	C		C			C		C	C			C	C		C	C
Stenotrophomonas maltophilia *	OTU-21								3											2			
Streptococcus salivarius	OTU-22																			16		19	
Unknown metabolism																							
Uncultured *Acidobacterium* sp.	OTU-23					3											2						
Uncultured bacterium	OTU-24															5							
Uncultured bacterium	OTU-25					1											1	1					
Uncultured Bacteroidetes	OTU-26													3				2			3		
Uncultured *Lysinibacillus* sp.	OTU-27	4																					
Uncultured α-Proteobacterium	OTU-28																4						
Uncultured β-Proteobacterium	OTU-29												1			7							

Table 2 footnote: * known CF pathogens.

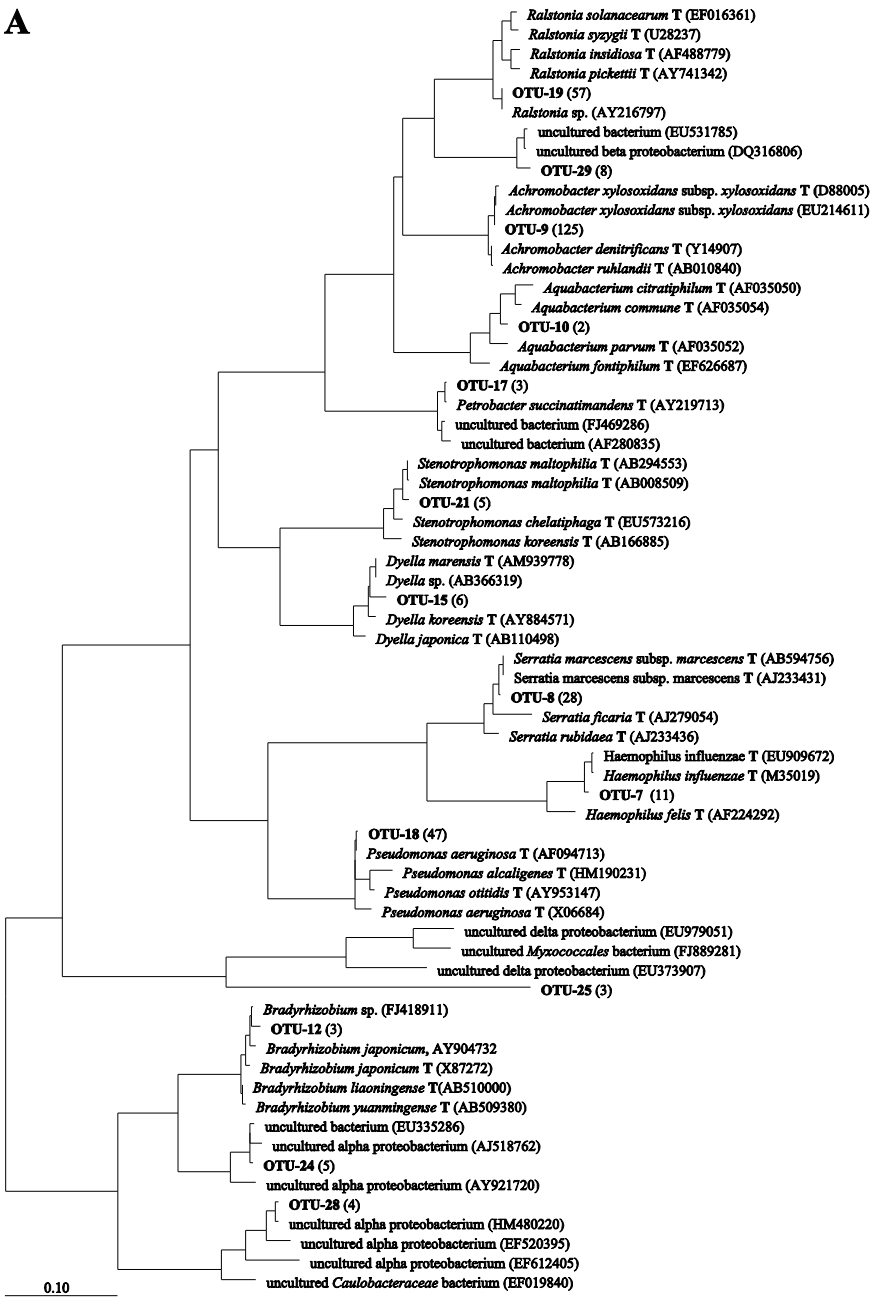
Table 3: Overview of patients with *P. acnes*, *P. aeruginosa* or staphylococci (both *S. aureus* and CNS). The microorganisms were found either by culture (marked C), in the clone libraries (marked M) or by qPCR (marked by number of CFU ± the standard deviation).

	Previous findings	CFU per gram sample
Patient 1	*P. acnes*^{M}	-
	P. aeruginosa$^{C, M}$	3.3*10^6 ±1%
	CNS^{C}	-
Patient 2	*P. aeruginosa*$^{C, M}$	1.3*10^6 ±6%
	S. aureus$^{C, M}$ and CNS^{C}	2.1*10^7 ±15%
Patient 3	*P. acnes*^{M}	-
Patient 4	*P. aeruginosa*^{C}	-
Patient 5	*S. aureus*^{M} and CNS^{C}	4.5*10^7 ±21%
Patient 6	CNS^{C}	-
Patient 7	*P. aeruginosa*$^{C, M}$	1.5*10^7 ±7%
Patient 8	*P. aeruginosa*^{C}	1.8*10^6 ±3%
	S. aureus^{M} and CNS^{C}	1.1*10^4 ±4%
Patient 9	*S. aureus*$^{C, M}$	1.1*10^9 ±16%
Patient 10	*P. acnes*^{M}	-
	CNS^{C}	-
Patient 11	*P. acnes*^{M}	3100 ±7%
	P. aeruginosa^{C}	3.6*10^4 ±6%
Patient 12	*P. acnes*^{M}	-
	CNS^{C}	-
Patient 13	*P. acnes*^{M}	-
	P. aeruginosa^{C}	-
	CNS^{C}	-
Patient 15	CNS^{C}	-
Patient 16	*P. acnes*^{M}	-
	P. aeruginosa^{C}	-
	CNS^{C}	-
Patient 17	*P. acnes*^{M}	-
	P. aeruginosa^{C}	-
	S. aureus^{M}	-
Patient 18	*P. acnes*^{M}	-
	P. aeruginosa^{C}	8.3*10^4 ±20%
	CNS^{C}	-
Patient 19	*S. aureus*^{M} and CNS^{C}	1.2*10^5 ±7%

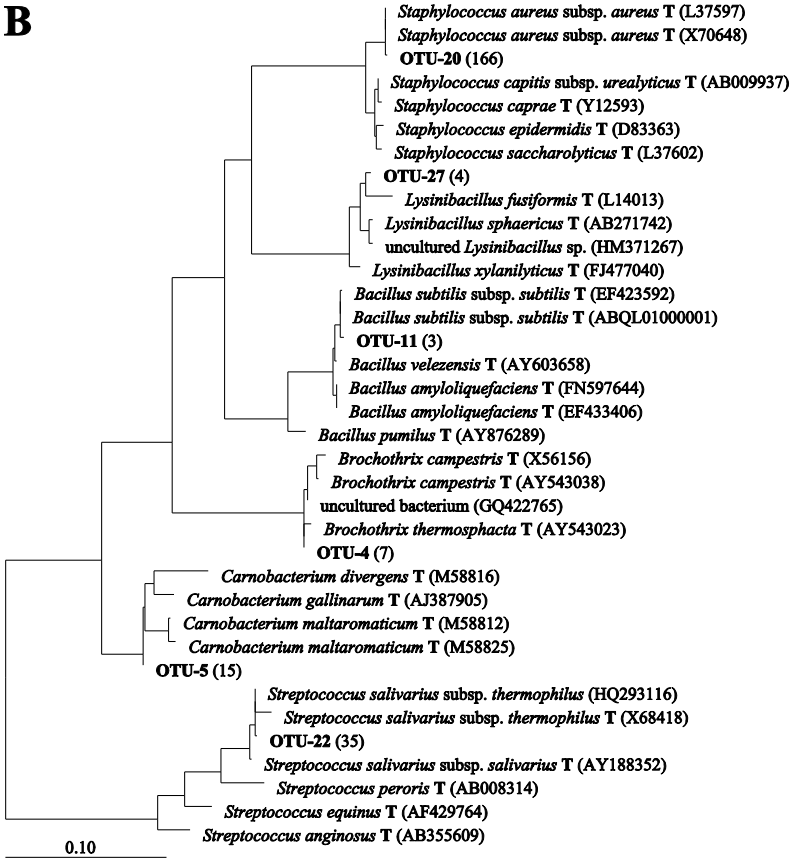
Table 3 footnote: - denotes a qPCR result below the detection limit.

Supporting information

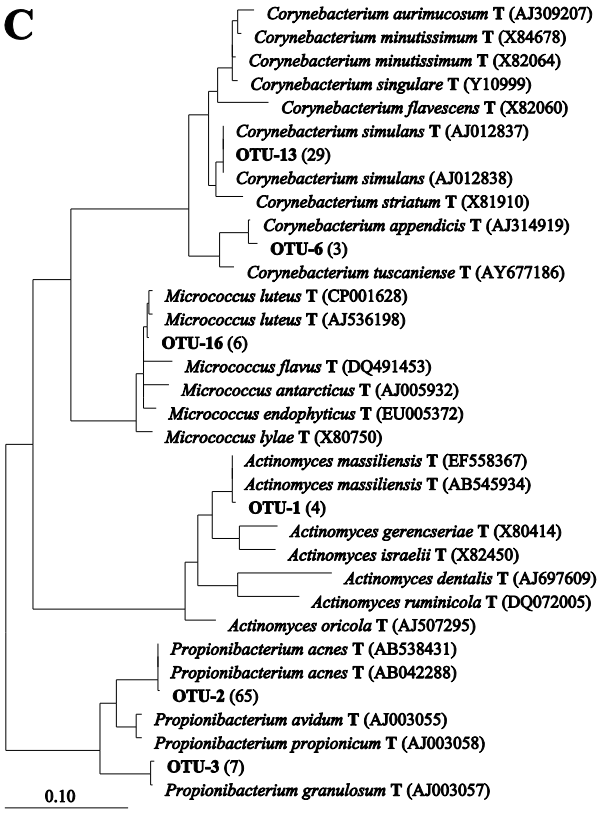
Figure S1: Maximum likelihood trees of the three major phyla found in sinuses of Danish CF patients. All trees were calculated with an outgroup consisting of 21 sequences from Thermotogae (not shown) and have a scale bar representing 10% estimated sequence deviation. The number in parenthesis indicates the number of clones belonging to the OUT and type strains are marked by **T**. In all 14 OTUs, corresponding to 307 clones, were assigned to Proteobacteria (A), 6 OTUs, corresponding to 230 clones, were assigned to Firmicutes (B) and 6 OTUs, corresponding to 114 clones, were assigned to Actinobacteria (C).


A
Ralstonia solanacearum T (EF016361)
Ralstonia syzygii T (U28237)
Ralstonia insidiosa T (AF488779)
Ralstonia pickettii T (AY741342)
OTU-19 (57)
Ralstonia sp. (AY216797)
uncultured bacterium (EU531785)
uncultured beta proteobacterium (DQ316806)
OTU-29 (8)
Achromobacter xylosoxidans subsp. xylosoxidans T (D88005)
Achromobacter xylosoxidans subsp. xylosoxidans (EU214611)
OTU-9 (125)
Achromobacter denitrificans T (Y14907)
Achromobacter ruhlandii T (AB010840)
Aquabacterium citratiphilum T (AF035050)
Aquabacterium commune T (AF035054)
OTU-10 (2)
Aquabacterium parvum T (AF035052)
Aquabacterium fontiphilum T (EF626687)
OTU-17 (3)
Petrobacter succinatimandens T (AY219713)
uncultured bacterium (FJ469286)
uncultured bacterium (AF280835)
Stenotrophomonas maltophilia T (AB294553)
Stenotrophomonas maltophilia T (AB008509)
OTU-21 (5)
Stenotrophomonas chelatiphaga T (EU573216)
Stenotrophomonas koreensis T (AB166885)
Dyella marensis T (AM939778)
Dyella sp. (AB366319)
OTU-15 (6)
Dyella koreensis T (AY884571)
Dyella japonica T (AB110498)
Serratia marcescens subsp. marcescens T (AB594756)
Serratia marcescens subsp. marcescens T (AJ233431)
OTU-8 (28)
Serratia ficaria T (AJ279054)
Serratia rubidaea T (AJ233436)
Haemophilus influenzae T (EU909672)
Haemophilus influenzae T (M35019)
OTU-7 (11)
Haemophilus felis T (AF224292)
OTU-18 (47)
Pseudomonas aeruginosa T (AF094713)
Pseudomonas alcaligenes T (HM190231)
Pseudomonas otitidis T (AY953147)
Pseudomonas aeruginosa T (X06684)
uncultured delta proteobacterium (EU979051)
uncultured Myxococcales bacterium (FJ889281)
uncultured delta proteobacterium (EU373907)
OTU-25 (3)
Bradyrhizobium sp. (FJ418911)
OTU-12 (3)
Bradyrhizobium japonicum, AY904732
Bradyrhizobium japonicum T (X87272)
Bradyrhizobium liaoningense T(AB510000)
Bradyrhizobium yuanmingense T (AB509380)
uncultured bacterium (EU335286)
uncultured alpha proteobacterium (AJ518762)
OTU-24 (5)
uncultured alpha proteobacterium (AY921720)
OTU-28 (4)
uncultured alpha proteobacterium (HM480220)
uncultured alpha proteobacterium (EF520395)
uncultured alpha proteobacterium (EF612405)
uncultured Caulobacteraceae bacterium (EF019840)
0.10

B


Staphylococcus aureus subsp. *aureus* **T** (L37597)
Staphylococcus aureus subsp. *aureus* **T** (X70648)
OTU-20 (166)
Staphylococcus capitis subsp. *urealyticus* **T** (AB009937)
Staphylococcus caprae **T** (Y12593)
Staphylococcus epidermidis **T** (D83363)
Staphylococcus saccharolyticus **T** (L37602)
OTU-27 (4)
Lysinibacillus fusiformis **T** (L14013)
Lysinibacillus sphaericus **T** (AB271742)
uncultured *Lysinibacillus* sp. (HM371267)
Lysinibacillus xylanilyticus **T** (FJ477040)
Bacillus subtilis subsp. *subtilis* **T** (EF423592)
Bacillus subtilis subsp. *subtilis* **T** (ABQL01000001)
OTU-11 (3)
Bacillus velezensis **T** (AY603658)
Bacillus amyloliquefaciens **T** (FN597644)
Bacillus amyloliquefaciens **T** (EF433406)
Bacillus pumilus **T** (AY876289)
Brochothrix campestris **T** (X56156)
Brochothrix campestris **T** (AY543038)
uncultured bacterium (GQ422765)
Brochothrix thermosphacta **T** (AY543023)
OTU-4 (7)
Carnobacterium divergens **T** (M58816)
Carnobacterium gallinarum **T** (AJ387905)
Carnobacterium maltaromaticum **T** (M58812)
Carnobacterium maltaromaticum **T** (M58825)
OTU-5 (15)
Streptococcus salivarius subsp. *thermophilus* (HQ293116)
Streptococcus salivarius subsp. *thermophilus* **T** (X68418)
OTU-22 (35)
Streptococcus salivarius subsp. *salivarius* **T** (AY188352)
Streptococcus peroris **T** (AB008314)
Streptococcus equinus **T** (AF429764)
Streptococcus anginosus **T** (AB355609)
0.10

C


Corynebacterium aurimucosum **T** (AJ309207)
Corynebacterium minutissimum **T** (X84678)
Corynebacterium minutissimum **T** (X82064)
Corynebacterium singulare **T** (Y10999)
Corynebacterium flavescens **T** (X82060)
Corynebacterium simulans **T** (AJ012837)
OTU-13 (29)
Corynebacterium simulans (AJ012838)
Corynebacterium striatum **T** (X81910)
Corynebacterium appendicis **T** (AJ314919)
OTU-6 (3)
Corynebacterium tuscaniense **T** (AY677186)
Micrococcus luteus **T** (CP001628)
Micrococcus luteus **T** (AJ536198)
OTU-16 (6)
Micrococcus flavus **T** (DQ491453)
Micrococcus antarcticus **T** (AJ005932)
Micrococcus endophyticus **T** (EU005372)
Micrococcus lylae **T** (X80750)
Actinomyces massiliensis **T** (EF558367)
Actinomyces massiliensis **T** (AB545934)
OTU-1 (4)
Actinomyces gerencseriae **T** (X80414)
Actinomyces israelii **T** (X82450)
Actinomyces dentalis **T** (AJ697609)
Actinomyces ruminicola **T** (DQ072005)
Actinomyces oricola **T** (AJ507295)
Propionibacterium acnes **T** (AB538431)
Propionibacterium acnes **T** (AB042288)
OTU-2 (65)
Propionibacterium avidum **T** (AJ003055)
Propionibacterium propionicum **T** (AJ003058)
OTU-3 (7)
Propionibacterium granulosum **T** (AJ003057)
0.10

Research paper 5

Thomsen, T.R., Aasholm, M.S., **Rudkjøbing, V.B.**, Saunders, A.M., Bjarnsholt, T., Givskov, M., Kirketerp-Møller, K., Nielsen, P.H. (2010). The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. *Wound Repair and Regeneration* 18, 38–49.

The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods

Trine R. Thomsen, PhD[1,2]; Martin S. Aasholm, MSc[1]; Vibeke B. Rudkjøbing, BSc[1]; Aaron M. Saunders, PhD[1,2]; Thomas Bjarnsholt, PhD[3]; Michael Givskov, Dr. Tech[3]; Klaus Kirketerp-Møller, MD[4]; Per H. Nielsen, PhD[1]

1. Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark,
2. The Danish Technological Institute, Chemistry and Water Technology, Århus, Denmark,
3. Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, and
4. Copenhagen Wound Healing Center, Bispebjerg Hospital, Bispebjerg, Denmark

Reprint requests:
Trine R. Thomsen, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark.
Tel: 45 7220 1828;
Fax: 45 9814 1808;
Email: trt@bio.aau.dk

Manuscript received: October 10, 2008
Accepted in final form: November 1, 2009

DOI:10.1111/j.1524-475X.2009.00561.x

ABSTRACT

The bacterial microbiota plays an important role in the prolonged healing of chronic venous leg ulcers. The present study compared the bacterial diversity within ulcer material from 14 skin graft operations of chronic venous leg ulcers using culture-based methods and molecular biological methods, such as 16S rRNA gene sequencing, fingerprinting, quantitative polymerase chain reaction, and fluorescence in situ hybridization. Each wound contained an average of 5.4 species but the actual species varied between wounds. The diversity determined by culture-based methods and the molecular biological methods was different. All the wounds contained *Staphylococcus aureus*, whereas *Pseudomonas aeruginosa* was in six out of 14 wounds. Molecular methods detected anaerobic pathogens in four ulcers that were not detected with anaerobic culture methods. Quantitative polymerase chain reaction was used to compare the abundance of *S. aureus* and *P. aeruginosa* at different locations in the ulcers and their numbers varied greatly between samples taken at different locations in the same ulcer. This should be considered when ulcers are investigated in routine clinical care. The differences between the results obtained with culture-based and molecular-based approaches demonstrate that the use of one approach alone is not able to identify all of the bacteria present in the wounds.

Chronic venous leg ulcers (CVLU) are a debilitating and often painful disease that affects approximately 1% of the world's population.[1,2] Apart from the human consequences, the treatment of wounds is expensive; in Denmark alone, wound treatment has been estimated to cost approximately two billion Danish kroner per year (~US$360 million),[2] and in the United Kingdom, France, and Germany an estimated 1.5–2% of the annual healthcare budget.[3,4]

The conditions leading to a CVLU are not fully understood; however, the primary cause is most likely insufficient valvular function of the veins in the legs causing increased hydrostatic pressure leading to edema of the subcutaneous tissue, which predispose to ulceration. This is linked to old age, obesity, height of the person, hereditary increased risk, number of births (more births lead to increased risk) and occupations in which the person is mainly standing. By removing edema with compression therapy, most CVLU will heal, but a number of ulcers will not despite effective treatment. In these cases, a well-documented and effective treatment is surgical debridement and split skin transplant.[2] Other treatments like topical negative pressure therapy have been found useful. Maggot debridement therapy have also proved promising, which involves having larvae from the fly *Lucilia sericata* removing necrotic tissue and bacteria from the wound, and in this way aiding the wound healing process.[5]

One of the factors affecting the effectiveness of wound healing therapies is the specific microorganisms that colonize the CVLU.[6] For example, the presence of *Pseudomonas aeruginosa* can retard the healing of wounds due to their ability to form biofilms.[6] Many studies describe biofilm as an important factor for the chronic behavior of chronic wounds,[6–10] and the spatial organization of these biofilms in a wound might be complex due to, for example, variations in environmental conditions and population composition.[11] Initial experiments by Bjarnsholt et al.[6] showed that *P. aeruginosa* in CVLU were assembled in microcolony-based structures unevenly distributed across the wound surface, and this uneven distribution might lead to insufficient sampling and wrong diagnosis.[6]

Until recently, the bacteria associated with CVLU have only been examined by culture-dependent methods by taking a swab or biopsy from the wound and using it as inoculate for various bacterial cultures. The emergence of molecular biology methods has illustrated that culture-dependent methods often underestimate the bacteria present, and especially ulcers with slow growing, fastidious, or anaerobic microbes.[9,12–14] Davies et al.[15] found that 40% of the organisms identified from CVLU by molecular biological methods could not be identified by culture-dependent methods, although most were species that are normally considered culturable.

The purpose of this study was to investigate the microbial diversity of chronic ulcers with molecular biological

Table 1. Summary of patient data*

Wound	Age of patient	Sex	Treatment of sample before extraction	Antibiotic treatment	Dressing at time of sampling	Duration of ulcer	Additional information
A	85	Male	DNA extracted from the entire wound	None	Nonsilver	12 months	
B	76	Male	DNA extracted from the entire wound	None	Nonsilver	6 months	Diabetic
C	54	Male	DNA extracted from the entire wound	None	Aquacell Ag	Years	
D	87	Female	DNA extracted from the entire wound	None	Nonsilver	4 months	
E	85	Female	Wound was cut into five parts and DNA extracted separately	None	Biatain AG	7 months	
F	71	Female	Wound was cut into five parts and DNA extracted separately	Sulfametizole due to urinary tract infect	Biatain AG	5 months	Diabetic
G	88	Female	DNA was extracted from six biopsies across the wound	None	Biatain AG	4 years	
H	82	Male	DNA was extracted from six biopsies across the wound	None	Nonsilver	6 months	Diabetic
I	81	Female	DNA was extracted from six biopsies across the wound	Phenoxymethylpenicillin until 2 months before sampling	Nonsilver	4 years	
J	78	Female	DNA was extracted from six biopsies across the wound	Phenoxymethyl-penicillin	Nonsilver	6 months	
K	65	Male	DNA was extracted from four biopsies across the wound	None	Nonsilver	6 months	Diabetic, impetigo
L	85	Female	DNA was extracted from four biopsies across the wound	None	Biatain AG	7 months	
M	69	Female	DNA was extracted from four biopsies across the wound	None	Nonsilver	6 months	
N	46	Male	DNA was extracted from four biopsies across the wound	None	Nonsilver	3 years	Sample from Achilles tendon
Average age	75.2						

*All DNA extractions were done using a DNeasy Blood and tissue kit except for the samples from wound F and wound E (center), which was extracted with an E.Z.N.A. Tissue DNA kit due to their greater size. Registered antibiotic treatment 3 months before sampling is mentioned.

methods and to compare these results with the conventional culture-dependent techniques. Furthermore, the spatial organization of bacteria in CVLU was examined.

MATERIALS AND METHODS

Patient population, sampling, and DNA extraction

The excision of biopsies and swabs of the wounds for culture-dependent and -independent experiments was performed by Copenhagen Wound Healing Center, Bispebjerg Hospital (Copenhagen, Denmark). Samples were obtained from patients diagnosed with chronic venous leg wounds just before surgical debridement and split skin transplant. In total, chronic wounds from 14 patients were investigated (named as wound A–N). The patients' age, sex, antibiotic treatment, dressings at the time of sampling, and additional information are described in Table 1. Patients with wound B, F, H, and K were also diagnosed with diabetes mellitus.

All ulcers were chronic and nonhealing despite optimal wound care and compression therapy. The duration of the ulcers are shown in Table 1. The patients were not receiving antibiotic treatment during the three months before sampling with three exceptions: Patient F was receiving sulfonametizole at the time of sampling, and patients J and I had received phenoxymethylpenicillin up until 3 days and 2 months before sampling, respectively. Five of the patients' wounds had been dressed with a silver-releasing dressing in the period before sampling (patient C, E, F, G, and L). The samples were collected with the patients' acceptance and in accordance with the biomedical project protocol (KA-20051011) approved by the Danish scientific ethical board.

On the day of surgery, the area surrounding the ulcer was swabbed with chlorhexidine in 70% alcohol but the surface of the ulcer was not disturbed. The excised wound material from the patient was transferred to a sterile Greiner tube and stored at $-20\,^{\circ}C$ until DNA extraction.

Before DNA extraction, the frozen wounds were thawed and cut to smaller pieces using sterile disposable scalpels. The total DNA content of wound F and E was extracted using an E.Z.N.A Tissue DNA kit (Omega Bio-Tek, VWR, Herlev, Denmark). Other wounds were cut to smaller pieces and were extracted using a DNeasy Blood and Tissue kit from Qiagen (Hilden, Germany). Both kits are based on proteinase K digestion for 2–4 hours.

Culture analysis

Identification of bacteria from the wounds by culturing was performed by the Department of Clinical Microbiology, Hvidovre Hospital, according to their standard protocols. Tissue samples were transported in sterile containers and swabs were transported in Stuart medium. Anaerobe culturing was performed on anaerobe plates (Statens Serum Institute [SSI], Copenhagen, Denmark) in a CO_2 atmosphere at $37\,^{\circ}C$ for 2 and 4 days. Aerobe culturing was performed on horse-blood agar (SSI) and Blue plates (SSI) for 1 and 2 days, respectively.

16S rRNA gene amplification

The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase with primers targeting conserved domains. The primers were 8F[16] and 1390R[17] and the samples were amplified according to Thomsen et al.[18] Negative controls including water and PCR mix were included for every five samples and were always negative indicating that there was no contamination of the reagents. Stringent procedures were generally used to avoid contamination, e.g., by using a PCR cabinet with UV light and all DNA handling was carried out with aerosol filter pipette tips to avoid cross contamination.

Cloning, sequencing, and phylogenetic studies

The amplified 16S rRNA gene products were purified with a Qiaquick PCR purification kit (Qiagen), according to the manufacturer's instructions. Cloning was performed using a TOPO TA Cloning® kit (Invitrogen, Taastrup, Denmark) for sequencing. Plasmids were purified using a Fastplasmid mini kit (Eppendorf, Horsholm, Denmark) and purified plasmids were amplified using M13 primers to test for inserts with the correct length. The plasmids were sequenced by Macrogen Inc. (Seoul, South Korea) using the M13F primer. The closest relative of the clones were identified by performing a BLAST search of the sequences at http://www.ncbi.nlm.nih.gov/blast. At least one representative clone from each species was additionally sequenced using the M13R primer, in order to obtain consensus sequences covering the entire length of the DNA fragments. Checks for chimeric sequences were conducted using the program BELLEROPHON.[19]

The ARB software[20] was used for the alignment of imported sequences with the FastAligner tool, and alignments were subsequently refined manually and phylogenetic analysis was performed. Only unambiguously aligned sequences were used for the calculation of trees using distance matrix, parsimony, and maximum likelihood approaches using default settings in the ARB software. The *Bacteria* sequence conservation filter of the ssu_jan04_corr_opt ARB database [available at http://www.arb-home.de]) in addition was applied. Phylogenetic trees were initially constructed using the consensus sequences representing the different groups of bacteria. Subsequently, partial sequences were added to the existing consensus trees by the "add species to existing tree" function in the ARB software. Priorly, a filter was carried out to define which positions to be used in adding the partial sequences (data not shown). Generally, the results obtained by the NCBI Blast Search corresponded well to the phylogenetic identifications. The coverage ratio (C) for each of the clone libraries were calculated with $C = (1 - (n1 \cdot N^{-1})) \cdot 100\%$ where $n1$ is the number of operational taxonomic units (OTUs) containing only one sequence and N is the total number of clones analyzed.[21]

Denaturant gradient gel electrophoresis (DGGE) fingerprinting

Amplification of samples for DGGE was performed using primers 341F-GC[22] and 907R.[17] The PCR products were

run on 8% polyacrylamide gels containing denaturant gradients of 30–70%, in 1×TAE buffer at 100 V overnight using the D-GENE™ gel system (Biorad) and stained with SYBR Gold (Invitrogen). The most intensive DGGE bands were excised and prepared for sequencing. The excised bands were reamplified with PCR, and the PCR products were thereafter purified using a NucleoSpin Extract II Machery Nagel and sequenced commercially by Macrogen Inc.

Quantitative PCR (qPCR)

Pure culture DNA was extracted using a FastDNA® Spin Kit for Soil (MP Biomedicals, LLC, Illkirch, France), according to the manufacturer's instructions. qPCR targeting the *nuc* gene[23] and *oprL* gene[24] was used to measure the amount of *Staphylococcus aureus* and *P. aeruginosa*, respectively. For each determination, triplicates of 20 µL reactions were run with each containing: 12.5 µL Brilliant® II SYBR® Green qPCR Master mix (Stratagene, AH Diagnostics, Aarhus, Denmark), 25 µg BSA, 10 µM of each primer, and 0.75 µM reference dye and 5 µL of template or standard. Reactions were run on an Mx3005P (Stratagene) for 10 minutes at 95 °C, 40 cycles of 30 seconds at 95 °C, 30 seconds at 62 °C (*nuc*)/ 62 °C (*oprL*), 60 seconds at 72 °C and 15 seconds, and SYBR data capture at 80 °C (*nuc*)/ 82 °C (*oprL*). For *S. aureus,* the specific product was separated at 79 °C and for *P. aeruginosa* at 90 °C. The specificity of the PCR reactions performed for each run was confirmed by the melting curve analysis and gel electrophoresis. Standard curves were prepared from serial dilutions of *S. aureus* (DSM 6148) and *P. aeruginosa* (DSM 1253) genomic DNA (5×10^{6}–5×10^{1}) in AE buffer (Qiagen). The limit of detection was 100 gene copies per PCR.

Fluorescence in situ hybridization (FISH)

After removal from the patient, the tissue sample was transferred to 4% neutral formaldehyde buffer and embedded in paraffin wax, cut into 4-µm–thick slides, and stored at room temperature. Before the hybridization, the paraffin was removed by xylene. The slides were treated using a Histology FISH Accessory Kit from DAKO cytomation according to the protocol. Hybridization was performed by covering the slide with 20 µL of hybridization buffer containing 0.9 M NaCl, 0.02 M Tris/HCl, 0.01% SDS, and formamide, depending on the requirement of the probes and probe mix (5 ng/µL). The probes used were an EUB mix (EUB-338,[25] EUB II-338,[26] and EUB III-338[26]) targeting most *Bacteria*; BET42a with GAM42a competitor[27] targeting most *Betaproteobacteria*; a mix of LGC354b, LGC354A, and LGC354C[28] targeting the *Firmicutes*, and probe Sau[29] targeting *S. aureus*. For more information about the probes, consult probeBase.[30] Lastly, the slides where treated with Vectashield hardset mounting medium with DAPI (4′,6-diamidino-2-phenylindole). Unspecific binding was examined by applying Non-EUB probes on a slide as described above. This revealed sporadic nonspecific binding but only with little signal intensity, and hence it was possible to use probes to examine CVLU. PNA FISH was performed as described previously.[10]

Nucleotide accession numbers

GenBank accession numbers for 16S rRNA gene consensus sequences determined in this study are EU931393-EU931450.

RESULTS

Culture analysis

Culture analysis of the 14 wounds (A–N) showed the presence of more than one species in all but one of the wounds (Tables 2 and 3). Although a diversity of other bacteria were isolated, *S. aureus* was detected in 13 wounds, *P. aeruginosa* in six, *Klebsiella oxytoca* in three, and *Enterococcus* sp. in three wounds. No obligate anaerobic species were detected in any of the wounds.

DGGE fingerprinting

The results of DGGE fingerprinting are shown in Tables 2 and 3, indicated by an "S." DGGE detected *S. aureus* in all of the wounds except wound C, despite *S. aureus* being detected by the culture methods. Wound E and F showed the presence of additional uncultured bacteria. DGGE showed that the wounds also contained a variety of anaerobic bacteria with multiple findings of species such as *Finegoldia magna, Anaerococcus vaginalis, Peptoniphilus asaccharolyticus, Peptoniphlus harei,* and *Peptostreptococcus anaerobius,* often with several of these species in the same wound. *P. aeruginosa* was detected in only one wound with DGGE fingerprinting despite its detection in six wounds using the culture methods. An average of 3.2 species per wound were detected using DGGE fingerprinting and 3.0 species per wound were detected using culture methods. In combination, DGGE and culture identified 5.4 species per wound.

Clone library and sequence analysis

To elucidate the bacterial diversity in the samples, clone libraries were constructed where the amplified 16S rRNA genes were inserted into cloning vectors, thereby a separation of the different fragments and its subsequent sequencing were possible. The sequences from the two clone libraries (clone library 1 from wounds A–F and clone library 2 from wounds G–N) were divided into OTUs using a similarity level of $>97\%$. A total of 60 clones were sequenced for clone library 1 and 94 clones for library 2. Table 4 shows the name and accession number of the closest relative for each OTU as identified by the phylogenetic analysis.

Clone library 1 showed many *S. aureus* and some *Alcaligenes* sp., *Anaerococcus* sp., *Stenotrophomonas* sp., *Enterococcus faecalis,* and *P. aeruginosa*. Clone library 2 showed a large amount of *S. aureus* and *P. aeruginosa*. Almost all OTUs have a similarity of $>97\%$ with their closest relatives. Only OTU 9 (uncultured *Anaerococcus*) in clone library 1 and OTU 10 *Helcococcus kunzii* in clone library 2 had a smaller similarity than 97% indicating that these OTUs had a lower phylogenetic resolution. The coverage ratio for the clone library 1 was 87.7% and for clone library 2 was 93.5%.

Table 2. A condensed overview of the bacteria found in wound A–F[1]

Species	Clone lib. 1	A	B	C	D	E	F
Staphylococcus aureus	+	S, C, 220 ± 6%	S, ND	C, ND	S, C	S,C,*	S,C,*
Pseudomonas aeruginosa	+	ND	ND	C, ND		C,*	C,*
Staphylococcus sp.	+	S			S	S,C	S,C
Stenotrophomonas maltophilia	+			S			
Alcaligenes sp.	+			S			
Enterococcus sp.	+	C					
Enterococcus faecalis	+		C			S	
Actinobaclulum schaalii	+					S	
Helcococcus kunzii	+						S
Finegoldia magna	+					S	
Staphylococcus cohnnii			S				
Corynebacterium amycolatum			S				
Achromobacter xylosoxidans				S			
Unidentified Gram-negative rod		C					
Proteus sp.			C				
Morganella morganii			C				
Klebsiella oxytoca					C		
Enterobacter cloacae							C
Peptoniphilus sp.						S	
Uncultured *Clostridia*						S	
Uncultured *Clostridia*							S
Uncultured *Porphyromonas*						S	
Uncultured *Bacterium*							S

[1]Bacteria identified from wounds A–F using culture-based methods (C) and sequencing of DGGE bands (S). Quantitative PCR data are presented for *S. aureus* and *P. aeruginosa* (copies/ng DNA ± standard error of the mean, n=3).
*The spatial orientation of bacteria was examined in wound D, E, and F revealing a diverse microbiota in wound E and F. Data for these two wounds are described in Table 5. Sequences also found in Clone library 1 are indicated with "+".
ND, not detected.

The consensus sequences in clone library 1 and 2 were used to produce phylogenetic trees to determine the detailed phylogenetic relationship of the 16S rRNA gene of the clones. A neighbor joining tree, a maximum parsimony tree, and a maximum likelihood tree all showed congruent phylogenetic relationships, and the maximum likelihood tree is shown in Figure 1. The locations on the tree confirm the BLAST identification of the sequences. The sequences are distributed into five phyla: *Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria,* and *Actinobacteria*. Similar bacteria were identified in the two clone libraries, although clone library 1 did not detect any bacteria from the phylum *Fusobacteria* and clone library 2 did not detect any *Bacteriodetes*. The clone libraries were dominated by sequences related to *S. aureus* and *P. aeruginosa*, but also contained many sequences from *E. faecalis, Alcaligenes faecalis,* and *Stenotrophomonas maltophilia*.

All 110 partial 16S rRNA gene sequences obtained from DGGE were added to the consensus maximum likelihood tree (data not shown) to confirm the result of the BLAST search. While the BLAST result was confirmed for most of the sequences, the phylogenetic analysis showed that it was not possible to distinguish the sequences identified as different *Alcaligenes* and *Ahcromobacter* species and no *Peptoniphilus* could be differentiated to more than the genus level. It also showed that the DGGE fingerprinting sequences most related to *Fusobacterium equinum* according to the BLAST were located closer to *Finegoldia gonidiaformans* on the tree. *F. gonidiaformans* was also found in clone library 2.

Quantitative PCR

The abundance of *S. aureus* and *P. aeruginosa* was found to vary considerably between the different wounds (Tables 2 and 3). While *S. aureus* could be detected by DGGE and by culturing in most samples, they were only above the limit of detection using the qPCR approach in four of the 14 ulcers investigated. *P. aeruginosa* could be quantified in three of the ulcers investigated.

Spatial location

To determine whether the bacterial composition varied throughout the wound, three wounds (D–F) were each

Table 3. A condesed overview of the bacteria found in wounds G–N*

Species	Clone lib. 2	Wounds G	H	I	J	K	L	M	N
Staphylococcus aureus	+	S, C, ND	S, C, 120 ± 14%	S, C, 5600 ± 13%	S, C, NT	S, C, NT	S, NT	S, C, 100 ± 5%	C, ND
Pseudomonas aeruginosa	+	C, 1400 ± 18%	C, ND	ND	NT	S, C, NT	NT	ND	ND
Alcaligenes sp.	+		S						
Proteus mirabillis	+			C					
Alcaligenes faecalis	+			C					
Enterococcus sp.	+			C					
Coagulase negative staphylococci	+				C	C			C
Staphylococcus epidermidis						S			
Peptoniphilus harei		S							S
Finegoldia magna		S					S		S
Fusobacerium equinum		S							
Peptostreptococcus anaerobius		S							
Peptoniphilus asaccharolyticus			S	S					S
Uncultured *Clostridia*				S					
Anaerococcus vaginalis							S	S	
Peptostreptococcus micros								S	
Corynebacterium sp.								S	C
Brevibacterium casei					S				
Gram-negative rod		C						C	
Morganella morganii		C							
Escherichia coli-like rod			C						
Hemolytic *Streptococcus*			C					C	
Klebsiella-like rod				C					
Klebsiella oxytoca				C					
Bacillus sp.					C				
Enterobacter cloacae								C	

*Bacteria identified from wounds G–N using culture-based methods (C) and sequencing of DGGE bands (S). Quantitative PCR data are presented for *S. aureus* and *P. aeruginosa* (copies/ng DNA ± standard error of the mean, n=3). Sequences also found in Clone library 2 are indicated with ''+''.
ND, not detected, NT, not tested.

divided in five parts and DNA was extracted from each of them. Each part was separately examined by DGGE fingerprinting and by subsequent sequencing of bands (Table 5). In wound D, only *S. aureus* could be detected by DGGE fingerprinting and it was present in all examined parts of the wound (data are not included in Table 5). Wound E was dominated by the aerobe *S. aureus*, the facultative aerobe *E. faecalis,* and the two anaerobes *Actinobaculum schaalii* and *F. magna,* and wound F was dominated by *S. aureus* and an uncultured *Clostridia* bacterium.

S. aureus and *P. aeruginosa* qPCR detected these species in all parts of wound E and F, except in subsample 3 in wound E (E3) (Table 5). The abundance of *S. aureus* and *P. aeruginosa* was, however, found to vary significantly depending on the location in the wound. This was particularly apparent for *P. aeruginosa,* which varied by three orders of magnitude in the various samples from wound F. Thus, not only the bacterial diversity but also the abundance of organisms were found to vary throughout the wound. To examine further the spatial organization of the CVLU, thin histological slides of wound H and another CVLU known to contain *P. aeruginosa* were produced and examined with FISH and PNA-FISH (Figure 2). It was found that the bacteria on the histological slides known to contain *P. aeruginosa* were located very locally (areas of

Table 4. Closest relatives of the bacterial OTUs in clone libraries

OTU	Number*	Species (BLAST)	Acc. number	Similarity (%)
Clone library 1				
1	[8/28]	*Staphylococcus aureus*	BX571856	97.1–100
2	[2/6]	*Alcaligenes* sp.	AY331576	99–100
3	[2/4]	*Anaerococcus* sp.	AM176522	99
4	[4/4]	*Stenotrophomonas* sp.	AM402950	99–100
5	[1/3]	Uncultured *Porphyromonas*	DQ130022	99–100
6	[2/3]	*Enterococcus faecalis*	DQ239694	99–100
7	[1/2]	*Pseudomonas aeruginosa*	EF064786	99–99.6
8	[1/1]	*Anaerococcus vaginalis*	AF542229	98
9	[0/1]	Uncultured *Anaerococcus*	DQ029049	95
10	[1/1]	*Enterobacter* sp.	EF088367	99
11	[1/1]	*Bacteroides tectus*	AB200228	99
12	[1/1]	*Actinobaculum schalli*	AF487680	98
13	[1/1]	*Helcococcus kunzii*	X69837	97
14	[1/1]	*Finegoldia magna*	AB109772	99
Total	57			
Clone library 2				
1	[7/46]	*Staphylococcus aureus*	DQ997837	98.8–99.9
2	[6/14]	*Pseudomonas* sp.	AY914070	98.7–99.0
3	[3/3]	Uncultured bacterium	EF511972	99.7–99.9
4	[1/3]	*Fusobacterium gonidoformans*	M58679	98.6–99.8
5	[2/2]	*Enterococcus faecalis*	DQ239694	99.8–100
6	[2/2]	*Acinetobacter junii*	AB101444	99.9
7	[1/2]	*Proteus mirabilis*	AF008582	98.6–99.8
8	[1/1]	*Actinobaculum schaalii*	AY957507	98.4
9	[1/1]	*Alcaligenes faecalis*	AY548384	97.2
10	[1/1]	*Helcococcus kunzii*	X69837	96.7
11	[1/1]	Uncultured bacterium	AM697030	98.2
12	[1/1]	Uncultured Clostridia	AY383733	99.7
Total	77			

approximately 150 μm) and nowhere else. This made it difficult to locate the area of infection if present. Wound H was examined to see if the bacteria found with DGGE fingerprinting could be located. It was possible to find small populations of *S. aureus* and *Alcaligenes* sp. using specific probes, thus confirming their presence but no large area of infection could be located.

DISCUSSION

There is an emerging body of evidence that bacteria play an important role in the persistence of chronic wounds. Using culture-based methods, the most frequently observed bacteria in CVLUs are *S. aureus*, *P. aeruginosa,* and *E. faecalis,* but the diversity is generally polymicrobial and heterogeneous.[31] To improve treatment of CVLUs, it is necessary to identify whether the most frequently detected bacteria are the critical causative agents or if other bacteria may also contribute to wound persistence. The choice of the analytical method, mode of sampling and the compositional variety of the wounds all play an important role in the results obtained from bacteriological studies. Some studies have been conducted to identify the important bacteria in wounds, however, the conclusions from the studies differ. Stephens et al.[8] focused on anaerobic bacteria and concluded that anaerobic bacteria play an important role in mediating the chronicity of CVLU. Gjodsbol et al.[32] in comparison suggested that *P. aeruginosa* is most important, rather than anaerobes, as it is *P. aeruginosa* that induces ulcer enlargement and delays healing. In the present study, it was examined how molecular methods could contribute to the characterization of the bacteria in CVLUs. As has been reported previously, the molecular biological methods uncovered a different and more diverse microbiota than the culture-based methods. Bacteria were detected that had not previously been identified from wounds but the potential virulence of these bacteria and their impacts on wound healing needs further investigation. Ultimately, the eventual significance of the different wound bacteria requires the determination of their pathogenesis and in order to do this, all of the bacteria that are present must be identified. The differences

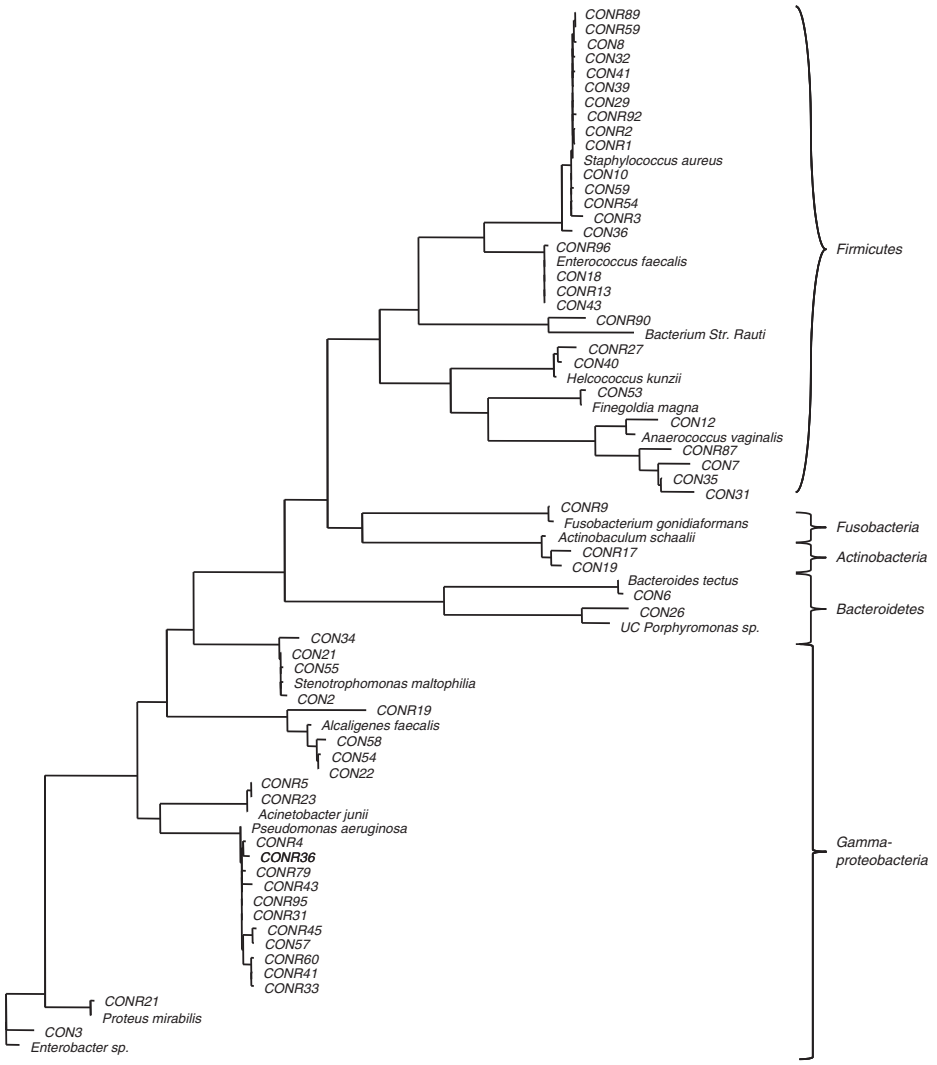


Figure 1. Maximum likelihood (AxML) tree of consensus sequences (1364 nt compared) of consensus sequences from clone library 1 (CON#) and 2 (CONR#). The scale bar represents a 10% deviation of sequence.

Table 5. A condensed overview of the spatial orientation of bacteria found in wounds E and F*

		Wound parts									
Species	Clone lib. 1	E, C	E, 3	E, 6	E, 9	E, 12	F, C	F, 3	F, 6	F, 9	F, 12
Pseudomonas aeruginosa		510 ± 18%	NT	760 ± 7%	47 ± 9%	280 ± 3%	920 ± 9%	300 ± 13%	8200 ± 8%	800 ± 10%	15 ± 5%
Staphylococcus aureus	+	S, 89 ± 11%	B, NT	B, 240 ± 10%	B, 310 ± 13%	S, 180 ± 8%	S, 200 ± 2%	S, 86 ± 8%	B, 290 ± 8%	B, 80 ± 5%	B, 93 ± 12%
Staphylococcus sp.	+	S	B	B	B	S	B	B	S	S	B
Enterococcus faecalis	+	S	S	S	S						
Enterococcus sp.			S							S	
Actinobaculum schaalii	+	S	B	B	B	B					
Helicococcus kunzii	+							S			
Finegoldia magna	+	B	B	B	B	S					
Peptoniphilus sp.				B		S					
Uncultured *Clostridia bacterium*						S					
Uncultured *Clostridia bacterium*							B	S		B	B
Uncultured *Porphyromonas* sp.		B		B		S					
Uncultured bacterium							S	B		B	

Besides wound E and F, clone library 1 represented wounds A–D.
*The spatial orientation of wounds E and F was examined by applying molecular methods on samples taken at the center (C), and at approximately 3, 6, 9, and 12 o'clock around the wounds' periphery. Bacteria were identified by sequencing DGGE bands (S) and putatively identified by comparison of bands to the sequenced bands at the same position on the gel (B). Sequences also found in Clone library 1 are indicated with ''+''. Quantitative PCR data are presented for *S. aureus* and *P. aeruginosa* (copies/ng DNA ± standard error of the mean, n=3). [I]NT, not tested; PCR, polymerase chain reaction.

between the results obtained with the culture-based and the molecular-based approaches demonstrate that the use of one of the methods alone might miss potentially important information about the bacteria present.

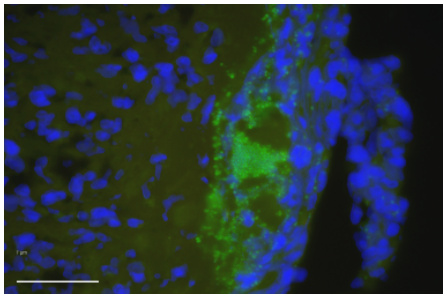


Figure 2. A PNA-FISH micrograph. The green color is a general probe for all bacteria and the picture was counter stained with DAPI, a DNA stain to visualize the localization of the host cells (blue).

Comparison of culture and molecular biological methods

All of the examined wounds contained a unique microbiota. The DGGE fingerprinting and culture method identified an average of 3.1 and 3.0 bacterial species per wound, respectively. Combined, 5.4 species were identified per wound. In accordance with previous reports, e.g.,[13] separate bands were observed in some lanes in the DGGE gels representing the same species. This may be due to more than one type of active 16S rRNA genes in the same species or the presence of different sub strains of the identified microorganisms differing in only one or a few base pairs. The presence of several species in the same wound complicates the task of determining which bacteria are mainly involved with infection. There might also be synergy between some species, e.g., predisposing or additive polymicrobial infections. For instance, species living in immunocompromised pockets created by different microorganisms are capable of killing leukocytes (like *P. aeruginosa*[6]).

The results of the culture experiments showed the presence of 12 different species in the analyzed wounds compared with 33 species found with molecular methods. None of the species found using culture methods were anaerobic. DGGE fingerprinting showed the presence of

anaerobic bacteria in wound G, H, I, M, and N. The anaerobic species are often overlooked by culture methods because they require longer culture times and previously lacked a valid identification scheme.[8] Many of the bacteria identified by DGGE fingerprinting have close relatives identified previously by culture experiments, and are therefore likely themselves to be culturable to some degree.

Some of the observed differences between the results obtained by the culture and the molecular methods could be due to the inability to differentiate species on the culture plates or that some specimens were collected as a biopsy and others using a swab. The differences may also be attributable to a fraction of the bacteria being dead or in a viable but unculturable state. This can be caused by the use of antibiotics[15]; however, only wound F was receiving antibiotics and indeed this wound showed the presence of only one species. The other two wounds, which had been treated with antibiotics until a short time before the study, both showed a diverse microbiota detected by culture methods. Based on these findings, there is no evidence of large amounts of residual genetic material from organisms no longer colonizing the ulcer bed.

Cultivation techniques have some limitations, but the molecular biological methods also have biases. These include amplification of naked DNA, unknown DNA recovery yields from extraction, differential amplification due to PCR primer bias, 16S rRNA copy number, and heterogeneity and co-migration of bands on DGGE fingerprinting. Some of the biases associated with, e.g., the DGGE approach were compensated by using the cloning approach, in which different primers were used with different specificities.

Diversity of CVLU bacteria

The clone library and DGGE analysis revealed a large diversity of bacteria of which some have not been associated previously with wounds: *Brevibacterium casei, Corynebacterium simulans*, *Corynebacterium amycolatum*. *A. schaalii, P. harei, F. gonidiaformans, Bacteroides tectus, Achromobacter xylosoxidans*, *A. faecalis,* and some uncultured bacteria. *B. casei* has been identified as an opportunistic pathogen in immunocompromised patients. The case reports by Reinert et al.[33] and Brazzola et al.[34] are examples, describing that *B. casei* needs a host with reduced immune system in order to initiate infection. Two other bacteria from phylum *Actinobacteria* (*C. simulans* and *C. amycolatum*) were also identified. The *Corynebacteria* are known as an aerobe and ubiquitous on human skin and are all opportunistic pathogens. *C. amycolatum* is frequently isolated from clinical specimens and infected wounds and it is resistant to most antibiotics[35] whereas *C. simulans* is a rare species found previously in blood and bile samples.[36] *A. schaalii* is a Gram-positive bacterium resembling normal skin flora and it is often overlooked by culture methods due to its slow growth in ambient air. Recently *A. schaalii* has been found as a pathogen in 10 cases of urinary infection.[37] *P. harei* belongs to the anaerobic Gram-positive family *Peptostreptococcaceae,* which is a heterogeneous family of opportunistic pathogens colonizing the skin and the mucosal surfaces of humans.[35] *H. kunzii* has been isolated previously from human skin and from diabetic foot wounds. It is mainly identified as a part of a polymicrobial community[38] but it has also been seen as the sole pathogen in a foot abscess.[39] The *Fusobacteria* are Gram-negative anaerobes found in the human gastrointestinal tract. Here, they are a part of the polymicrobial flora but they are also involved in a variety of different diseases.[40] The phylum *Fusobacterium* is often associated with chronic wounds.[41] *F. gonidiaformans* is a rare type of *Fusobacterium* species isolated previously from infected dog bites[42] and from skin infections.[43] In both surveys, the *F. gonidiaformans* constituted a very small percentage of the isolated bacteria. *A. xylosoxidans* and *A. faecalis* are both aerobe Gram-negative *Betaproteobacteria* from the *Alcaligenaceae* family. They are ubiquitous in the environment but rarely involved with human disease. They have been isolated from blood cultures of various immunosuppressed patients[44] and also appeared in a recent study of chronic wounds by Dowd et al.[12] The uncultured *Porphyromonas* (DQ130022) was identified previously from the forearm of a healthy human[45] and the uncultured bacterium (AY958901) was identified from the vaginal epithelium of a healthy woman.[46]

Phylogenetic analysis showed that the 33 different species belonged to six phyla. Both in terms of the number of different species and the number of identified clones, the *Proteobacteria* and the *Firmicutes* (Clostridia) were the dominating phyla. Gao et al.[45] examined the skin flora of healthy forearms in a large molecular biological study. They found that the dominating phylum was the *Actinobacteria,* although the *Firmicutes* and *Proteobacteria* were also present in high numbers. Healthy skin seems to be the only human environment where *Actinobacteria* are dominating.[45] In comparison, the inner mucosal surfaces of humans (e.g., colon and oral cavity) are dominated by *Firmicutes* and *Proteobacteria*.[45] This difference is probably due to environmental changes such as humidity and changes in pH value.

Eleven of the species were confirmed with both the cloning approach and DGGE fingerprinting. There was not a complete overlap between the findings of the two molecular methods and a reason for this might be that the DNA from the wounds was pooled before cloning on basis of the intensities of the bands on a gel. Another explanation might be that the primers used in the two methods had different affinity. Differences between the findings of the applied methods were also seen by Dowd et al.[12]

This study also indicated the presence of a varied anaerobic flora dominated by *F. magna* and *P. asaccharolyticus,* which were found in three wounds each. Table 3 (representing wound G, M, and N) also shows that the anaerobic species were often located in the same wound. This suggests that anaerobic pockets were present in the wound and that there is a possible synergistic effect between them. Stephens et al.[8] tested the effects of *P. vaginalis, F. magna,* and *P. asaccharolyticus* on cellular wound healing responses and found that they caused delayed reepitheliazation and defective extracellular matrix reorganization and angiogenesis in vitro. These are all important steps in wound healing. They also compared this with the effect of *P. aeruginosa* and found that this had less detrimental effect compared with the anaerobes.

Spatial orientation of bacteria in CVLU

The results from the DGGE approach investigating the spatial orientation of the bacteria in three wounds

illustrated that if only one biopsy from a wound was analyzed it would most likely not represent the bacterial composition of the entire wound. The qPCR results demonstrated that the abundance of *S. aureus* and *P. aeruginosa* also varied depending on the different locations in the wound. The technique is rapid and has recently been used to determinate *Pseudomonas* in a chronic wound within few hours, enabling fast decisions on treatment.[47] In addition, multiple biopsies from the same wound can also indicate which species of bacteria are most important for the infection as these are probably present in large numbers all over the wound. Furthermore, it supports the claim that the bacteria found in wounds are located in niches, which covers their needs. Using FISH, we detected bacteria in microcolonies also known as biofilms (Figure 2), which might explain how the bacteria survive inside the wound bed. This correlates with the finding that in some CVLU, *P. aeruginosa* live in large biofilms underneath the wound surface.[6] Antibacterial dressings, e.g., silver containing dressings are likely to influence the bacterial flora on the surface of the wounds. However, as the PNA-FISH pictures show that the bacteria reside deep in the tissue, it is not likely that bacteria will be influenced by the dressings. Furthermore, all swabs were taken after thoroughly surgical revision far away from local antimicrobial dressings. This indicates that the diversity was probably not influenced by the dressing, but by other factors such as antibiotics and difference in skin flora. The FISH technology increases the understanding of the pathology of bacteria in chronic wounds and how it might impact therapies.

This study compared the bacterial flora of different types of wound material from 14 skin graft operations of CVLU. Results from the culture methods were compared with the results from the molecular biological methods, which showed that the flora of the wounds varied, as did the number of *S. aureus* and *P. aeruginosa* investigated by qPCR. Each wound contained multiple species but apart from that the methods detected rather different floras. An average of 5.4 species were found in each wound by the methods combined. All of the wounds contained *S. aureus* but *P. aeruginosa* was also frequent. The molecular biological methods detected a varied anaerobic flora in four of the wounds and species not found previously in CVLU were identified. All of these were known pathogens. No anaerobes or new species were detected with culture methods. It was also found that the wound flora was different and that the number of the pathogens *S. aureus* and *P. aeruginosa* varied, depending on which location and depth of the wound was examined. Three wounds were examined and they showed that some species were present all over while some were only present in parts of the wounds. This emphasizes the need for multiple samplings when examining wounds, and swabs and biopsies each have specific advantages as sampling technologies.

qPCR is a promising fast method for fast characterization of the bacteria present in ulcers, and importantly the running cost is comparable with the cultivation techniques. The next important step is to elucidate the bacteria that contribute to the pathogenicity of these chronic wounds. This information could be used to develop the optimal sampling, identification, and treatment regimes.

ACKNOWLEDGMENTS

The Danish Technical Research Council supported this study under the innovation consortia ''BIOMED.'' We thank Jane Ildal, Aalborg University, for valuable technical assistance and Bo Jørgensen, Copenhagen Wound Healing Center, for collecting samples. The authors would also like to thank AdvanDx Inc., Woburn, MA, USA for supplying the PNA probes for the FISH experiments. TB received financial support from The Carlsberg Foundation and Lundbeck Foundation (the role of biofilms in chronic infections).

REFERENCES

1. Trent J, Falabella A, Eaglstein W, Kirsner R. Venous ulcers: pathophysiology and treatment options. *Ostomy Wound Manage* 2005; 51: 38–54.
2. Gottrup F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. *Am J Surg* 2004; 187 (5A): 38S–43S.
3. Laing W. *Chronic venous diseases of the leg*. London: Office of Health Economics, 1992.
4. Ruckley C. Socioeconomic impact of chronic venous insufficiency and leg ulcers. *Angiology* 1997; 48: 67–9.
5. Church JCT, Courtenay M. Maggot debridement therapy for chronic wounds. *Lower Extremity Wounds* 2002; 1: 129–34.
6. Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Rasmussen TB, Krogfelt K, Høiby N, Givskov M. Why chronic wounds won't heal: a novel hypothesis. *Wound Repair Regen* 2008; 16: 2–10.
7. Edwards R, Harding KG. Bacteria and wound healing. *Cur Op Infect Dis* 2004; 17: 91–6.
8. Stephens P, Wall I, Wilson M, Hill K, Davies C, Hill C, Harding K, Thomas D. Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro. *Br J Derm* 2003; 148: 456–466.
9. James G, Swogger E, Wolcott R, Pulcin iE, Secor P, Sestrich J, Costerton JW, Stewart PS. Biofilms in chronic wounds. *Wound Repair Regen* 2008; 16: 37–44.
10. Kirketerp-Moller K, Jensen P, Fazli M, Madsen K, Pedersen J, Moser C, Tolker-Nielsen T, Hoiby N, Givskov M, Bjarnsholt T. The distribution, organization and ecology of bacteria in chronic wounds. *J Clin Microbiol* 2008; 46: 2717–22.
11. Tolker-Nielsen T, Molin S. Spatial organization of microbial biofilm communities. *Microb Ecol* 2000; 40: 75–84.
12. Dowd S, Sun Y, Secor P, Rhoads D, Wolcott B, James G, Wolcott R. Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol* 2008; 8: 43.
13. Andersen A, Hill K, Stephens P, Thomas D, Jorgensen B, Krogfelt K. Bacterial profiling using skin grafting, standard culture and molecular bacteriological methods. *J Wound Care* 2007; 16: 171–5.
14. Hill KE, Davies CE, Wilson MJ, Stephens P, Harding KG, Thomas DW. Molecular analysis of the microflora in chronic venous leg ulceration. *J Med Microbiol* 2003; 52: 365–9.
15. Davies CE, Hill KE, Wilson MJ, Stephens P, Hill CM, Harding KG, Thomas DW. Use of 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis for analysis of

the microfloras of healing and nonhealing chronic venous leg ulcers. *J Clin Microbiol* 2004; 42: 3549–3557.
16. Juretschko S, Timmermann G, Schmid M, Schleifer K-H, Pommerening-Röser A, Koops HP, Wagner M. Combined molecular and conventional analysis of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrosospira*-like bacteria as dominant populations. *Appl Environ Microbiol* 1998; 64: 3042–3051.
17. Lane DJ *16/23S rRNA sequencing*. In: *Nucleic acid techniques in bacterial systematics*, Stackebrandt E, Goodfellow M, editors. Chichester: Wiley, 1991: 113–175.
18. Thomsen TR, Ramsing NB, Finster K. Biogeochemical and molecular signatures of anaerobic methane oxidation in a marine sediment. *Appl Environ Microbiol* 2001; 67: 1646–56.
19. Hugenholtz P, Huber T. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *Int J Syst Evol Microbiol* 2003; 53: 289–293.
20. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H. ARB: a software environment for sequence data. *Nucleic Acids Res* 2004; 32: 1363–71.
21. Juretschko S, Loy A, Lehner A, Wagner M. The microbial community composition of a nitrifying-dentrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *J Syst Appl Microbiol* 2002; 25: 84–99.
22. Muyzer G, De Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993; 59: 695–700.
23. Brakstad O, Aasbakk K, Maeland J. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of amplification of the nuc gene. *J Clin Microbiol* 1992; 30: 1654–60.
24. Jaffe R, Lane J, Bates C. Real-time identification of *Pseudomonas aeruginosa* direct from clinical samples. *J Clin Lab Anal* 2001; 15: 131–7.
25. Amann R, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 1995; 59: 143–69.
26. Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* 1999; 22: 434–44.
27. Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* 1992; 15: 593–600.
28. Meier H, Amann R, Ludwig W, Schleifer K-H. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. *Syst Appl Microbiol* 1999; 22: 186–96.
29. Kempf V, Trebesius K, Autenrieth I. Fluorescent In situ hybridization allows rapid identification of microorganisms in blood cultures. *J Clin Microbiol* 2000; 38: 830–8.
30. Loy A, Horn M, Wagner M. probeBase—an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Res* 2003; 31: 514–6.
31. Davies C, Wilson M, Hill K, Stephens P, Hill C, Harding K, Thomas D. Use of molecular techniques to study microbial diversity in the skin: chronic wounds reevaluated. *Wound Repair Regen* 2001; 9: 332–340.
32. Gjodsbol K, Christensen J, Karlsmark T, Jorgensen B, Klein B, Krogfelt K. Multiple bacterial species reside in chronic wounds: a longitudinal study. *Int Wound J* 2006; 3: 225–31.
33. Reinert RR, Schnitzler N, Haase G, Lütticken R, Fabry U, Schaal KP, Funke G. Recurrent Bacteremia due to *Brevibacterium casei* in an immunocompromised patient. *Eur J Clin Microbiol Infect Dis* 1995; 14: 1082–6.
34. Brazzola P, Zbinen R, Rudin C, Schaad UB, Heininger U. *Brevibacterium casei* sepsis in an 18-year-old female with AIDS. *J Clin Microbiol* 2000; 38: 3513–4.
35. Murray MAP, Rosenthal KS, Pfaller MA. *Med Microbiol*. 5th ed. Philadelphia, PA: Elsevier Mosby, 2005.
36. Bernard KA, Munro C, Wiebe D, Ongsansoy E. Characteristics of rare or recently described Corynebacterium species recovered from human clinical material in Canada. *J Clin Microbiol* 2002; 40: 4375–81.
37. Reinhard M, Prag J, Kemp M, Andreasen K, Klemmensen B, Højlyng N, Sørensen SH, Christensen JJ. Ten cases of *Actinobaculum schalii* infection: clinical relevance, bacterial identification, and antiobiotic susceptibility. *J Clin Microbiol* 2005; 43: 5306–8.
38. Haas J, Jernick L, Scardina R, Teruya J, Caliendo A, Ruoff K. Colonization of skin by *Helcococcus kunzii*. *J Clin Microbiol* 1997; 35: 2759–61.
39. Riegel P, Lepargneur J. Isolation of *Helcococcus kunzii* from a post-surgical foot abscess. *Int J Med Microbiol* 2003; 293: 437–9.
40. Johnson C, Wexler H, Becker S, Garcia M, Finegold S. Cell-wall-defective variants of *Fusobacterium*. *Antimicrobial Agents Chem* 1988; 33: 369–72.
41. Hernandez R. The use of systemic antibiotics in the treatment of chronic wounds. *Dermatologic Therapy* 2006; 19: 326–37.
42. Talan D, Citron J, Abrahamian F, Moran G, Goldstein E. Bacteriologic analysis of infected dog and cat bites. *N Eng J Med* 1999; 340: 85–92.
43. Goldstein EJC, Citron DM, Merriam CV, Warren Y, Tyrrell KL, Gesser RM. General microbiology and in vitro susceptibility of anaerobes isolated from complicated skin and kinstructure infections in patients enrolled in a comparative trial of ertapenem versus piperacillin-tazobactam. *Clin Infec Dis* 2002; 35 (Suppl.): S119–25.
44. Aisenberg G, Rolston K, Safdar A. Bacteremia caused by *Achromobacter* and *Alcaligenes* species in 46 patients with cancer (1989–2003). *Cancer* 2004; 101: 2134–40.
45. Gao Z, Tseng C, Pei Z, Blaser MJ. Molecular analysis of human forearm superficial skin bacterial biota. *Proc Natl Acad Soc* 2007; 104: 2927–2932.
46. Hyman RW, Fukushima M, Diamond L, Kumm J, Giudice LC, Davies RW. Microbes on the human vaginal epithelium. *Proc Natl Acad Soc* 2005; 102: 7952–7.
47. Wolcott R, Dowd S. A rapid molecular method for characterizing bacterial bioburden in chronic wounds. *J Wound Care* 2009; 17: 513–6.

Research paper 6

Xu, Y., **Rudkjøbing, V.B.**, Simonsen, O., Pedersen, C., Lorenzen, J., Schønheyder, H.C., Nielsen, P.H., Thomsen, T.R. (2012). Bacterial diversity in suspected prosthetic joint infections: An exploratory study using 16S rRNA gene analysis. *FEMS Immunology & Medical Microbiology* 65, 291–304.

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Bacterial diversity in suspected prosthetic joint infections: an exploratory study using 16S rRNA gene analysis

Yijuan Xu[1,2], Vibeke Børsholt Rudkjøbing[1], Ole Simonsen[3], Christian Pedersen[4], Jan Lorenzen[2], Henrik Carl Schønheyder[5], Per Halkjær Nielsen[1] & Trine Rolighed Thomsen[1,2]

[1]Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark; [2]Life Science Division, The Danish Technological Institute, Aarhus, Denmark; [3]Department of Orthopedic Surgery, Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark; [4]Department of Orthopedic Surgery, Viborg Hospital, Viborg, Denmark; and [5]Department of Clinical Microbiology, Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark

Correspondence: Trine Rolighed Thomsen, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark/The Danish Technological Institute (DTI), Life Science Division, Kongsvang Allé 29, DK-8000 Aarhus C, Denmark. Tel.: +45 72201828; fax: +45 96350558; e-mail: trt@bio.aau.dk

Received 15 September 2011; revised 20 February 2012; accepted 20 February 2012. Final version published online 22 March 2012.

DOI: 10.1111/j.1574-695X.2012.00949.x

Editor: Thomas Bjarnsholt

Keywords
prosthetic joint infection; biofilm; 16S rRNA gene; clone library; phylogeny; quantitative PCR.

Abstract

Formation of biofilm is a prominent feature of prosthetic joint infections (PJIs) and constitutes a challenge to current sampling procedures and culture practices. Molecular techniques have a potential for improving diagnosis of biofilm-adapted, slow-growing and non-culturable bacteria. In this exploratory study we investigated the bacterial diversity in specimens from 22 patients clinically suspected of having PJIs. Bacteriological cultures were performed according to standard practice. A total of 55 specimens from 25 procedures ('specimen sets') were submitted to broad range 16S rRNA gene PCR, cloning, sequencing and phylogenetic analysis. More than 40 bacterial taxa within six phyla were identified in 14 specimen sets originating from 11 patients. Direct observation of biofilm was made in selected specimens by fluorescence *in situ* hydridization. 16S rRNA gene analysis and bacteriological cultures were concordant for 15/25 specimen sets (60%; five positive, 10 negative); additional taxa were detected in four sets by gene analysis, and discrepant results were obtained for six sets, five of which were negative on culture. Polymicrobial communities were revealed in 9/14 sets by gene analysis and 1/10 sets by culture ($P < 0.05$). Although our study was not conclusive, these findings are consistent with a primary role of biofilm formation in PJIs.

Introduction

Joint replacement is one of the most common surgical procedures in industrialized countries. In Denmark the combined incidence of primary hip and knee arthroplasties was 280 per 100 000 inhabitants in 2008 (DHAR, 2011; DKAR, 2011). Revisions accounted for 40 additional operations per 100 000 inhabitants (DHAR, 2011; DKAR, 2011). The main causes for revisions are aseptic biomechanical failure and infection (Trampuz *et al.*, 2003). After primary arthroplasty the cumulative prevalence of infection is estimated to be 0.5–2% (Spangehl *et al.*, 1999; Zimmerli *et al.*, 2004; Kurtz *et al.*, 2008; Pulido *et al.*, 2008) and it is even higher after surgical revision (Trampuz & Zimmerli, 2008). The burden of morbidity and the economic costs associated with prosthetic joint infections (PJIs) are significant (Hebert *et al.*, 1996; Lavernia *et al.*, 2006). Both diagnosis and treatment of PJI remain complex, which can to a large extent be attributed to protected growth of bacteria in biofilms (Trampuz *et al.*, 2003; Trampuz & Widmer, 2006). The biofilm mode of growth renders bacteria resistant to the host immune system and most antimicrobial agents (Stewart & Costerton, 2001).

Culture techniques have been the mainstay for the diagnosis of PJIs, with synovial fluid and surgical periprosthetic soft tissue biopsies being the preferred specimen types (Bauer *et al.*, 2006). Nevertheless, culture-based methods often fail to demonstrate bacterial agents in patients with a high likelihood of PJI (Zimmerli *et al.*, 2004; Mikkelsen *et al.*, 2006; Berbari *et al.*, 2007; Trampuz *et al.*, 2007). This has called for reconsideration of

sampling and laboratory procedures. Biofilms on the surface of the prosthesis may be important because this niche can remain undetected when biopsies are taken from periprosthetic tissues or the synovial membrane (Gomez & Patel, 2011). Sonication has proved effective for dislodgement of biofilms from removed prostheses or prosthetic components (Trampuz *et al.*, 2007) but even with these precautions, biofilm bacteria may grow poorly on agar plates (if at all), and some bacteria may be viable but non-culturable (Zimmerli *et al.*, 2004; Costerton, 2005).

To overcome these limitations, culture-independent molecular methods have been introduced (Costerton, 2005; Fenollar *et al.*, 2006; Vandercam *et al.*, 2008). Still, the number of published PJI studies using molecular methods remains small. Complex bacterial communities are a hallmark of biofilm infections and in this study we have specifically addressed bacterial diversity in samples from patients suspected of PJI. Broad range 16S rRNA gene PCR, cloning, sequencing, phylogeny and quantitative PCR (qPCR) were applied to different types of specimens with the aim of helping to devise effective strategies for the diagnosis of PJI.

Methods

This exploratory non-interventional study was conducted within the framework of 'PRIS', a Danish multidisciplinary project on prosthesis-related infection and pain. The 'PRIS' project was approved by the regional research ethics committee for North Denmark (N-20110022).

Patients and sampling procedures

Specimens for bacterial DNA analysis were obtained in parallel with specimens for bacteriological culture in 22 patients with suspected PJI during a planned diagnostic procedure – a preoperative aspiration of synovial fluid ($n = 11$), a surgical revision ($n = 9$) or both ($n = 2$). Four patients had a hip prosthesis and 18 a knee prosthesis. Except for the surgeon's suspicion of infection, no fixed criteria were set for inclusion of patients.

Sampling was carried out once in 20 patients and three and two times in one patient each (nos 1 and 2, respectively). Both patients had a preoperative aspiration of synovial fluid and subsequent removal of the prosthesis within 10 days. Patient 1 had a previous specimen set obtained during debridement with retention of the prosthesis 7 months earlier. The median time (interquartile range) from implantation of the prosthesis to the diagnostic procedure was 4.5 months (1–12 months); if more than one procedure was performed, the first defined the insertion period.

Periprosthetic surgical biopsies (approximately 0.15 cm^3) were taken under sterile conditions with separate instruments and placed in sterile tubes (Greiner Bio-One, Germany); biopsies for culture were stored in Stuart transport medium (SSI Diagnostika, Denmark). Specimens from the surface of the prosthesis (approximately 2–5 cm^2) were obtained with a flocked swab placed in Amies transport medium (ESwab, Copan, Italy); the material was released from the swab and the medium subsequently analyzed. Prostheses or spacers removed during revision were placed in sterile containers of the appropriate size. All specimens were transported within a few hours to the laboratory at ambient temperature.

DNA extraction

Biopsies of soft tissue or spongious bone were cut into small pieces under sterile conditions. Removed prostheses or spacers were either sampled with an ESwab or submitted to sonication (42 kHz ± 6%, 10 min) in autoclaved MilliQ water. Subsequently, the sonication fluid was centrifuged (6000 ***g***, 10 min) and the pellet was resuspended in 1–5 mL of diethylpyrocarbonate (DEPC)-treated water. For one patient (no. 2B) both procedures were performed.

In two patients (nos 1B and 3) extraction of total DNA was performed with DNeasy® Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's protocol. For all other patients, bacterial DNA was extracted with MolYsis Basic (Molzym, Germany) followed by DNeasy® Blood & Tissue kit according to the manufacturers' protocols. Unlike the DNeasy® Blood & Tissue kit, which resulted in a mixture of eukaryotic and prokaryotic DNA, MolYsis Basic pretreatment enabled the selective preparation of prokaryotic DNA from intact cells, significantly lowering the background in PCR analyses. Before extraction with MolYsis Basic, 150 μL of DEPC-treated water were added to biopsies. Aliquots (200 μL) of synovial fluid, Amies transport medium and sonication fluid were processed directly. DNA was eluted in 200 μL of DEPC-treated water.

16S rRNA gene PCR amplification

The 16S rRNA gene was amplified in nearly full length using universal bacterial primers 5′-AGAGTTTGATCCT GGCTCA-3′ (26F) and 5′-GACGGGCGGTGTGTACAA-3′ (1390R) (Lane, 1991) according to Thomsen *et al.* (2001). The amplified DNA was subjected to agarose gel electrophoresis. Stringent procedures were employed to prevent contamination. Each reaction mixture excluding DNA template was prepared in a Biocap™ (Erlab, France) with UV light exposure for at least 10 min before each PCR setup. DNA templates were added to the reaction mixtures in a separate room, where post-PCR analysis was also carried out. Negative and positive controls were included within each batch of specimens. Positive controls

contained the standard reaction mixture with DNA extracted from an activated sludge sample, whereas negative controls contained DEPC-treated water instead of specimen.

Cloning and sequencing

After the 16S rRNA gene PCR products were confirmed to be of the correct size by agarose gel electrophoresis, the PCR products were purified with Nucleospin Extract II columns (Machery-Nagel, Germany) according to the manufacturer's instructions. The PCR fragments were then ligated into the pCT 4-TOPO-plasmid and transformed into One Shot® TOP10 chemically competent *Escherichia coli* as described in the TOPO TA Cloning® Kit for Sequencing (Invitrogen) protocol. The transformed cells were spread on Luria–Bertani agar containing 50 μg mL^{-1} kanamycin and 50 μg mL^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and incubated overnight at 37 °C.

From each clone library, 24–48 colonies were randomly selected and the plasmids were amplified using rolling circle amplification with IllustraTM TempliPhi Kit (GE Healthcare, UK) according to the manufacturer's instructions. Presence of an insert of the correct size was analyzed by PCR using M13 primers followed by agarose gel electrophoresis. The plasmids were sequenced by Macrogen Inc. (South Korea) in both directions using the M13 primers.

Phylogenetic analysis

A consensus sequence was compiled by assembling the forward and reverse sequences for each clone and trimming vector sequences in CLC Main Workbench (CLC bio, Denmark). Sequences were checked for chimeras using the MALLARD software package (Ashelford *et al.*, 2006). The BLASTN function was used for initial identification of the closest relatives of the consensus sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) with standard parameters except that 'Nucleotide collection' was the chosen database and 'Entrez Query' was limited to 'Bacteria [ORGN]'. Afterwards, the consensus sequences were aligned using SINA Web Aligner (Pruesse *et al.*, 2007) and imported into the ARB software package (Ludwig *et al.*, 2004) for taxonomic lineage assignment using the non-redundant SSU Ref database from SILVA Release 106 as reference database. The sequences were assigned based on their position after parsimony insertion into the database using a filter which was defined by applying the SAI sequence 'pos_var_ssuref: bacteria', using only sequences between *E. coli* nucleotides 27–1390, and omitting hypervariable portions of the rRNA gene. The consensus sequences and their closest relatives in the database were then selected to calculate phylogenetic trees using neighbor-joining, maximum parsimony and maximum likelihood methods.

Additionally, all clones having a 16S rRNA gene sequence similarity of more than 97% with each other were grouped into an operational taxonomic unit (OTU), roughly corresponding to the bacterial species level (Juretschko *et al.*, 2002). Only representative sequences from each OTU were selected to construct the phylogenetic trees. The coverage ratio (C) for each of the clone libraries was calculated using the equation $C_{coverage} = [1 - (N_{singletons} \cdot N_{total}^{-1})] \cdot 100\%$, where $N_{singletons}$ is the number of OTUs containing only one sequence and N_{total} is the total number of 16S rRNA gene clones analyzed (Juretschko *et al.*, 2002).

The non-redundant, near full-length 16S rRNA gene sequences representing each OTU obtained in this study were deposited in GenBank under the accession numbers JN584679–JN584724.

Quantitative PCR

Quantification of *Propionibacterium acnes* in specimens positive by the 16S rRNA gene clone library approach was done with qPCR according to Eishi *et al.* (2002). The target sequence was a 131-bp portion of the *P. acnes* 16S rRNA gene. The primers were PA-F (5′-GCGTGAGT GACGGTAATGGGTA-3′) and PA-R (5′-TTCCGACGC GATCAACCA-3′), and the TaqMan probe was PA-TAQ (5′-AGCGTTGTCCGGATTTATTGGGCG-3′). Triplicate 25 μL qPCR reactions were run containing 5 μL of a DNA specimen, 12.5 μL Brilliant® II QPCR Master Mix (Stratagene), 38 nM ROX (Stratagene), 1 μg μL^{-1} bovine serum albumin (Sigma, Germany), 100 nM of each primer and 40 nM of the probe (Eishi *et al.*, 2002). Reactions were run on an Mx3005P (Stratagene) with 5 min at 95 °C, 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The DNA standard was synthesized plasmid containing the 131-bp target gene (GenScript). The standard curve was prepared from serial dilution of the plasmid ($2 \cdot 10^0 \rightarrow 2 \cdot 10^7$ copies μL^{-1}). In all, 0–11 copies of the *P. acnes* target gene were detected in the controls without template, and the lower detection limit of the assay was therefore set to be 50 copies per reaction.

Fluorescence *in situ* hybridization (FISH)

Fluid samples (synovial fluid and Amies transport medium) were fixed in ethanol (50% v/v) for detection of Gram-positive bacteria (Roller *et al.*, 1994) and paraformaldehyde (40 g L^{-1}) for detection of Gram-negative bacteria (Amann *et al.*, 1990). The samples were analyzed by FISH using a universal bacterial peptide nucleic acid (PNA) probe according to the manufacturer's instructions (UNIBAC; AdvanDx, Inc., Woburn, MA). Visualization

was carried out with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

Bacterial culture

All bacteriological cultures were performed in the Department of Clinical Microbiology, Aalborg Hospital. Synovial fluid was centrifuged at approximately 1400 ***g*** and the pellet was used for Gram stain and inoculation. Aerobic culture was done on 5% horse blood agar and chocolate agar at 35 °C in 5% CO_2 (incubation period: 4 days); anaerobic culture was done on 10% horse blood agar for 4 days, chocolate agar enforced with menadione and cysteine for 6 days, and in semisolid thioglycollate agar for 4 days (all media were from SSI Diagnostika).

Tissue biopsies were cut into smaller pieces and imprints were made on the agar media listed above (for further details see Kamme & Lindberg, 1981). Incubation temperature and time were as described above. Interpretive criteria were in accordance with Kamme & Lindberg (1981). Culture from at least three biopsies of one or more phenotypically identical bacteria was deemed to be a significant finding; the number of colony forming units was not a criterion in itself, as enrichment culture was performed for each biopsy and contributed equally to the result. Identification to species level or a provisional group was done according to Murray *et al.* (2007). Coagulase-negative staphylococci and coryneform rods were identified with API Staph and API Coryne, respectively (bioMérieux, France). Hemolytic streptococci were grouped by agglutination for Lancefield antigens A, B, C and G. If a good identification was not obtained, provisional names were retained in the final report.

Data analysis

Any number of specimens obtained concurrently by either joint aspiration or surgical revision was defined as the unit of observation and was referred to as a 'specimen set' ($n = 25$).

Information on bacteriological cultures was retrieved from the laboratory information system after completion of molecular analyses whereby blinding was obtained *de facto*.

Differences in proportions were assessed by the Fisher exact test (2-tailed) with $P < 0.05$ deemed to be statistically significant.

Results

16S rRNA gene analysis

A total of 55 specimens were available for 16S rRNA gene analysis and PCR was positive for 25 specimens from 14 different sets and 11 patients (Table 1). Specimens of synovial fluid were positive in two patients and intraoperative specimens in 12. A clone library was constructed for each positive specimen, giving 25 clone libraries and 666 consensus sequences of high sequence quality. A total of 41 OTUs were formed based on 16S rRNA gene sequence similarity. Except for one bone specimen, all clone libraries had a coverage ratio above 85%, indicating that the majority of the microorganisms in the specimens were detected (for more details, see Supporting Information, Data S1).

The phylogenetic trees constructed from consensus sequences were robust, as congruent phylogenetic relationships were obtained by neighbor-joining, maximum parsimony and maximum likelihood methods. Sequences were distributed into six phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria* and *Fusobacteria*, with the majority of the sequences belonging to the first three phyla (Table 1). Maximum likelihood trees of *Proteobacteria*, *Firmicutes* and *Actinobacteria* are shown in Figs 1–3.

Table 1 shows that the most frequent species were *Staphylococcus epidermidis* and *P. acnes*, each were detected in six specimen sets. However, the majority of the identified species were detected only in a single patient. Multiple species were detected per specimen set in nine patients. Of note, in four specimen sets (patients 4, 6, 8 and 11) some species were present in all PCR-positive specimens, whereas other species were only detected in some specimens. The presence of *P. acnes* was confirmed by the specific Taqman qPCR assay in six of nine specimens and in four of six patients (Table 2). Both sonication and sampling by ESwab were applied to the prosthesis from patient 2B yielding the same species, namely *S. epidermidis*.

The polymicrobial communities comprised a broad range of bacteria, some of which have rarely been reported from clinical specimens, e.g. *Wautersiella falsenii*, *Dietzia cinnamea* and *Propioniferax innocua*. Among the OTUs there were 10 uncultured taxa, whose closest known relatives were determined by phylogenetic analysis (Figs 1–3).

Comparison of 16S rRNA gene analysis with culture reports

Results obtained by 16S rRNA gene analysis and conventional bacterial culture are summarized in Table 3. Results were concordant in 15 of the 25 specimen sets (five positive and 10 negative). In four cases the culture report was corroborated by 16S rRNA gene analysis; however, the analysis revealed multiple additional species. Results were discrepant for six specimen sets (gene analy-

Table 1. Overview of the positive 16S rRNA gene PCR and clone library results. All patients had knee prostheses except patient 8, who had a hip prosthesis. Three and two specimen sets were obtained from patients 1 and 2, respectively. Clones with a 16S rRNA gene sequence similarity of more than 97% were grouped into an OTU. For each patient, the number of clones belonging to an OTU is given with the sample origins indicated in different colours. Blue: bone biopsy; orange: periprosthetic biopsy; green: synovial fluid; magenta: prosthesis or spacer

			Aspiration of synovial fluid		Debridement with retention of prosthesis							Implantation of new prosthesis*		Removal of prosthesis		
			Patient no.		Patient no.							Patient no.		Patient no.		
Phylum	OTU	Species	1A	2A	1B	3	4	5	6	7	8	9	10	1C	2B	11
Proteobacteria	1	*E. coli*			1											
	2	*Pseudomonas* sp.									3					
	3	Uncultured *Rhodoferax* sp.														12
																5
	4	Uncultured *Curvibacter* sp.			1	4										
	5	Uncultured *Betaproteobacteria*														3
	6	*Alcaligenes faecalis/Achromobacter xylosoxidans* ssp. *xylosoxidans*					1									
	7	Uncultured *Burkholderia* sp.		2			2	1								
	8	*Betaproteobacteria*														4
	9	Uncultured *Methylobacillus* sp.										2				
	10	Uncultured *Neisseria* sp.					1									
	11	*Stenotrophomonas maltophilia*										2				
	12	*Sphingomonas* sp.					1					2				
							1									
Bacteroidetes	13	*Prevotella* sp.						2								
	14	Uncultured *Bergeyella* sp.									1					
	15	*Wautersiella falsenii*														1
	16	Uncultured *Bacteroidetes*														5
Cyanobacteria	17	Uncultured bacteria						3								
Fusobacteria	18	*Fusobacterium nucleatum*						1								
Actinobacteria	19	*Corynebacterium tuberculostearicum*			16							1				
		Corynebacterium accolens						1				3				
	20	*Corynebacterium aurimucosum*							2							
	21	*Corynebacterium amycolatum*							17							
									18							
	22	*Corynebacterium lipophilofavum*				1										
	23	*Corynebacterium pseudodiphtheriticum*						1								
	24	*Corynebacterium durum*						1				1				
	25	*Corynebacterium* sp.							7							
	26	*Dietzia cinnamea*				1										

Table 1. (continued)

Phylum	OTU	Species	Aspiration of synovial fluid		Debridement with retention of prosthesis							Implantation of new prosthesis*		Removal of prosthesis		
			Patient no.		Patient no.							Patient no.		Patient no.		
			1A	2A	1B	3	4	5	6	7	8	9	10	1C	2B	11
	27	*Propionibacterium acnes*				6	1	11				27	22			7
							5									7
							7									
	28	*Propionibacterium granulosum*					1									
	29	*Propionibacterium avidum*										3				
	30	*Propioniferax innocua*			1											
	31	*Micrococcus luteus*				6		2								
	32	*Rothia mucilaginosa*						1								
	33	*Rothia* sp.									1					
	34	*Kocuria* sp.			1											
Firmicutes	35	*Streptococcus sanguinis*					1									
		Streptococcus mitis									1					
	36	*Streptococcus salivarius*				3										
	37	*Streptococcus dysgalactiae* ssp.					13	13		45						
		equisimilis					26									
							2									
							8									
	38	*Streptococcus agalactiae*							5							
									9							
	39	Uncultured *Lactobacillus*		1												
	40	*Staphylococcus epidermidis*	25	37				2	10					27	51	
									1							
		Staphylococcus hominis										1				
		Staphylococcus caprae				1										
	41	*Staphylococcus aureus*			1						30					
					1						26					
											17					
											69					
Total no. of clones		666	25	40	22	22	70	39	69	45	148	42	22	27	51	44

*Implantation of new prosthesis. In this procedure the antibiotic-impregnated cement spacer was removed before a new prosthesis was inserted.

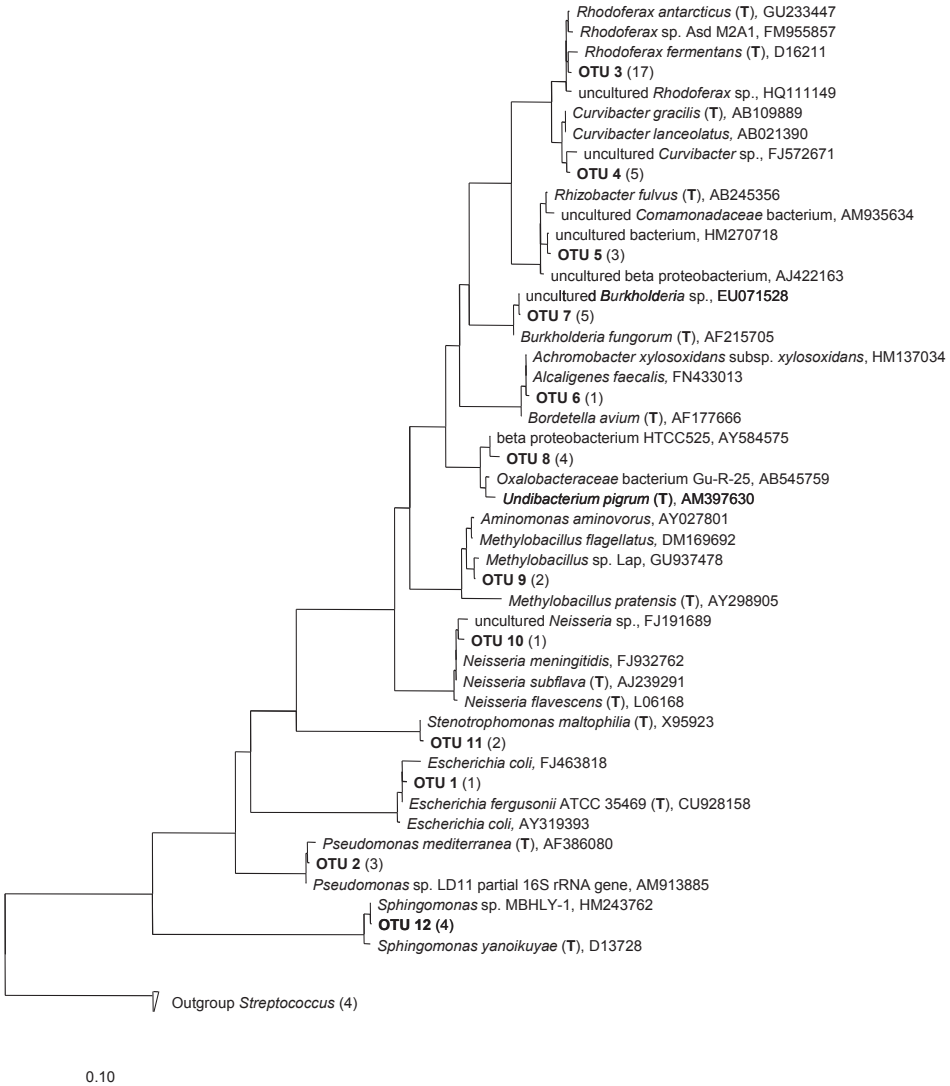


Fig. 1. Maximum likelihood tree of *Proteobacteria*. Twelve OTUs, corresponding to 48 clones (consensus sequences), were assigned to *Proteobacteria*. For simplicity, only representative sequences from each OTU were used in tree calculation. The outgroup consists of four sequences from streptococci. The scale bar represents 10% estimated sequence deviation. The number in parentheses indicates the number of clones belonging to the OTU. Type strains are marked by (T).

sis positive and culture negative for five and the reverse result for one).

In general, the culture reports fell short of the precise species diagnoses obtained by 16S rRNA gene analysis. Accordingly, only six species or provisional groups were identified by conventional phenotypic methods as compared with 45 species by gene analysis. Nonetheless, *S. epidermidis* was the most common species using either culture or the molecular approach.

The gene analysis revealed a mixed bacterial flora in more positive specimen sets (9/14; 64%) compared with conventional culture (1/10; 10%); the difference was statistically significant (Fisher exact test, 2-tailed, $P = 0.013$).

Findings in three patients pointed to a heterogeneous distribution of bacteria (Table 1). Thus, in patient 8, *Staphylococcus aureus* was cultured from periprosthetic biopsies and confirmed by molecular analysis. Nevertheless, three additional species were detected in the specimen from a prosthesis and from a bone biopsy. A mixed flora was found by 16S rRNA gene analysis in patients 1B and 5, in either Amies transport medium (ESwab from the prosthesis) or a bone biopsy, whereas a tissue biopsy was negative in both. Nonetheless, culture of periprosthetic biopsies revealed a single species in both cases.

Fluorescence *in situ* hybridization

PNA-FISH was performed with a universal bacterial probe on nine selected specimens that were 16S rRNA gene PCR-positive (from patients 4, 5, 8 and 9, respectively). Both single cells and microcolonies/biofilms were visualized. Figure 4 features a large microcolony of coccoid bacteria sampled with the flocked swab from the surface of the prosthesis (patient 8). The observation correlated with the finding of *S. aureus* by 16S rRNA gene analysis and culture.

Discussion

In this study of patients with suspected PJI, notably higher bacterial diversity was detected by broad range 16S

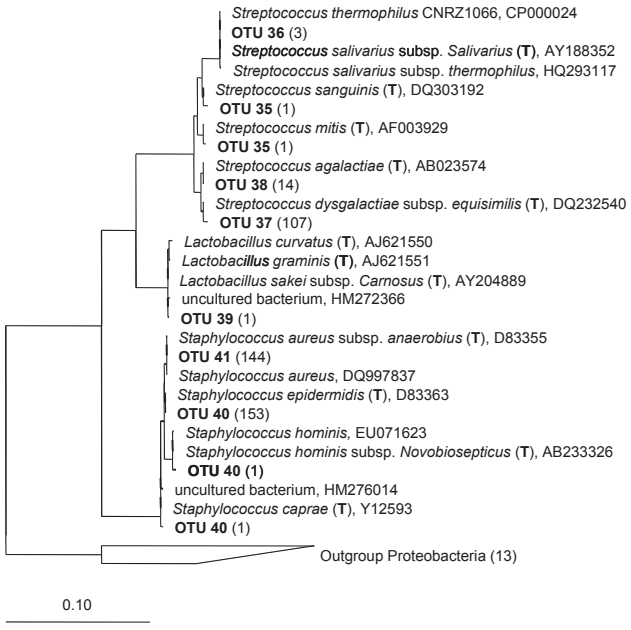


Fig. 2. Maximum likelihood tree of *Firmicutes*. Seven OTUs, corresponding to 426 clones (consensus sequences), were assigned to *Firmicutes*. For simplicity, only representative sequences from each OTU were used in tree calculation. The outgroup consists of 13 sequences from *Proteobacteria*. The scale bar represents 10% estimated sequence deviation. The number in parentheses indicates the number of clones belonging to the OTU. Type strains are marked by (T).

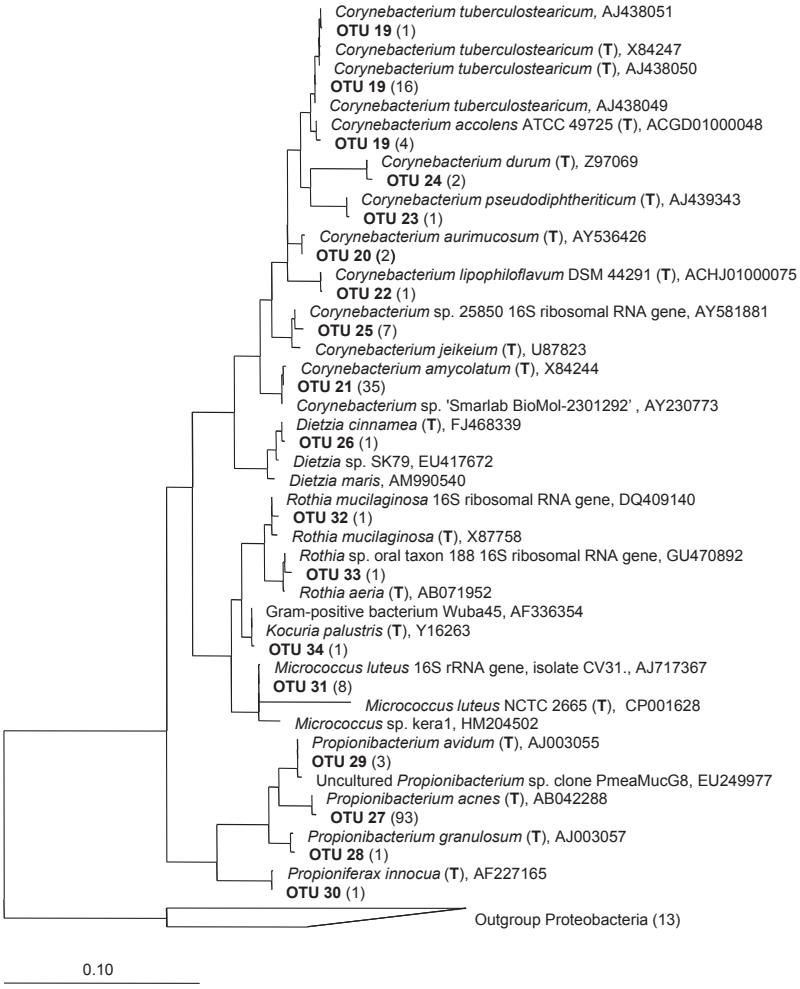


Fig. 3. Maximum likelihood tree of *Actinobacteria*. Sixteen OTUs, corresponding to 179 clones (consensus sequences), were assigned to *Actinobacteria*. For simplicity, only representative sequences from each OTU were used in tree calculation. The outgroup consists of 13 sequences from *Proteobacteria*. The scale bar represents 10% estimated sequence deviation. The number in parentheses indicates the number of clones belonging to the OTU. Type strains are marked by (T).

rRNA gene analysis than with conventional bacteriological culture. Still, there was a fair agreement between results obtained by culture and molecular analysis (Table 3). It is noteworthy that 10 sets of specimens concurred in being negative in both diagnostic setups.

Figures 1–3 highlight the many exotic bacteria detected in this study. The various *Proteobacteria* have an acknowledged environmental distribution and occur regularly in clinical specimens, although their clinical significance is often doubtful (Murray *et al.*, 2007). The flavobacterium

Table 2. Quantification of *Propionibacterium acnes* by Taqman qPCR in specimens found positive by the 16S rRNA gene clone library approach

Sample	Average ± STD (copies μL^{-1} DNA extract)
Patient 3	
Periprosthetic biopsy	–
Patient 4	
Bone	23 ± 3
Periprosthetic biopsy	34 ± 8
Flocked swab (prosthesis)	28 ± 6
Patient 5	
Flocked swab (prosthesis)	65 ± 22
Patient 9	
Periprosthetic biopsy	16 ± 4
Patient 10	
Sonication fluid (prosthesis)	111 ± 39
Patient 11	
Sonication fluid (prosthesis)	–
Bone	–

– indicates that *P. acnes* was not detected in the sample or the number was below the detection limit of the assay.

W. falsenii was first described in 2006 and multiple clinical isolates, including blood isolates, were included in the first publication (Kampfer *et al.*, 2006). The actinobacterial genus *Dietzia* is very similar to *Rhodococcus* and may be an emerging pathogen with a role in PJI (Pidoux *et al.*, 2001; Koerner *et al.*, 2009). *Propioniferax* (formerly *Propionibacterium*) *innocua* is a member of the skin flora in humans and has not yet, to our knowledge, been assigned a pathogenic role (Yokota *et al.*, 1994). It should not be precluded that exotic bacteria may have been a regular presence in clinical samples and have now become detectable with new techniques. Studies of intravenous catheters and wounds point in that direction (Larsen *et al.*, 2008; Thomsen *et al.*, 2010). A better understanding of the pathogenic potentials of these less described bacteria in a polymicrobial biofilm is essential for management of such infections, and currently different theories exist in the literature. Burmølle *et al.* (2010) suggested that the presence of a bacterium does not necessarily imply that it contributes to the pathogenesis of the infection, and requires treatment. However, different microorganisms may act synergistically in a polymicrobial infection (Brogden *et al.*, 2005) and some authors advocate that bacterial diversity in itself promotes the persistence of chronic infections (Ehrlich *et al.*, 2005) and increased pathogenicity, e.g. in wounds (Bowler, 2003). The total number of different bacterial species present, rather than some particular species, was found to correlate positively with impaired wound healing (Edwards & Harding, 2004). Further studies are warranted to determine the function, interaction and clinical implications of the exotic bacteria as well as the polymicrobial flora detected by 16S rRNA gene analysis. However, circumstances strongly suggest that they are part of a complex biofilm community that is not sampled and/or cultured properly with conventional methods. Most likely the specific growth requirements of these bacteria are not met by standard culture conditions and overgrowth by other pathogens may be an additional problem. It was not possible to assess the significance of each identified species in the current study. To fulfil that aim, systematic application of broad range molecular techniques is required in patients suspected of PJI.

PNA-FISH was applied to selected specimens to obtain visual support for the organization of bacteria into biofilms, but the current results should be regarded as preliminary. It was clear, however, that some bacteria were present in microcolonies or pieces of biofilms.

This study was conceived as an exploratory study and the molecular work-up of specimens was more extensive than would be practical for routine diagnosis. The use of clone libraries would probably be too cumbersome for clinical use but it was pivotal for the demonstration of bacterial diversity in this study. Even without a firm basis for clinical interpretation, our study provides useful guidance for handling of specimens from orthopedic implants. The use of the MolYsis DNA extraction kit made it safe to conclude that the preparations of DNA originated from intact and viable bacteria (Horz *et al.*, 2008; Handschur *et al.*, 2009). The first preparatory step comprised lysis of human cells while leaving bacterial cells unaffected, and the following DNase treatment degraded human DNA as well as DNA from dead microorganisms. This approach mitigates the impact of high amounts of human DNA and PCR inhibitors, which have previously been found to impede studies of, for example, synovial fluid (van der Heijden *et al.*, 1999). Moreover, the origin of DNA from viable bacteria should make the results of 16S rRNA gene analysis directly comparable with culture reports.

The intraoperative sampling from the metal surface of the prosthesis or spacer with a flocked swab was an option when the prosthesis was retained, but the procedure was also applicable in the molecular laboratory as an alternative to sonication. An experimental study with biofilm formed by Gram-positive bacteria on metal discs has previously shown that sampling by scraping is less effective compared with sonication (Bjerkan *et al.*, 2009). The flocked swab merits consideration especially for intraoperative use, because it is easy to handle and bacteria are eluted quantitatively to the medium (Van Horn *et al.*, 2008). However, sonication should be considered the best option for *in vitro* use (Bjerkan *et al.*, 2009).

In this study, *P. acnes* was detected in six patients by 16S rRNA gene analysis but was not isolated by culture

Table 3. Overview of results obtained by culture-based methods and 16S rRNA gene analysis

Patient no.	Culture	16S rRNA gene analysis
Concordance of positive results		
1A	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
1C	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
2B	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
6	Hemolytic *streptococcus* group B, coagulase-negative *staphylococcus*, coryneform rods	*Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Corynebacterium amycolatum*, *Corynebacterium aurimucosum*, *Corynebacterium* sp.
7	Hemolytic *streptococcus* group G	*Streptococcus dysgalactiae* ssp. *equisimilis*
Partial concordance of positive results		
1B	Coryneform rods	Uncultured *Curvibacter* sp., *Corynebacterium tuberculostearicum*, *Propioniferax innocua*, *Staphylococcus aureus*, *Kocuria* sp., *Escherichia coli*
2A	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*, uncultured *Burkholderia* sp., *Pseudomonas* sp., uncultured *Lactobacillus*
5	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*, *Micrococcus luteus*, *Streptococcus dysgalactiae* ssp. *equisimilis*, *Corynebacterium pseudodiphthericum*, *Corynebacterium accolens*, *Corynebacterium durum*, *Rothia mucilaginosa*, uncultured *Burkholderia* sp., uncultured *Cyanobacterium*, *Prevotella* sp., *Fusobacterium nucleatum*, *Propionibacterium acnes*
8	*Staphylococcus aureus*	*Staphylococcus aureus*, *Streptococcus mitis*, *Rothia* sp., *Pseudomonas* sp., uncultured *Bergeyella* sp.
Concordance of negative results		
12–21	Negative	Negative
Discordance of results: PCR positive and culture negative results		
3	Negative	*Staphylococcus caprae*, *Micrococcus luteus*, *Dietzia cinnamea*, *Corynebacterium lipophiloflavum*, uncultured *Curvibacter* sp., *Streptococcus salivarius*, *Propionibacterium acnes*
4	Negative	*Streptococcus dysgalactiae* ssp. *equisimilis*, *Streptococcus sanguinis*, *Sphingomonas* sp., uncultured *Burkholderia* sp., *Neisseria* sp., *Alcaligenes faecalis/Achromobacter xylosoxidans* ssp. *xylosoxidans*, *Propionibacterium acnes*, *Propionibacterium granulosum*
9	Negative	*Staphylococcus hominis*, *Corynebacterium accolens*, *Corynebacterium durum*, *Corynebacterium tuberculostearicum*, *Sphingomonas* sp., *Stenotrophomonas maltophilia*, uncultured *Methylobacillus* sp., *Propionibacterium acnes*, *Propionibacterium avidum*
10	Negative	*Propionibacterium acnes*
11	Negative	Uncultured *Rhodoferax* sp., *Wautersiella falsenii*, uncultured *Betaproteobacteria*, uncultured *Bacteroidetes*, *Propionibacterium acnes*
Discordance of results: PCR negative and culture positive results		
22	Coagulase-negative *staphylococcus*	Negative

from any of the specimen sets, which may be due to a relatively short incubation period for anaerobic media (4 and 6 days, respectively) (Lutz *et al.*, 2005). As the qPCR method can facilitate detection of pathogens within hours, the *P. acnes*-specific qPCR assay was chosen to test the feasibility of this method for PJI diagnosis. The discrepant results obtained for *P. acnes* with clone libraries and Taqman qPCR assay are most likely due to a lower sensitivity of the qPCR assay, but unfortunately contamination during broad range 16S rRNA gene PCR cannot be precluded.

Currently, there are few studies with broad range 16S rRNA gene analysis that allow a direct comparison with our results. Vandercam *et al.* (2008) analyzed biopsies, swabs or aspirates from 34 patients suspected of PJI and found one patient with a polymicrobial flora comprising two species. Fenollar *et al.* (2006) analyzed bone or joint specimens from 525 patients, 155 of whom had either a hip or knee prosthesis. A total of 121 specimens were positive by either PCR or culture. Although results were not analyzed separately for prosthetic implants, it is interesting that a subset of specimens had a polymicrobial flora (with two to eight bacteria). The bacterial spectrum was wide and included approximately 20 exotic bacteria, most of which were anaerobes.

There are a number of important limitations to our study. A number of potential sources for contamination with microbial DNA exist despite the precautions taken when handling and processing the clinical specimens. The number of patients was small and no fixed criteria were

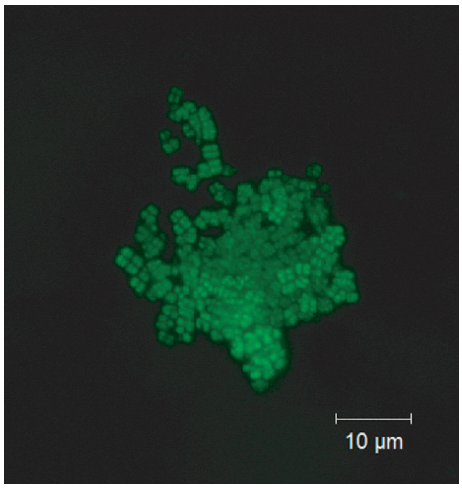


Fig. 4. A large microcolony of coccoid bacteria sampled with a flocked swab (ESwab) from the surface of the prosthesis. The sample was stained with a universal bacterial PNA-FISH probe (UniBac; AdvanDx). *Staphylococcus aureus* infection was confirmed by culture and 16S rRNA gene analysis in the patient (no. 8).

set for inclusion except the suspicion of PJI. Culture methods may not have been optimal with regard to duration of incubation of anaerobic media. Likewise, the phenotypic speciation of bacteria was not as precise as that obtainable by 16S rRNA gene analysis. The flocked swabs used intraoperatively were not submitted for culture because they were not part of the diagnostic routine. While each culture report for surgical biopsies was based on five specimens (Kamme & Lindberg, 1981; Mikkelsen *et al.*, 2006), most 16S rRNA gene analyses were carried out on one specimen per anatomic site. Even with the best precautions contamination can occur, and the finding of bacterial species that have not previously been associated with PJI should be interpreted with caution.

The inference concerning biofilm formation in the PJIs studied was indirect, and the visualization of bacteria by PNA-FISH and confocal microscopy was carried out with selected specimens only. These limitations not withstanding, our study strongly suggests that 16S rRNA gene analysis can detect a more diverse bacterial flora than conventional culture methods. However, 16S rRNA gene analysis combined with cloning as carried out this study is labor-intensive and time-consuming and therefore not applicable for routine diagnosis.

Considering these results, the location and composition of biofilms in PJIs should be addressed more directly in future studies. This can be done by new intraoperative sampling strategies and the use of newer and faster molecular techniques such as direct 16S rRNA gene sequencing combined with the use of the software RIPSEQ (Kommedal *et al.*, 2009) or the IBIS T5000 Biosensor System (Costerton *et al.*, 2011).

Acknowledgements

We are indebted to the orthopedic surgeons and nurses for their cooperation. We thank Susanne Bielidt and Masumeh Chavoshi for their valuable technical assistance, and Lone Heimann Larsen and Mette Mølvadgaard for their constructive criticism. The ESwab kits were provided by COPAN, Italy. This study partly fulfilled the requirements for an MSc degree for Yijuan Xu at Aalborg University. The study was supported by a grant for the PRIS Innovation Consortium from the Danish Agency of Science and Technology (no. 09-052174).

Authors' contributions

Y.X. and T.R.T. were responsible for the conception and design of the study. Y.X. carried out the molecular experiments. Y.X., V.B.R., T.R.T., O.S., C.P. and H.C.S. participated in the collection and assembly of data. Y.X., V.B.R., T.R.T., O.S., C.P., P.H.N. and H.C.S. contributed to data analysis and interpretation. Y.X. and H.C.S. prepared the first draft of the manuscript. All authors read and approved the final manuscript.

References

Amann RI, Krumholz L & Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* **172**: 762–770.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* **72**: 5734–5741.

Bauer TW, Parvizi J, Kobayashi N & Krebs V (2006) Diagnosis of periprosthetic infection. *J Bone Joint Surg Am* **88**: 869–882.

Berbari EF, Marculescu C, Sia I, Lahr BD, Hanssen AD, Steckelberg JM, Gullerud R & Osmon DR (2007) Culture-negative prosthetic joint infection. *Clin Infect Dis* **45**: 1113–1119.

Bjerkan G, Witsø E & Bergh K (2009) Sonication is superior to scraping for retrieval of bacteria in biofilm on titanium and steel surfaces *in vitro*. *Acta Orthop* **80**: 245–250.

Bowler PG (2003) The 10(5) bacterial growth guideline: reassessing its clinical relevance in wound healing. *Ostomy Wound Manage* **49**: 44–53.

Brogden KA, Guthmiller JM & Taylor CE (2005) Human polymicrobial infections. *Lancet* **365**: 253–255.

Burmølle M, Thomsen TR, Fazli M *et al.* (2010) Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol* **59**: 324–336.

Costerton JW (2005) Biofilm theory can guide the treatment of device-related orthopaedic infections. *Clin Orthop Relat Res* **437**: 7–11.

Costerton JW, Post JC, Ehrlich GD *et al.* (2011) New methods for the detection of orthopedic and other biofilm infections. *FEMS Immunol Med Microbiol* **61**: 133–140.

DHAR (2011) Danish Hip Arthroplasty Register. Annual report 2009. http://www.dhr.dk/ENGLISH.htm (accessed 28 April 2011).

DKAR (2011) Danish Knee Arthroplasty Register. Annual report 2009. www.dkar.dk/ (accessed 28 April 2011).

Edwards R & Harding KG (2004) Bacteria and wound healing. *Curr Opin Infect Dis* **17**: 91–96.

Ehrlich GD, Hu FZ, Shen K, Stoodley P & Post JC (2005) Bacterial plurality as a general mechanism driving persistence in chronic infections. *Clin Orthop Relat Res* **437**: 20–24.

Eishi Y, Suga M, Ishige I *et al.* (2002) Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J Clin Microbiol* **40**: 198–204.

Fenollar F, Roux V, Stein A, Drancourt M & Raoult D (2006) Analysis of 525 samples to determine the usefulness of PCR amplification and sequencing of the 16S rRNA gene for diagnosis of bone and joint infections. *J Clin Microbiol* **44**: 1018–1028.

Gomez E & Patel R (2011) Laboratory diagnosis of prosthetic joint infection, Part II. *Clin Microbiol Newsl* **33**: 63–70.

Handschur M, Karlic H, Hertel C, Pfeilstöcker M & Haslberger AG (2009) Preanalytic removal of human DNA eliminates false signals in general 16S rDNA PCR monitoring of bacterial pathogens in blood. *Comp Immunol Microbiol Infect Dis* **32**: 207–219.

Hebert CK, Williams RE, Levy RS & Barrack RL (1996) Cost of treating an infected total knee replacement. *Clin Orthop Relat Res* **331**: 140–145.

Horz HP, Scheer S, Huenger F, Vianna ME & Conrads G (2008) Selective isolation of bacterial DNA from human clinical specimens. *J Microbiol Methods* **72**: 98–102.

Juretschko S, Loy A, Lehner A & Wagner M (2002) The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Syst Appl Microbiol* **25**: 84–99.

Kamme C & Lindberg L (1981) Aerobic and anaerobic bacteria in deep infections after total hip arthroplasty: differential diagnosis between infectious and non-infectious loosening. *Clin Orthop Relat Res* **154**: 201–207.

Kampfer P, Avesani V, Janssens M, Charlier J, De Baere T & Vaneechoutte M (2006) Description of *Wautersiella falsenii* gen. nov., sp. nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter*. *Int J Syst Evol Microbiol* **56**: 2323–2329.

Koerner RJ, Goodfellow M & Jones AL (2009) The genus *Dietzia*: a new home for some known and emerging opportunist pathogens. *FEMS Immunol Med Microbiol* **55**: 296–305.

Kommedal O, Kvello K, Skjastad R, Langeland N & Wiker HG (2009) Direct 16S rRNA gene sequencing from clinical specimens, with special focus on polybacterial samples and interpretation of mixed DNA chromatograms. *J Clin Microbiol* **47**: 3562–3568.

Kurtz SM, Lau E, Schmier J, Ong KL, Zhao K & Parvizi J (2008) Infection burden for hip and knee arthroplasty in the United States. *J Arthroplasty* **23**: 984–991.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley and Sons, New York.

Larsen MKS, Thomsen TR, Moser C, Hoiby N & Nielsen PH (2008) Use of cultivation-dependent and -independent techniques to assess contamination of central venous catheters: a pilot study. *BMC Clin Pathol* **8**: 10.

Lavernia C, Lee DJ & Hernandez VH (2006) The increasing financial burden of knee revision surgery in the United States. *Clin Orthop Relat Res* **446**: 221–226.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Lutz M-F, Berthelot P, Fresard A, Cazorla C, Carricajo A, Vautrin A-C, Fessy M-H & Lucht F (2005) Arthroplastic and osteosynthetic infections due to *Propionibacterium acnes*: a retrospective study of 52 cases, 1995–2002. *Eur J Clin Microbiol Infect Dis* **24**: 739–744.

Mikkelsen DB, Pedersen C, Højbjerg T & Schønheyder HC (2006) Culture of multiple peroperative biopsies and diagnosis of infected knee arthroplasties. *APMIS* **114**: 449–452.

Murray PR, Baron EJ, Jorgensen JH, Landry ML & Pfaller MA eds (2007) *Manual of Clinical Microbiology*, 9th edn. American Society of Microbiology, Washington, DC.

Pidoux O, Argenson JN, Jacomo V & Drancourt M (2001) Molecular identification of a *Dietzia maris* hip prosthesis infection isolate. *J Clin Microbiol* **39**: 2634–2636.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Pulido L, Ghanem E, Joshi A, Purtill JJ & Parvizi J (2008) Periprosthetic joint infection: the incidence, timing, and predisposing factors. *Clin Orthop Relat Res* **466**: 1710–1715.

Roller C, Wagner M, Amann R, Ludwig W & Schleifer KH (1994) *In situ* probing of gram-positive bacteria with high DNA G + C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**(Pt 10): 2849–2858.

Spangehl MJ, Masri BA, O'Connell JX & Duncan CP (1999) Prospective analysis of preoperative and intraoperative

investigations for the diagnosis of infection at the sites of two hundred and two revision total hip arthroplasties. *J Bone Joint Surg Am* **81**: 672–683.
Stewart PS & Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358**: 135–138.
Thomsen TR, Finster K & Ramsing NB (2001) Biogeochemical and molecular signatures of anaerobic methane oxidation in a marine sediment. *Appl Environ Microbiol* **67**: 1646–1656.
Thomsen TR, Aasholm MS, Rudkjøbing VB, Saunders AM, Bjarnsholt T, Givskov M, Kirketerp-Møller K & Nielsen PH (2010) The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. *Wound Repair Regen* **18**: 38–49.
Trampuz A & Widmer AF (2006) Infections associated with orthopedic implants. *Curr Opin Infect Dis* **19**: 349–356.
Trampuz A & Zimmerli W (2008) Diagnosis and treatment of implant-associated septic arthritis and osteomyelitis. *Curr Infect Dis Rep* **10**: 394–403.
Trampuz A, Osmon DR, Hanssen AD, Steckelberg JM & Patel R (2003) Molecular and antibiofilm approaches to prosthetic joint infection. *Clin Orthop Relat Res* **414**: 69–88.
Trampuz A, Piper KE, Jacobson MJ *et al.* (2007) Sonication of removed hip and knee prostheses for diagnosis of infection. *N Engl J Med* **357**: 654–663.
van der Heijden IM, Wilbrink B, Vije AE, Schouls LM, Breedveld FC & Tak PP (1999) Detection of bacterial DNA in serial synovial samples obtained during antibiotic treatment from patients with septic arthritis. *Arthritis Rheum* **42**: 2198–2203.
Van Horn KG, Audette CD, Tucker KA & Sebeck D (2008) Comparison of 3 swab transport systems for direct release and recovery of aerobic and anaerobic bacteria. *Diagn Microbiol Infect Dis* **62**: 471–473.
Vandercam B, Jeumont S, Cornu O, Yombi J-C, Lecouvet F, Lefèvre P, Irenge LM & Gala J-L (2008) Amplification-based DNA analysis in the diagnosis of prosthetic joint infection. *J Mol Diagn* **10**: 537–543.
Yokota A, Tamura T, Takeuchi M, Weiss N & Stackebrandt E (1994) Transfer of *Propionibacterium innocuum* Pitcher and Collins 1991 to *Propioniferax* gen. nov. as *Propioniferax innocua* comb. nov. *Int J Syst Bacteriol* **44**: 579–582.
Zimmerli W, Trampuz A & Ochsner PE (2004) Prosthetic-joint infections. *N Engl J Med* **351**: 1645–1654.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Overview of coverage ratio of each done library.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Supporting file no 1.

Y. Xu et al.: Bacterial diversity in suspected prosthetic joint infections: an exploratory study using 16S rRNA gene analysis

Overview of coverage ratio of each clone library

The coverage ratio of each clone library (25 in total) is given in the table. The numbers are given with the number of analysed clones followed by coverage ratio in brackets. The 16S rRNA gene PCR negative samples are indicated as "-", while the samples unavailable for analysis are indicated by "N/A". Generally 24-48 clones were selected from each clone library. However, several clone libraries have less than 24 clones,. This was either due to a low sequence quality of the clones or fewer than 24 colonies formed in the clone library. For patient no. 1B, only 1 good sequence was obtained from synovial fluid, and the coverage ratio of the bone sample was only 76% based on 21 sequences. Attempts to make new clone libraries from these two samples failed due to difficulty in obtaining new 16S rRNA gene PCR products (samples from patient no. 1B were not extracted with MolYsis Basic). All the remaining clone libraries had coverage ratio above 85%, indicating that the majority of the microorganisms in the samples were detected.

Patient no.	Bone	Periprosthelic biopsy	Synovial fluid	Flocked swab (prosthesis)	Sonication fluid: Prosthesis or spacer	Flocked swab (spacer)
1A	N/A	N/A	25 (100%)	N/A	N/A	N/A
1B	21 (76%)	-	1 (100%)	-	N/A	N/A
1C	-	-	-	27 (100%)	N/A	N/A
2A	N/A	N/A	40 (98%)	N/A	N/A	N/A
2B	-	-	-	34 (100%)	17 (100%)	N/A
3	-	22 (91%)	-	-	N/A	-
4	29 (90%)	9 (89%)	13 (100%)	19 (89%)	N/A	N/A
5	-	-	-	39 (85%)	N/A	-
6	-	27 (96%)	-	N/A	42 (100%)	N/A
7	-	-	-	-	N/A	45(100%)
8	20 (85%)	26 (100%)	30 (100%)	33 (100%)	N/A	40 (100%)
9	-	42 (93%)	-	N/A	N/A	-
10	-	-	-	N/A	22 (100%)	N/A
11	31 (100%)	-	-	N/A	13 (92%)	N/A