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NEW METHODS FOR DETECTION, SUSCEPTIBILITY TESTING AND BIOFILM ERADICATION OF DIFFICULT-TO-DIAGNOSE AND DIFFICULT-TO-TREAT MICROORGANISMS

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NEW METHODS FOR DETECTION, SUSCEPTIBILITY TESTING AND BIOFILM ERADICATION OF DIFFICULT ORGANISMS

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Abbreviations

AMB	Amphotericin B
ANI	Anidulafungin
ATCC	American type culture collection
BA	Blood agar
BHI	Brain heart infusion
CAMBH	Cation-adjusted muller-hinton broth
CAS	Caspofungin
CEF	Ceftriaxone
CFU	Colony forming unit
CLI	Clindamycin
CLSI	Clinical laboratory standards institute
C_{max}	Maximum (peak) concentration
C_{min}	Minimum (through) concentration
DAP	Daptomycin
EPS	Extracellular polymeric substances
EUCAST	European committee on antimicrobial suceptibility testing
GC	Growth control
GEN	Gentamicin
His	Histidine
i.p.	Intraperitoneal
Ile	Isoleucine
Leu	Leucine
LEVO	Levofloxacin
MBC	Minimal bactericidal concentration
MBEC	Minimal biofilm eradication concentration
McF	McFarland
MEC	Minimal effective concentration
MHIC	Minimal heat inhibitory concentration
MIC	Minimal inhbitory concentration
NaCl	0.9% sodium chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEN G	Penicillin G
POS	Posaconazole
RIF	Rifampin
SD	Standard deviation
SDB	Sabouraud dextrose broth
SEM	Standard error mean
Ser	Serine
T_{max}	Time at which Cmax is achieved
TSB	Tryptic soy broth

Tyr	Tyrosine
Val	Valine
VAN	Vancomycin
VOR	Voriconazole

Summary

The diagnosis and treatment of infectious diseases are today increasingly challenged by the emergence of difficult-to-manage situations, such as infections associated with medical devices and invasive fungal infections, especially in immunocompromised patients. The aim of this thesis was to address these challenges by developing new strategies for eradication of biofilms of difficult-to-treat microorganisms (treatment, **part 1**) and investigating innovative methods for microbial detection and antimicrobial susceptibility testing (diagnosis, **part 2**).

The **first part** of the thesis investigates antimicrobial treatment strategies for infections caused by two less investigated microorganisms, *Enterococcus faecalis* and *Propionibacterium acnes*, which are important pathogens causing implant-associated infections. The treatment of implant-associated infections is difficult in general due to reduced susceptibility of bacteria when present in biofilms.

We demonstrated an excellent *in vitro* activity of gentamicin against *E. faecalis* in stationary growth-phase and were able to confirm the activity against "young" biofilms (3 hours) in an experimental foreign-body infection model (cure rate 50%). The addition of gentamicin improved the activity of daptomycin and vancomycin *in vitro*, as determined by time-kill curves and microcalorimetry. *In vivo*, the most efficient combination regimen was daptomycin plus gentamicin (cure rate 55%). Despite a short duration of infection, the cure rates were low, highlighting that enterococcal biofilms remain difficult to treat despite administration of newer antibiotics, such as daptomycin.

By establishing a novel *in vitro* assay for evaluation of anti-biofilm activity (microcalorimetry), we demonstrated that rifampin was the most active antimicrobial against *P. acnes* biofilms, followed by penicillin G, daptomycin and ceftriaxone. In animal studies we confirmed the anti-biofilm activity of rifampin (cure rate 36% when administered alone), as well as in combination with daptomycin (cure rate 63%), whereas in combination with vancomycin or levofloxacin it showed lower cure rates (46% and 25%, respectively). We further investigated the emergence of rifampin resistance in *P.* acnes *in vitro*. Rifampin resistance progressively emerged during exposure to rifampin, if the bacterial concentration was high (10^8 cfu/ml) with a mutation rate of 10^{-9}. In resistant isolates, five point mutations of the *rpoB* gene were found in cluster I and II, as previously described for staphylococci and other bacterial species.

The **second part** of the thesis describes a novel real-time method for evaluation of antifungals against molds, based on measurements of the growth-related heat production by isothermal microcalorimetry. Current methods for evaluation of antifungal agents against molds, have several limitations, especially when combinations of antifungals are investigated. We evaluated the activity of amphotericin B, triazoles (voriconazole, posaconazole) and echinocandins (caspofungin and anidulafungin) against *Aspergillus* spp. by microcalorimetry. The presence of amphotericin B or a triazole delayed the heat production in a concentration-dependent manner and the minimal heat inhibition concentration (MHIC) was determined as the lowest concentration inhibiting 50% of the heat produced at 48 h. Due to the different mechanism of action echinocandins, the MHIC for this antifungal class was determined as the lowest concentration lowering the heat-flow peak with 50%. Agreement within two 2-fold dilutions between MHIC and MIC or MEC (determined by CLSI M38A) was 90% for amphotericin B, 100% for voriconazole, 90% for posaconazole and 70% for caspofungin. We further evaluated our assay for antifungal susceptibility testing of non-*Aspergillus* molds. As determined by microcalorimetry, amphotericin B was the most active agent against Mucorales and *Fusarium* spp., whereas voriconazole was the most active agent against *Scedosporium* spp. Finally, we evaluated the activity of antifungal combinations against *Aspergillus* spp. Against *A. fumigatus*, an improved activity of amphotericin B and voriconazole was observed when combined with an echinocandin. Against *A. terreus*, an echinocandin showed a synergistic activity with amphotericin B, whereas in combination with voriconazole, no considerable improved activity was observed.

Resumé

Aujourd'hui, les problèmes des maladies infectieuses concernent l'émergence d'infections difficiles à traiter, telles que les infections associées aux implants et les infections fongiques invasives chez les patients immunodéprimés. L'objectif de cette thèse était de developper des stratégies pour l'éradication des biofilms bactériens (**partie 1**), ainsi que d'étudier des méthodes innovantes pour la détection microbienne, pour l'établissement de nouveaux tests de sensibilité (**partie 2**).

Le traitement des infections associées aux implants est difficile car les biofilms bactériens peuvent résister à des niveaux élevés d'antibiotiques. A ce jour, il n'y a pas de traitement optimal défini contre des infections causées par des bactéries de prévalence moindre telles que Enterococcus *faecalis* ou *Propionibacterium acnes.* Dans un premier temps, nous avons démontré une excellente activité *in vitro* de la gentamicine sur une souche de *E. faecalis* en phase stationnaire de croissance Nous avons ensuite confirmé l'activité de la gentamicine sur un biofilm précoce en modèle expérimental animal à corps étranger avec un taux de guérison de 50%. De plus, les courbes de bactéricidie ainsi que les résultats de calorimétrie ont prouvé que l'ajout de gentamicine améliorait l'activité *in vitro* de la daptomycine, ainsi que celle de la vancomycine. *In vivo*, le schéma thérapeutique le plus efficace était l'association daptomycine/gentamicine avec un taux de guérison de 55%.

En établissant une nouvelle méthode pour l'évaluation de l'activité des antimicrobiens vis-à-vis de micro-organismes en biofilm, nous avons démontré que le meilleur antibiotique actif sur les biofilms à *P. acnes* était la rifampicine, suivi par la penicilline G, la daptomycine et la ceftriaxone. Les études conduites en modèle expérimental animal ont confirmé l'activité de la rifampicine seule avec un taux de guérison 36%. Le meilleur schéma thérapeutique était au final l'association rifampicine/daptomycine avec un taux de guérison 63%. Les associations de rifampicine avec la vancomycine ou la levofloxacine présentaient des taux de guérisons respectivement de 46% et 25%.

Nous avons ensuite étudié l'émergence *in vitro* de la résistance à la rifampicine chez *P. ac*nes. Nous avons observé un taux de mutations de 10^{-9}. La caractérisation moléculaire de la résistance chez les mutant-résistants a mis en évidence l'implication de 5 mutations ponctuelles dans les domaines I et II du gène *rpoB*. Ce type de mutations a déjà été décrit au préalable chez d'autres espèces bactériennes, corroborant ainsi la validité de nos résultats.

La deuxième partie de cette thèse décrit une nouvelle méthode d'évaluation de l'efficacité des antifongiques basée sur des mesures de microcalorimétrie isotherme. En utilisant un microcalorimètre, la chaleur produite par la croissance microbienne peut être mesurée en temps réel, très précisément.

Nous avons évalué l'activité de l'amphotéricine B, des triazolés et des échinocandines sur différentes souches de *Aspergillus* spp. par microcalorimétrie. La présence d'amphotéricine B ou de triazole retardait la production de chaleur de manière concentration-dépendante. En revanche, pour les échinochandines, seule une diminution le pic de « flux de chaleur » a été observé. La concordance entre la concentration minimale inhibitrice de chaleur (CMIC) et la CMI ou CEM (définie par CLSI M38A), avec une marge de 2 dilutions, était de 90% pour l'amphotéricine B, 100% pour le voriconazole, 90% pour le pozoconazole et 70% pour la caspofongine. La méthode a été utilisée pour définir la sensibilité aux antifongiques pour d'autres types de champignons filamenteux. Par détermination microcalorimétrique, l'amphotéricine B s'est avéré être l'agent le plus actif contre les Mucorales et les *Fusarium* spp.. et le voriconazole le plus actif contre les *Scedosporium* spp. Finalement, nous avons évalué l'activité d'associations d'antifongiques vis-à-vis de *Aspergillus* spp. Une meilleure activité antifongique était retrouvée avec l'amphotéricine B ou le voriconazole lorsque ces derniers étaient associés aux échinocandines vis-à-vis de *A. fumigatus*. L'association échinocandine/amphotéricine B a démontré une activité antifongique synergique vis-à-vis de *A. terreus*, contrairement à l'association échinocandine/voriconazole qui ne démontrait aucune amélioration significative de l'activité antifongique.

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PART I.

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Chapter 1. General introduction.

Bacterial biofilms.

Bacteria can exist as single cells, also called planktonic, or in sessile aggregates, which is commonly referred to as a biofilm growth mode. The definition of a bacterial biofilm, as for medical microbiology, is a "coherent cluster of bacterial cells imbedded in a matrix, which are more tolerant to most antimicrobials and the host defense, than planktonic bacterial cells" [1]. The life cycle of a biofilm, illustrated in figure 1, can be divided into three main stages as follows; bacterial adhesion to an abiotic or biotic surface and aggregation of cells (*1*), bacterial proliferation and biofilm maturation, including production of the extracellular matrix (*2*), and detachment and dispersal of planktonic bacteria (*3*) [2].

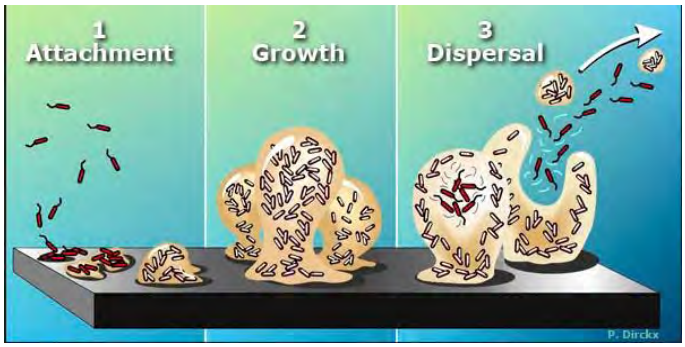

Figure 1. The biofilm life cycle. A biofilm starts to form when bacteria attach to a surface, (*1*); the biofilm matures through growth of the bacterial cells and production of the extracellular matrix (*2*); eventually planktonic bacteria can detach and disperse from the biofilm (*3*).

From: *biofilmbook.hypertextbookshop.com*

The biofilm matrix, composed by extracellular polymeric substances (EPS), immobilizes the bacteria and keeps them in close proximity, allowing cell-to-cell communication, also known as quorum sensing [3]. Through quorum sensing, bacteria secrete and detect autoinducer molecules, in a cell-density dependent manner, which will influence their behavior, including production of the EPS and virulence factors [4]. In addition, the matrix creates a scavenging system, serving as a nutrient supply and protecting bacteria from the host defense and antimicrobial agents [3, 5]. A biofilm is a dynamic system which maintains balance through growth and dispersal. Dispersal of planktonic cells, either as single cells or microcolonies, is

important in a medical perspective since bacteria can spread from the infection site to the whole body [1].

The biofilm mode of growth represents a survival strategy, and the biofilm bacteria can be up to 1000-fold more resistant to antimicrobials than their planktonic counterparts [6]. However, once bacteria detach from the biofilm they generally become susceptible again, which suggests that the antimicrobial tolerance in biofilm is not due to common resistance mechanisms, such as efflux pumps, target mutations or modifying enzymes [6]. Several hypotheses behind the mechanism of tolerance in bacterial biofilms exist [7]. Studies show that the penetration of antimicrobials into the biofilm can be delayed by interactions with components of the biofilm matrix and that some antimicrobials can become even fully inactivated by chelating enzymes [5]. Even though most antimicrobials are capable to successfully penetrate the matrix they might be unable to kill metabolically inactive bacteria, located in the nutrient- and oxygen-deprived layers of the biofilm [5]. Another type of bacteria contributing to the antimicrobial resistance are the so-called persister cells, representing a sub-population of spore-like cells present in a non-growing stationary phase [8]. Persister cells can escape most antimicrobial agents targeting cellular processes, such as cell wall synthesis, DNA replication and translation, taking place only in physiologically active bacteria.

Implant-associated infections.

Due to a higher median age of the population, suffering from degenerative diseases, and an emerging technology in the field on medical devices, implants are increasingly used to improve or replace parts or functions of the human body [9]. One of the most successful and frequently used types of implant is the prosthetic joint. Only in Switzerland, around 20 000 hip and 16 000 knee prosthesis are implanted every year (Swiss Implant Registry, www.siris-

implant.ch). Infections associated with implants are rare, ranging from 1-5% depending on type of device, but are difficult to diagnose and to treat [10]. The treatment is associated with high costs, due to the need of several surgical interventions and prolonged hospital stay. An implant can be infected exogenously, during surgery or wound healing, or hematogenously via the bloodstream any time after implantation [10].

Microbiology.

Prosthetic-joint infections can be classified according to time of manifestation. Early and delayed infections of exogenous origin manifest within 3 months and between 3 months and 2 years after implantation, respectively, whereas late infections manifest after 2 years [9]. Early infections are in general caused by highly virulent bacteria, such as *Staphylococcus aureus* and *Escherichia coli*, whereas delayed infections are mainly caused by low-virulent bacteria, such as *S. epidermidis* and *Propionibacterium acnes*. *S. aureus* causes most of the late infections of hematogenous origin [10]. The distribution of species isolated from prosthetic-joint infections is presented in figure 2. In around 11% of cases no microorganism is detected [11].

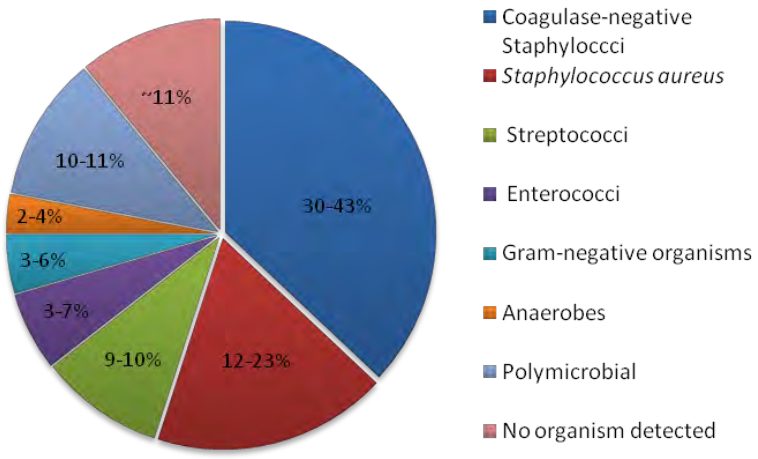

Figure 2. Species distribution of bacteria isolated from prosthetic-joint infections. Adapted from [11].

During the last decades, most research in the field of biofilm and implant-associated infections has been focusing on staphylococci. Consequently, there has been important progress, both in the understanding of staphylococcal biofilm biogenesis, and in improvement and optimization of antimicrobial treatment of staphylococcal biofilm infections. Regarding the less frequently found species, such as enterococci and anaerobes, including *P. acnes*, the optimal treatment regimens have not yet been defined.

Enterococcus faecalis.

Enterococci are opportunistic pathogens increasingly recognized as the cause of healthcare-acquired infections, especially in endocarditis, bacteremia and urinary tract infections [12]. They are Gram-positive facultative anaerobic cocci naturally colonizing the gastrointestinal and female genital tract and the oral cavity [12]. *E. faecalis* is the most common entercoccal species, accounting for 80 - 90% of infections, followed by *E. faecium*, which is more often associated with antimicrobial resistance [12]. In comparison with other Gram-positive bacteria, enterococci are relatively low virulent, but can persist in harsh conditions, such as low pH, high salt concentrations and extreme temperatures, especially when present in biofilm [13].

Enterococcal biofilms have been detected on a wide range of medical devices, including orthopedic implants, intravascular catheters, biliary stents, silicone gastrostomy devices and ocular lenses [14]. In *E. faecalis*, several proteins have been identified to play important roles in the biofilm biogenesis (figure 3). In the early steps of biofilm formation, surface proteins, such as enterococcal surface protein (Esp) and adhesion to collagen by *E. faecalis* (Ace) contribute to adhesion of bacteria, whereas aggregation substance (AS) promotes the aggregation of replicating bacteria [15]. In addition, a surface pili composed by proteins coded by the *epb* locus (*Endocarditis and biofilm associated pili*) has been demonstrated as important for the biofilm formation of *E. faecalis* [16]. When the bacterial density is

sufficient, the quorum sensing locus *frc* (*E. faecalis regulator*) will activate the secretion of proteases that together with Esp and Ace will lead to maturation of the biofilm [14]. Another gene that was shown to be essential for successful infection in mice, including biofilm formation on inert surfaces and invasion of phagocytes, is the *bopD* (*biofilm on plastic surface D*) gene [13]. From the mature biofilm cytolysins and proteases, such as gelatinase, are secreted and can cause tissue damage by lysing host cells, including polymorphonuclear leukocytes [14].

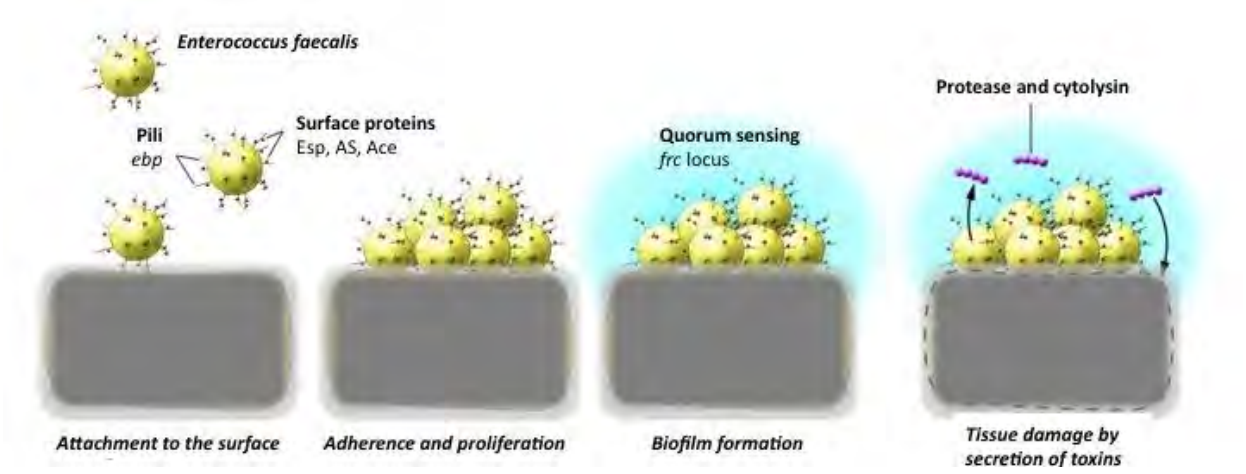

Figure 3. Factors implicated in the biofilm formation of *E. faecalis*. Proteins involved in the bacterial attachment includes Esp (enterococcal surface protein), Ace (adhesion to collagen by *E. faecalis*), AS (aggregation substance) and epb (endocarditis and biofilm associated pili).In the mature biofilm quorum sensing is mediated by the *frc* (*E. faecalis regulator*) locus. From the biofilm proteases and cytolysin can be secreted causing tissue damage. Adapted from .

Enterococci cause subacute infections of prosthetic joints that are particularly difficult to treat and associated with high failure rates [9]. The reasons for treatment failure are not entirely clear, but could be explained by virulence factors associated with biofilm formation described above, and the unavailability of antimicrobial agents active against enterococcal biofilms [9]. A few studies demonstrated activity of the staphylococcal anti-biofilm agent rifampin against enterococcal biofilms *in vitro*, in combination with ciprofloxacin and linezolid [17] and *in vivo*, in combination with tigecycline [18]. However, based on clinical experience of

treatment failure associated with the use of the drug, rifampin is currently not recommended for treatment of enterococcal implant-associated infections [9]. In addition, enterococci may acquire genes encoding resistance against β-lactams, glycopeptides, aminoglycosides and oxazolidinones, which further complicates the treatment of infections [12].

Propionibacterium acnes.

P. acnes is a Gram-positive, slow-growing, facultative anaerobe that is found in the skin, the sebaceous glands, the oral cavity, the large intestine, the conjunctiva and the external ear canal [19]. *P. acnes* is primarily known as the major cause of inflammatory acne, but is also a common pathogen causing deep-seated invasive infections associated with implanted devices, such as shoulder prosthesis, neurosurgical shunts, deep brain stimulators and cardiac devices [20-22].

P. acnes is a low-virulent organism, but its ability form biofilm on different biomaterials is considered as an important virulence factor for the pathogenesis of infections caused by this organism [23]. However, in contrast to staphylococci and enterococci, little is known about the molecular mechanism involved in the biofilm biogenesis. A fibronectin-binding protein found on the surface of the bacterium was shown to facilitate the attachment to the plasma protein film found on many implanted materials [24].

The relevance of *P. acnes* in foreign-body infections may be underestimated for several reasons. Isolation of this anaerobic organism is difficult and, due to slow growth, up to 14 days of incubation is recommended for detection [25]. However, whether late growth in an enriched growth media reflects infection or only contamination is not always obvious. In order to confirm infection, *P. acnes* should be isolated in a pure culture from multiple specimens. Additionally, there is not much data on the spectrum of clinical presentations of *P. acnes* implant-associated infections [23, 25]. Infection typically occurs exogenously during

surgery but due to the low virulence of the bacteria, clinical manifestations may be delayed for months and even, rarely, for years [20]. A hematogenous route of infection is unusual, but was demonstrated in a rabbit model of total joint replacement [26] and in a case-report on a prosthetic hip infection (Mihailesku R, Trampuz A, and Borens O, presented at the 30th Annual Meeting of The European Bone and Joint Infection Society (EBJIS) in Copenhagen, Denmark September, 15-17 2011, poster Nr. 29).

P. acnes is highly susceptible to several antimicrobials and infections are in general treated with penicillins, cephalosporins or clindamycin [27]. Besides the intrinsic resistance to metrodinazole, antimicrobial resistance in *P. acnes* is rare but has been reported for antimicrobials used for topical treatment of acne, such as clindamycin and erythromycin [28]. For eradication of *P. acnes* biofilms, penicillin G and linezolid in combination with rifampin showed the best activity *in vitro* [29]. In addition, to the protective effect of the biofilm, it has been shown that *P. acnes* can escape the immune response by resisting phagocytosis and surviving inside macrophages [30].

Diagnosis and treatment of implant-associated infections.

The diagnosis of implant-associated infections is based on an evaluation of clinical symptoms in combination with analysis of microbiological and histopathological samples, and laboratory analysis, including leukocyte count in blood and synovial fluid [31]. In recent years, new methods and techniques have been proposed, allowing an improved and faster diagnosis, with high sensitivity and specificity. The conventional diagnostic method of culturing peri-prosthetic tissue samples frequently fails in recovering and detecting biofilm bacteria, as the biofilm is mainly attached to the implant [31]. Bacterial biofilms can be dislodged from the explanted device by sonication, and the resulting sonication fluid can be cultured or analyzed by molecular methods. This approach was shown to be more sensitive than peri-prosthetic

tissue culture for the microbiologic diagnosis of prosthetic joint infection, especially in patients who had received antimicrobial therapy before surgery [32]. New rapid non-culture based molecular assays for bacterial identification include multiplex and 16S rDNA polymerase chain reaction (PCR) [31, 33] and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) [31].

Conventional antimicrobial susceptibility tests determine susceptibility of actively replicating bacteria, and may thus not be predictive for the treatment outcome in the presence of a biofilm. It was shown that an *in vitro* bactericidal activity against non-growing stationary-phase bacteria could better predict the treatment outcome in an implant-associated infection animal model [34]. Different *in vitro* methods for evaluation of antimicrobial agents for eradication of biofilms have been described. The majority of methods are based on a static microplate assay, where biofilms are formed in the wells of a microplate, followed by an antimicrobial challenge and quantification of the remaining biofilm by different methods [35-37]. Microplate assays allow screening of multiple isolates and drugs, and have shown to be convenient for fast-growing bacteria forming solid biofilms, such as *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* [37]. However, for more slow-growing bacteria, such as *P. acnes*, these assays have not been widely used, which may be due to problems of reproducibility and intra-experimental variations, as was shown for the use of crystal violet for staining of *P. acnes* biofilm [38]. Existing methods for antimicrobial susceptibility testing of biofilms are too cumbersome and time-consuming for clinical practice, and are currently only suitable for research purposes.

Eradication of an implant-associated infection often includes removal of all foreign-body material, and its replacement after several weeks (if needed). This approach requires several surgical interventions and causes considerable soft tissue damage and bone stock loss, in the case of a prosthetic joint [9]. Therefore, new treatment options using antimicrobials with

increased activity against biofilms are being investigated, potentially allowing successful eradication of implant-associated infections without removal of the device.

Antimicrobial therapy.

Due to reduced antimicrobial susceptibility of bacteria in biofilm, a long-term, high-dose therapy is required to cure implant associated infections. Table 1 summarizes the recommended choice of antimicrobial agent depending on the causative pathogen [9, 11].

Based on *in vitro*, animal and clinical data, the optimal antimicrobial therapy has been defined for staphylococci and includes the use of rifampin [39]. Another antibiotic that has shown good activity against staphylococcal biofilm *in vitro* and *in vivo*, and is currently clinically evaluated for the treatment of prosthetic joint infections, is the lipopeptide daptomycin [40].

Table 1. Antimicrobial treatment of prosthetic joint infections.

Microorganism	**Antimicrobial agent**	
	First 2-4 weeks	**Following 8-10 weeks**
Staphylococci	rifampin plus (flu)cloxacillin	rifampin plus levofloxacin or other fluoroquinolone
Methicillin-resistant staphylococci	rifampin plus vancomycin or daptomycin	rifampin plus levofloxacin or other fluoroquinolone or daptomycin or teicoplanin or fusidic acid or cotrimoxazole or minocycline
Streptococci	penicillin G or ceftriaxone	amoxicillin
Enterococci	penicillin G or amoxicillin or ampicillin or daptomycin plus aminoglycoside	amoxicillin
Penicillin-resistant enterococci	vancomycin or daptomycin plus aminoglycoside	
Gram-negatives	ciprofloxacin, cefepime or ceftazidime plus aminoglycoside (nonfermenters)	ciprofloxacin
Anaerobes	clindamycin	clindamycin
Polymicrobial	amoxicillin/clavulanic acid or piperacillin/tazobactam or imipenem or meropenem	individual regimen

Adapted from [9, 11].

Rifampin.

Rifampin is a semisynthetic derivate of rifamycin, approved for treatment of tuberculosis but also widely used in combination therapy for different staphylococcal infections, including prosthetic and native valve endocarditis, chronic osteomyelitis and prosthetic joint infections [41]. Rifampin acts by binding to the β-subunit of the bacterial DNA-dependent RNA polymerase inhibiting the transcription initiation, which confers its bactericidal action [42]. The potent activity of rifampin against mycobacteria is due to the ability of the drug to enter the host cells, and high concentrations of rifampin has been detected in macrophages, endothelial cells and neutrophils [41]. Rifampin also penetrates well into biofilms, which is the most important mechanism, supported by strong clinical data, for using the antimicrobial in the treatment of biofilm infections. Other hypotheses behind the anti-biofilm activity of rifampin, based on *in vitro* and *in vivo* data, includes a reduced bacterial adherence to the foreign material, an improved activity of other antimicrobials when used in combination, and activity against stationary-phase bacteria present in the biofilm [41].

Rifampin is always administered in combination with another active antimicrobial to prevent a rapid emergence of resistance, which has been described in a wide range of species, including *S. aureus* [43], *E. coli* [44], *Streptococcus pneumoniae* [45] and *Mycobacterium tuberculosis* [46]. Resistance is in general due to alterations in the *rpoB* gene, encoding the β-subunit of the RNA polymerase. Rifampin resistance can emerge through point mutations, insertions or deletions in the conserved regions cluster I-III or near the N-terminal of the *rpoB* [47]. In the majority of cases resistance is due to point mutations. Other more rare mechanisms of resistance include duplication of the target, RNA polymerase binding proteins and modification of rifampin or its permeability into the cell [47].

Daptomycin.

Daptomycin is a cyclic lipopeptide with rapid bactericidal activity against Gram-positive bacteria, including multi-resistant organisms [48]. The mechanism of action, illustrated in figure 4, involves a calcium-dependent insertion of the lipophilic tail of the molecule into the bacterial cytoplasmic membrane (*1*), oligomerization of the molecule (*2*) leading to membrane polarization, potassium efflux and eventually rapid cell death without lysis (*3*) [49].

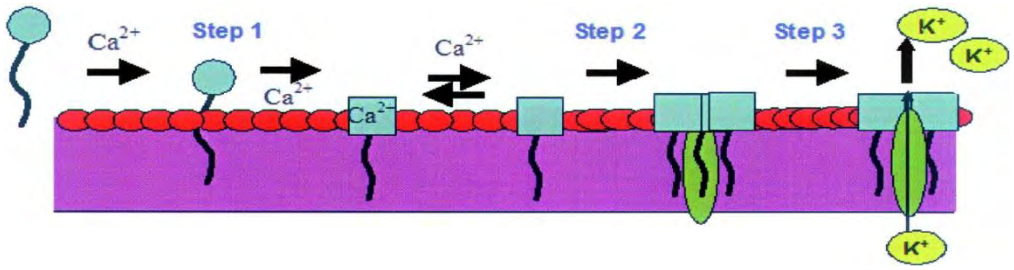

Figure 4. Mechanism of action of daptomycin. Insertion of the lipophilic tail in a calcium-dependent manner (*1*); oligomerization of the molecule and formation of a trans-membrane pore (*2*); membrane depolarization, potassium efflux and cell death (*3*) From [50].

Daptomycin was discovered in the 1980s but clinical trials were stopped due to muscle toxicity, which was observed when the drug was administered twice daily at a dose of 4 mg/kg. In 2003, daptomycin was approved for the treatment of complicated skin and soft tissue Gram-positive infections at a daily dose of 4 mg/kg and in 2006 for treatment of bacteremia and right-sided endocarditis caused by *S. aureus*, at a dose of 6 mg/kg [48]. However, clinical trials, retrospective studies and case reports on the use of higher doses (up to 12 mg/kg), have reported safety and tolerability, and an improved efficiency for the treatment of staphylococcal bone and joint infections, bacteremia and endocarditis [51]. In addition, *in vitro* effects, such as an increased rapidity of the bactericidal activity and suppression of the emergence of daptomycin resistance, were reported, in presence of higher concentrations of the drug [51]. Daptomycin is suitable for once daily dosing due to its

concentration-dependent activity, half-life of 8 h and demonstrated post-antibiotic effect up to 6.6 h [48].

Daptomycin resistance is currently rare, but has been described in staphylococci and enterococci. Mutations in genes encoding for protein implicated in the phospholipid biosynthesis, have been detected in laboratory and clinical *S. aureus* strains exhibiting a decreased susceptibility to daptomycin [52]. These mutations are thought to induce a reduction in the net-negative charge of the cell membrane, causing an electropulsion of daptomycin [52]. In *E. faecalis*, three deletions in different genes have been demonstrated to play a role in daptomycin resistance *in vivo* [53]. Two genes encode for enzymes of the phospholipid synthesis; *cls* encoding for cardiolipin synthase and *GdpD*, encoding for glycerophosphoryl diester phosphodiesterase. The third gene encodes a membrane protein, LiaF, which is thought to be involved in the stress response to antimicrobials acting on the bacterial cell envelope [53].

Animal models of implant-associated infections.

The key advantage of studying biofilm infections using animal models is the presence of a physiological environment and the immune system of the host. *In vivo* studies are especially important for evaluation of antimicrobial agents, as pharmacokinetic and pharmacodynamic factors may influence the interaction between the drug and the microorganism, and the drug efficacy [54]. Among the different types of biofilm infections, foreign-body infections are convenient to study by inserting an implant, which is consequently infected, locally or hematogenously. After a preferred duration of infection, the foreign-body can be explanted and the presence of a biofilm can be evaluated by different methods, such as examination by confocal scanning microscopy of bacterial cells and the extracellular matrix stained with specific dyes, or examination by high-resolution electronic microscopy [54]. When studying

the efficiency of an antimicrobial treatment, the foreign-body is aseptically explanted after the end of therapy and the presence of biofilm bacteria evaluated by CFU-count of bacteria detached from the surface by sonication or scraping, or if possible, culture of the whole implant. Currently, the most studied animal models are models of catheter-associated urinary tract infection, ventilator-associated pneumonia or infection of different materials implanted subcutaneously or intraperitoneally on the animal [54].

The tissue-cage infection model.

With the primary goal to study host factors implicated in foreign-body infections, a guinea-pig model with subcutaneous implants (tissue cages) was developed by Zimmerli et al. [55, 56]. Interesting findings on the immunological level were a deficiency in phagocytic activity of neutrophils present in sterile tissue-cage fluid, an inability in killing of catalase-positive bacteria indicating a defective oxygen-dependent killing mechanism, and a low local level of the cytokine tumor necrosis factor alpha [57].

The model was further adapted for investigation of antimicrobial treatment regimens against bacterial biofilm infections, and was later also modified for the use in rats and mice. In comparison with other more complex foreign-body infection models, the tissue-cage model does not require refined surgical skills [56]. Briefly, for the guinea-pig model, four regularly perforated Teflon cylinders (figure 5B), allowing accumulation of inflammatory fluid, bacterial inoculation and pharmacokinetic studies, are implanted on the flanks of male albino guinea-pigs (figure 5A). After wound healing, approximately three weeks after implantation, cages are infected by injection of a well-defined bacterial inoculum [56]. The minimal infective dose needed to achieve a stable infection of the cage in guinea pigs is in the range of 10^2-10^3 CFU for staphylococci. In rat or mouse models, the minimal infective dose is higher

and immunosupression may be needed to prevent spontaneous healing of the infection [58, 59].

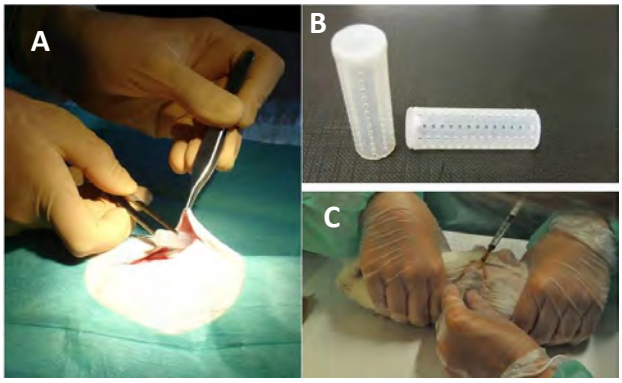

Figure 5. Guinea-pig foreign-body infection model.
A. Subcutaneous insertion of cages.
B. Tissue-cages (Teflon cylinders).
C. Aspiration of tissue-cage fluid.

Different treatment regimens for biofilm infections have been evaluated using tissue-cage infection models. A limitation when using guinea pigs is their intolerance to β-lactam antimicrobials and clindamycin, which both cause lethal diarrhea. Additionally, guinea pigs only support short-term therapy up to 4 days. Rats are more suited for studying chronic infections and long-term therapy, but the infective dose needs to be increased to avoid spontaneous healing [56, 59]. When using a novel antimicrobial, a pharmacokinetic profile can be established by aspirating of tissue-cage fluid after intraperitoneal injection of different doses of the drug. Most antimicrobials are administered twice daily using doses that will achieve drug levels in the tissue-cage fluid, equal to the levels obtained in human serum. For evaluation of the treatment efficacy the tissue-cages are explanted after the end of therapy and the cure rate is determined by dividing the number of culture-negative cages by the total number of cages in the treatment group. Additionally, the antimicrobial activity against planktonic bacteria present in the fluid within the cage can be evaluated by aspirating cage fluid before, during and after treatment (figure 5C) [56].

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Chapter 2. Aim of the study.

The general aim of the study was investigate novel antimicrobial combinations for the eradication of biofilms of difficult-to-treat microorganisms *in vitro* and in a foreign-body infection model using guinea pigs.

The aim of the first part (chapter 3) was to investigate the activity of daptomycin and vancomycin, alone and in combination with gentamicin, against planktonic and adherent *E. faecalis in vitro* and *in vivo*.

The aim of the second part (chapter 4) was to investigate the activity of rifampin alone and in combination with other antimicrobials against *P. acnes* biofilm *in vitro* and *in vivo*.

Finally, in the last part (chapter 5) we investigated the *in vitro* emergence of rifampin resistance in *P. acnes*, and characterized the molecular background in resistant isolates.

Chapter 3.

Gentamicin improves the activities of daptomycin and vancomycin against *Enterococcus faecalis in vitro* and in an experimental foreign-body infection model.

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Gentamicin Improves the Activities of Daptomycin and Vancomycin against *Enterococcus faecalis In Vitro* and in an Experimental Foreign-Body Infection Model▽

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For enterococcal implant-associated infections, the optimal treatment regimen has not been defined. We investigated the activity of daptomycin, vancomycin, and gentamicin (and their combinations) against *Enterococcus faecalis in vitro* and in a foreign-body infection model. Antimicrobial activity was investigated by time-kill and growth-related heat production studies (microcalorimetry) as well as with a guinea pig model using subcutaneonsly implanted cages. Infection was established by percutaneous injection of *E. faecalis* in the cage. Antibiotic treatment for 4 days was started 3 h after infection. Cages were removed 5 days after end of treatment to determine the cure rate. The MIC, the minimal bactericidal concentration (MBC) in the logarithmic phase, and the MBC in the stationary phase were 1.25, 5, and >20 μg/ml for daptomycin, 1, >64, and >64 μg/ml for vancomycin, and 16, 32, and 4 μg/ml for gentamicin, respectively. *In vitro*, gentamicin at subinhibitory concentrations improved the activity against *E. faecalis* when combined with daptomycin or vancomycin in the logarithmic and stationary phases. In the animal model, daptomycin cured 25%, vancomycin 17%, and gentamicin 50% of infected cages. Iu combination with geutamicin, the cnre rate for daptomycin increased to 55% and that of vancomycin iucreased to 33%. In couclusiou, daptomycin was more active than vancomycin against adherent *E. faecalis*, and its activity was further improved by the addition of gentamicin. Despite a short duration of infection (3 h), the eure rates did not exceed 55%, highlighting the difficulty of eradicating *E. faecalis* from implants already in the early stage of implant-associated infection.

Implant-associated infections are caused by microorganisms growing attached on the device surface as biofilms (12, 31). The biofilm mode of growth represents a survival strategy through which microorganisms can attach and better resist antibiotics and the host defense system. In a biofilm, microorganisms can be up to 1,000-fold more resistant to antimicrobials tban their planktonic counterparts (27). Therefore, infections in the presence of an implant are persistent and difficult to eradicate. Often, removal of all foreign-body material is performed, and it is replaced after several weeks, if needed. This approach causes considerable soft tissue damage and bone stock loss and requires repeated surgical interventions and a prolonged hospital stay (40). Therefore, new treatment options using antibiotics with increased activity against biofilms are being investigated, potentially allowing successful eradication of implant-associated infections without device removal.

Enterococci are increasingly recognized as the cause of health care-acquired infections, especially in immunocompromised hosts, such as critically ill patients and recipients of intravascular and extravascular implants (20, 25). Enterococci cause 3 to 10% of periprosthetic joint infections, and the treatment is challenging due to high failure rates and common relapses (22, 40). Reasons for the common treatment failure of enterococcal implant-associated infections are not entirely clear. They may include the low killing rate of enterococci by β-lactam antibiotics and glycopeptides, antimicrobial tolerance, persistence in host cells (potentially mediated by the enterococcal surface proteins), and unavailability of potent antibiofilm agents, such as rifampin, which is active against staphylococcal biofilms (15, 26, 40). In addition, enterococci may acquire genes encoding resistance against β-lactams, glycopeptides, aminoglycosides, and oxazolidinones, which further complicates the treatment of enterococcal infections (17, 23, 38).

Daptomycin may be a treatment option for enterococcal biofilms due to its mechanism of action, particularly its rapid killing and concentration-dependent activity (9). We investigated the activity of daptomycin and vancomycin, alone and in combination with gentamicin, against planktonic and adherent *Enterococcus faecalis in vitro* and in a foreign-body infection model using guinea pigs. This model was previously used to evaluate treatment regimens of implant-associated infections caused by staphylococci and *Escherichia coli* (1, 5, 16, 30, 36, 39) but has not yet been employed for enterococcal infections.

(Parts of the results of the present study were presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Helsinki, Finland, 16 to 19 Sep-

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tember 2009, and at the 50th Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC], Boston, MA, 12 to 15 September 2010.)

MATERIALS AND METHODS

Study organism. All experiments were performed with *E. faecalis* strain ATCC 19433. The strain has been shown to form biofilms (7). Bacteria were stored at −70°C by using a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). To prepare the inoculum, one bead was spread on a blood agar plate and incubated at 37°C. One colony was resuspended in 5 ml of tryptic soy broth (TSB) and incubated at 37°C. Overnight cultures were adjusted to a turbidity of McFarland 0.5 (corresponding to ~5×10^7 CFU/ml). The exact inoculum was determined by CFU counting. For the infection inoculum, the overnight culture of *E. faecalis* was washed twice with sterile pyrogen-free 0.9% saline.

Antimicrobial agents. Daptomycin for injection was supplied by Novartis Pharma Schweiz AG (Bern, Switzerland). A stock solution of 50 mg/ml was prepared in sterile and pyrogen-free 0.9% saline. Vancomycin was delivered by Teva Pharma AG (Aesch, Switzerland) as 10 mg of powder in ampoules. The stock solution of 50 mg/ml was prepared in sterile and pyrogen-free 0.9% saline. Gentamicin was purchased from Essex Chemie AG (Lucerne, Switzerland) as a 40-mg/ml solution.

Antimicrobial susceptibility. The MICs and minimal bactericidal concentrations (MBCs) for daptomycin, vancomycin, and gentamicin were determined by the broth macrodilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (10). A standard inoculum of ~3×10^5 CFU/ml was used. Serial 2-fold dilutions of the antimicrobials were prepared in cation-adjusted Mueller-Hinton broth (CAMHB) for the logarithmic growth phase. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth. The MBC_{log} was defined as the lowest antimicrobial concentration which killed ≥99.9% of the initial bacterial count (i.e., ≥3 $\log_{10}$ CFU/ml) in 24 h (10). For stationary-growth-phase bacteria, MBC_{stat} was determined by using phosphate-buffered saline (PBS) supplemented with 0.1% TSB (34). Growth media for daptomycin studies were supplemented with 50 mg/liter Ca^{2+}. All experiments were performed in triplicate.

Time-kill studies. The activities of daptomycin, vancomycin, and gentamicin were investigated by time-kill studies under logarithmic- and stationary-growth-phase conditions. The initial inoculum was 5×10^5 CFU/ml. Medium inoculated with bacteria only served as the growth control. CAMHB was used for the logarithmic growth phase, and PBS supplemented with 0.1% TSB was used for the stationary growth phase. Growth media for daptomycin studies were supplemented with 50 mg/liter Ca^{2+}. Tubes were incubated under static conditions at 37°C, and aliquots were taken after 0, 2, 4, 6, 8, and 24 h. To avoid antibiotic carry-over, samples were washed with sterile 0.9% saline and appropriate dilutions were spread on Muller-Hinton agar. The experiments were performed in triplicate, and means and standard deviations were plotted in a semilogarithmic graph. A bactericidal effect was defined as a ≥3-$\log_{10}$ (≥99.9%) reduction of the initial bacterial count in 24 h.

Microcalorimetry. To test the growth inhibition by antimicrobials and their combinations, measurements of bacterial heat production were performed with a 48-channel batch calorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, New Castle, DE). Microcalorimetry is a highly sensitive and accurate method for measurement of heat production generated by microbial growth. The effect of antibiotics on bacterial growth can be evaluated by the delay of heat flow and reduction of the heat flow peak compared to results with the growth control without the presence of antibiotics (2, 3, 6, 28, 29, 33). Calorimeter ampoules with 3 ml of CAMHB supplemented with Ca^{2+} containing daptomycin, vancomycin, or gentamicin at 0.125× MIC, 0.25× MIC, 0.5× MIC, and 1× MIC were inoculated with 3×10^5 CFU/ml. In combination studies, daptomycin and vancomycin at 0.25× MIC were combined with gentamicin in concentrations of 0.125× MIC, 0.25× MIC, and 0.5× MIC. The heat flow at 37°C was measured for 24 h. The results were plotted as heat flow over time.

Animal model. Male albino guinea pigs (Charles River, Sulzfeld, Germany) were kept in the Animal Facility of the University Hospital Lausanne, Lausanne, Switzerland, and the experiments were performed according to the regulations of Swiss veterinary law. Guinea pigs were weighed daily to monitor their well-being during the experiment. Short-term studies (4-day treatment) were performed in a foreign-body cage model for guinea pigs developed by Zimmerli et al. (41). Four sterile polytetrafluorethylene (Teflon) cages with 130 regularly spaced perforations 1 mm in diameter (Angst-Pfister AG, Zürich, Switzerland) were subcutaneously implanted in flanks of the guinea pigs (450 to 550 g) under aseptic conditions. After complete wound healing (approximately 2 weeks after implantation), the sterility of the cages was confirmed by culturing aspirated cage fluid on blood agar plates. The bacterial inoculation was performed only in initially sterile cages.

Pharmacokinetic studies. Pharmacokinetic studies for daptomycin and vancomycin were performed in previous studies (5, 16). For gentamicin pharmacokinetic studies, cage fluid was aspirated from noninfected animals over 24 h (2, 4, 6, 10, and 24 h) following intraperitoneal administration of a single dose of gentamicin at 10 mg/kg. Three guinea pigs were used; i.e., 12 cages. At each time point, 150-μl aliquots of cage fluid were aspirated from two cages from each animal (i.e., six replicates per time point). The collected fluid was centrifuged ($2,100 \times g$ for 7 min) at 4°C and the supernatant was stored at −20°C until further analysis. The means ± standard deviations (SD) of the peak concentration (C_{max}), the time to reach C_{max} (T_{max}), and the trough concentration at 24 h after dosing (C_{min}) were determined.

Determination of drug concentrations. Gentamicin concentrations in cage fluid were determined by an agar plate diffusion bioassay with *Bacillus subtilis* strain ATCC 6051 as the indicator organism. Mueller-Hinton agar (Difco, BD, Le Pont de Claix, France) was suspended in sterile pyrogen-free water, and the mixture was autoclaved. The medium was then cooled to 45°C, inoculated with the overnight culture of the indicator organism (100 μl per 165 ml of medium), and poured into large assay plates (30 cm by 30 cm). Calibration curves were plotted for the assay plate, and the regression-fitting equation was extrapolated. The standard solutions were prepared in 0.9% NaCl by preparing 2-fold serial dilutions. One hundred microliters of the cage fluid samples and duplicates of the gentamicin standard solutions were spotted into holes punched in the assay plates, and the plates were incubated overnight at room temperature, followed by incubation at 37°C for 24 h. The diameter of the inhibition zone was measured with calipers. The bioassay detection limit corresponded to the gentamicin 0.5× MIC of the indicator organism (i.e., 0.5 μg/ml).

Antimicrobial treatment. Cages were infected by percutaneous inoculation of 200 μl of *E. faecalis* containing 3×10^4 CFU. The exact infection inoculum was determined by colony counts. Three hours after infection, quantitative cultures of aspirated cage fluid were performed, followed by the start of antimicrobial treatment. In each of the following five treatment regimens, three animals were randomized, each having 4 cages (i.e., 12 cages per treatment regimen): control group (no antibiotic treatment); daptomycin, 40 mg/kg of body weight; daptomycin, 40 mg/kg plus gentamicin 10 mg/kg; vancomycin, 15 mg/kg; vancomycin, 15 mg/kg plus gentamicin 10 mg/kg; and gentamicin, 10 mg/kg. All antimicrobial agents were given intraperitoneally twice daily, except daptomycin, which was given once daily. The duration of treatment was 4 days.

Antimicrobial efficacy on planktonic and adherent *E. faecalis*. Cage fluid was aspirated before the start of treatment, during treatment (immediately before the application of the last dose), and 5 days after completion of treatment. The bacterial counts were expressed as $\log_{10}$ CFU/ml of cage fluid. The quantification limit for bacteria in the cage fluid was set to 200 CFU/ml (>10 CFU in 50 μl of a 10-fold dilution). The clearance rate (as a percentage) was defined as the number of cage fluid cultures without growth of *E. faecalis* divided by the total number of cage fluid cultures in the individual treatment group. To determine the antimicrobial efficacy against adherent bacteria, animals were sacrificed 5 days after completion of treatment, and cages were explanted under aseptic conditions and incubated in 5 ml TSB. After a 48-h incubation of the cages in TSB, aliquots of 100 μl were spread on a blood agar plate and incubated at 37°C for additional 48 h. The treatment efficacy against adherent bacteria was expressed as the cure rate (as a percentage), defined as the number of cages without *E. faecalis* growth divided by the total number of cages in the individual treatment group.

Statistical calculations. Comparisons for continuous variables were performed by the Mann-Whitney U test. For all tests, differences were considered significant when P values were <0.05. The graphs in the figures were plotted with Prism (version 5.0a) software (GraphPad Software, La Jolla, CA).

RESULTS

***In vitro* antimicrobial susceptibility.** Table 1 summarizes the *in vitro* susceptibility of *E. faecalis*. Daptomycin and vancomycin had low MIC values (1.25 and 1 μg/ml, respectively) but did not kill the bacteria in the stationary growth phase. Daptomycin showed bactericidal activity in the logarithmic phase (MBC_{log}, 5 μg/ml), and gentamicin was killing *E. faecalis* in

TABLE 1. *In vitro* susceptibility of *E. faecalis* strain ATCC 19433 against daptomycin, vancomycin, and gentamicin as determined by broth macrodilution[a]

Antimicrobial	MIC (μg/ml)	MBC_{log} (μg/ml)	MBC_{stat} (μg/ml)
Daptomycin	1.25	5	>20
Vancomycin	1	>64	>64
Gentamicin	16	32	4

[a] MBC_{log}, minimal bactericidal concentration in the logarithmic growth phase; MBC_{stat}, minimal bactericidal concentration in the stationary growth phase. The results are mean values from triplicate measurements.

both the logarithmic (MBC_{log}, 32 μg/ml) and the stationary (MBC_{stat}, 4 μg/ml) growth phases.

Time-kill studies. Figure 1 demonstrates killing of bacteria in the logarithmic growth phase. Daptomycin and gentamicin (Fig. 1A and C) show a dose-dependent activity at concentrations from 0.25× to 16× MIC, both being bactericidal (i.e., 3-log reduction) at 4× MIC. Vancomycin (Fig. 1B), on the other hand, showed no bactericidal effect at concentrations of up to 16× MIC. Figure 2 shows the antimicrobial activity against bacteria in the stationary phase. Daptomycin showed no bactericidal effect at up to 16× MIC (Fig. 2A), whereas vancomycin reduced bacterial counts by approximately 3-log at 1× to 16× MIC (Fig. 2B). Gentamicin was the most active antimicrobial agent, being bactericidal at 0.5× MIC (Fig. 2C). When combining daptomycin at 0.25× MIC with gentamicin at different subinhibitory concentrations, the antimicrobial activity was increased against *E. faecalis* in the logarithmic phase (Fig. 3A). This additive effect was less pronounced when combining vancomycin and gentamicin (Fig. 3B). In the stationary phase, gentamicin improved the killing of *E. faecalis* by daptomycin and vancomycin (Fig. 3C and D), with a time-kill curve resembling that of gentamicin alone.

***In vitro* activity on *E. faecalis* evaluated by microcalorimetry.** The effect of daptomycin, vancomycin, and gentamicin at subinhibitory concentrations and 1× MIC on *E. faecalis* growth-related heat production was investigated using a batch calorimeter. The drugs were tested alone (Fig. 4A to C) and in combination with gentamicin (Fig. 4D and E) at subinhibitory concentrations. Since bacteria in the stationary phase do not produce detectable heat, the experiment was performed only with logarithmic phase bacteria. A fixed concentration of daptomycin and vancomycin at 0.25× MIC was combined with gentamicin at 0.125×, 0.25×, and 0.5× MIC.

The growth control had a maximal heat flow peak of 315 μW at 5.6 h of incubation. The activity of the used antibiotics resulted in decreased and delayed peaks of the heat flow curves. At 1× MIC, heat production was completely inhibited for 24 h by all antibiotics. Daptomycin alone was more active in the suppression of growth-related heat production of *E. faecalis* than vancomycin and gentamicin alone using the same concentrations. Whereas a delay in the heat flow peak was observed for daptomycin already at 0.125× MIC (Fig. 4A), the inhibitory effect of vancomycin (Fig. 4B) and gentamicin (Fig. 4C) was present only at 0.5× MIC.

The addition of gentamicin at a low concentration (0.125× MIC) enhanced the antienterococcal activity of both daptomycin and vancomycin (Fig. 4 D and E), whereas gentamicin alone showed a minimal antimicrobial effect at this concentration (Fig. 4C). The combination of daptomycin and gentamicin was more active in the suppression of growth-related heat production than the combination of vancomycin and gentamicin using the same fractional concentrations of the MIC.

Pharmacokinetics of gentamicin in the cage fluid. After an intraperitoneal application of gentamicin (10 mg/kg), a C_{max} (mean ± SD) of 6.2 ± 1.6 μg/ml was reached after 2 h (T_{max}).

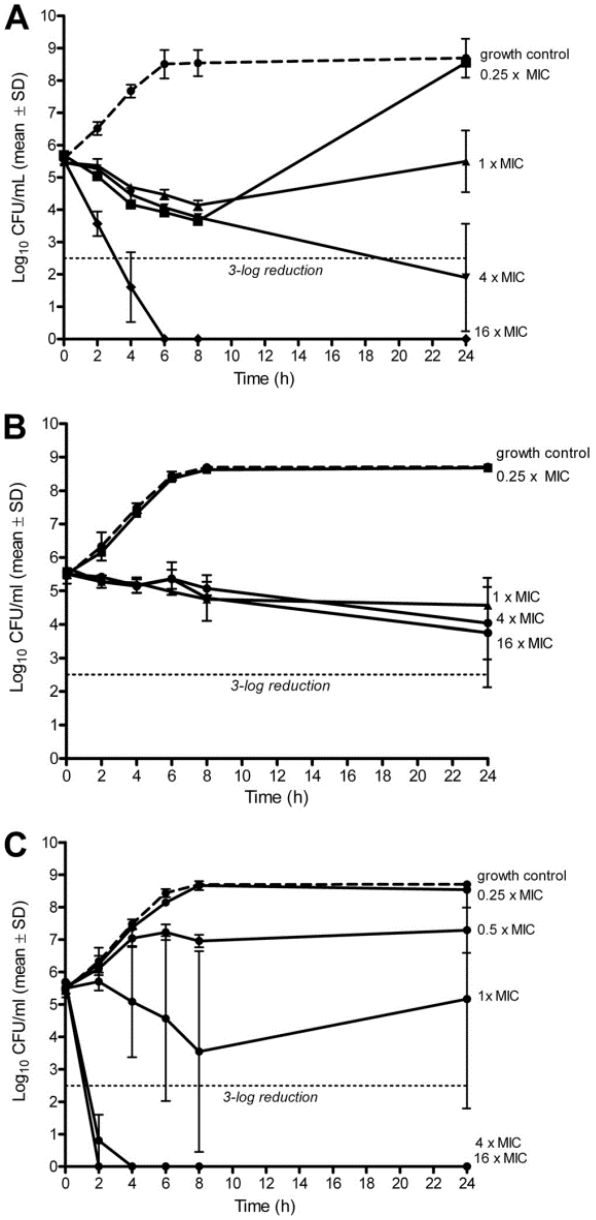

FIG. 1. Time-kill studies for daptomycin (A), vancomycin (B), and gentamicin (C) in logarithmic growth phase. Values represent means and standard deviations (SD) of triplicate measurements. The limit of detection was 200 CFU/ml. The horizontal dashed line represents the reduction of 3 $\log_{10}$ CFU/ml of the initial inoculum. The dashed line represents the growth control.

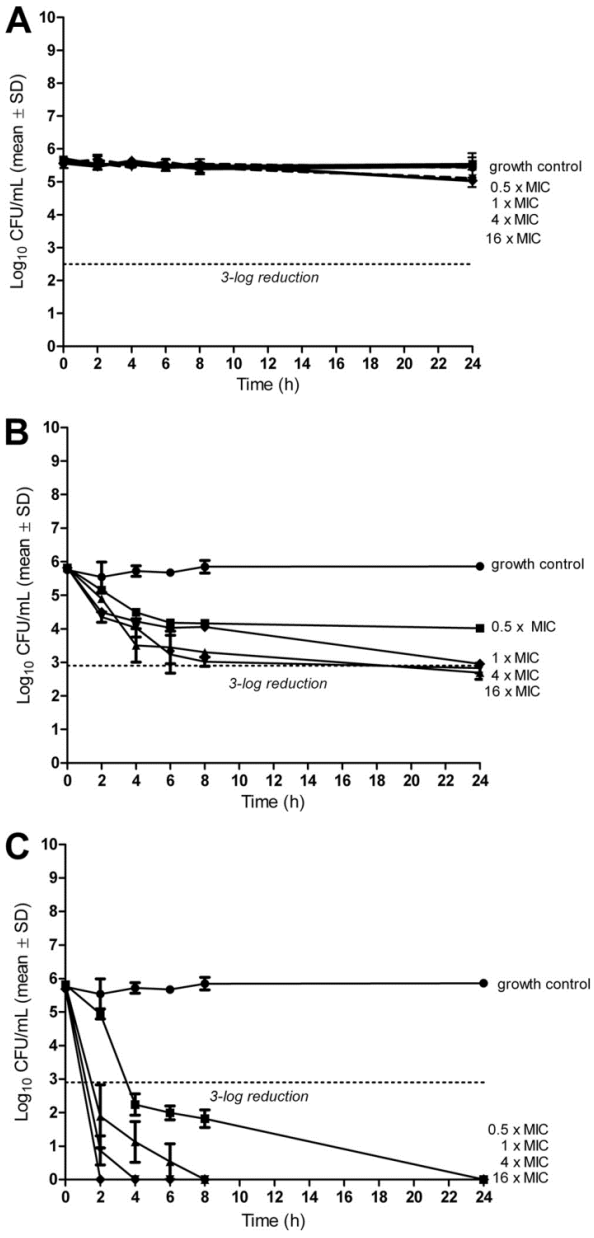

FIG. 2. Time-kill studies for daptomycin (A), vancomycin (B), and gentamicin (C) in stationary growth phase. Values represent means and standard deviations (SD) of triplicate measurements. The limit of detection was 200 CFU/ml. The horizontal dashed line represents the reduction of 3 $\log_{10}$ CFU/ml of the initial inoculum.

The concentration of gentamicin in the cage fluid 24 h after application (C_{min}) was 0 μg/ml.

***In vivo* activity on planktonic *E. faecalis*.** Figure 5 shows the bacterial load in the cage fluid throughout the experiment. After 3 h of infection, before the start of treatment, the bacterial load in the aspirated cage fluid was 3.5 $\log_{10}$. No spontaneous clearance of planktonic bacteria from the cage fluid in the untreated group was observed, and the bacterial load remained unchanged during the experiment (~3.5 $\log_{10}$). Five days after completion of therapy, the planktonic bacteria in the cage fluid had decreased by 1.9 $\log_{10}$ with daptomycin, by 2.5 $\log_{10}$ with vancomycin, and by 2.6 $\log_{10}$ with gentamicin. The combination of gentamicin and daptomycin was the most efficient regimen in killing planktonic *E. faecalis*, with complete clearance of the cage fluid (Fig. 6A). When a combination of gentamicin with vancomycin was used, planktonic bacteria decreased by 1.8 $\log_{10}$ and clearance of planktonic bacteria was achieved in 50% of cage fluid samples.

***In vivo* activity on adherent *E. faecalis*.** Figure 6B shows the cure rates of different antibiotic regimes against adherent bacteria. Without antibiotics (control animals), the cure rate was 0%. Daptomycin, vancomycin, and gentamicin as monotherapy eradicated the adherent bacteria in 25%, 17%, and 50% of cages, respectively. In combination with gentamicin, the cure rate increased to 55% (daptomycin) and 33% (vancomycin).

DISCUSSION

The management of implant-associated infections is challenging, and clinical experience is limited, especially with retention of the infected device (40). Therefore, we compared the *in vitro* and *in vivo* activities of daptomycin and vancomycin, alone and in combination with gentamicin, against planktonic and adherent *E. faecalis*. The chosen *E. faecalis* strain is representative of the current epidemiological situation, with susceptibility to lipopeptides and glycopeptides, without demonstrating high-level resistance to gentamicin (MIC, 16 μg/ml). However, the prevalence of multiresistant enterococcal strains may increase in the future. Therefore, daptomycin was chosen as one of the treatment regimens. Daptomycin is a promising agent due to its mechanism of action, particularly its rapid killing and concentration-dependent activity (9). According to CLSI (11) and EUCAST (http://www.eucast.org/) guidelines, the majority of current clinical isolates of enterococci are susceptible to daptomycin (MIC, ≤4 μg/ml). Moreover, daptomycin is not associated with cross-resistance to other antimicrobials and is also active against most resistant isolates, including vancomycin-resistant enterococci (VRE) (9). However, the risk of emergence of daptomycin resistance during treatment of enterococcal infections is of concern. The genetic basis for daptomycin resistance in enterococci was recently reviewed (21).

The *in vitro* experiments using time-kill studies and measurement of growth-related bacterial heat production (microcalorimetry) showed an improved antibacterial activity of daptomycin and vancomycin when in combination with gentamicin. This effect was observed in the logarithmic phase, whereas in the stationary phase, the killing effect of gentamicin combination represented mostly the pronounced killing effect of gentamicin alone. Based on these *in vitro* results, gentamicin was included in the treatment combination regimens in the experimental studies in the guinea pig model. The animal model was adapted from the previously described setting (1, 16, 30, 39) by reducing the infection inoculum to 3×10^4 CFU/cage and shortening the duration of infection to 3 h. Despite the fact that we have not demonstrated the formation of biofilm on explanted cages, we assume that adherent bacteria in biofilm

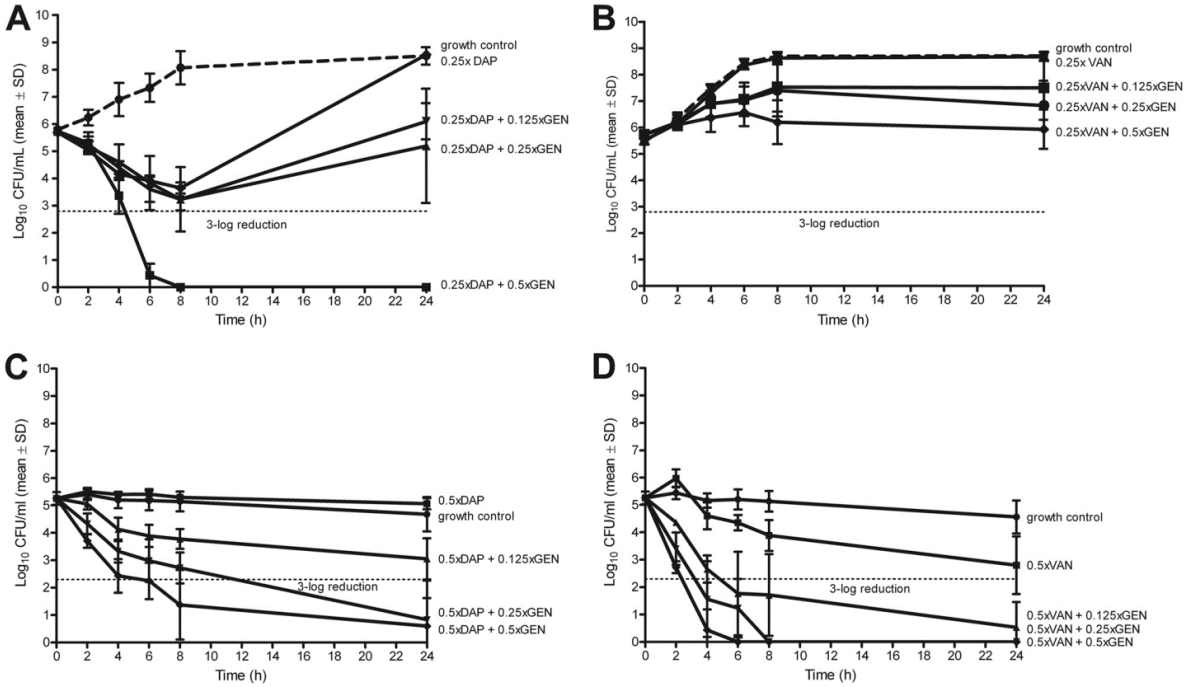

FIG. 3. Time-kill curves of *E. faecalis* in logarithmic (A and B) and stationary (C and D) growth phase with daptomycin (DAP; A and C) and vancomycin (VAN; B and D) combined with gentamicin (GEN) at different concentrations. Values represent means and standard deviations (SD) of triplicate measurements. The horizontal dashed line represents the reduction of 3 $\log_{10}$ CFU/ml of the initial inoculum.

are responsible for frequent treatment failure. With the 24-h duration of infection, no cure of infected cages was observed (data not shown). Under adapted experimental conditions (using a short infection duration), cure rates did not exceed 55%. This highlights the propensity of *E. faecalis* to rapidly cause persistent infections, which are difficult to eradicate despite a low MIC of all tested antimicrobial drugs. In staphylococcal infection, cure rates were significantly improved by combina-

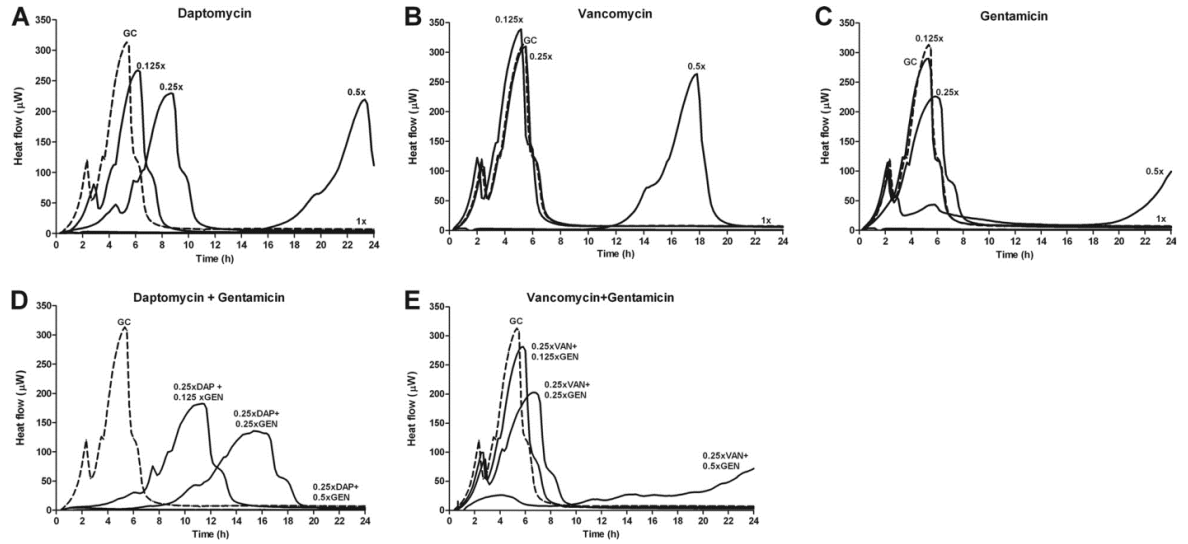

FIG. 4. Calorimetric measurements of heat produced by *E. faecalis* during 24 h exposed to different concentration of daptomycin (DAP; A), vancomycin (VAN; B), gentamicin (GEN; C) and their combinations (D and E). All drugs were measured at concentrations of 0.125× MIC, 0.25× MIC, 0.5× MIC and 1× MIC. The combinations were tested with fixed concentrations of daptomycin and vancomycin (0.25× MIC) with three concentrations of gentamicin (0.125×, 0.25×, and 0.5× MIC). GC, growth control (dashed line).

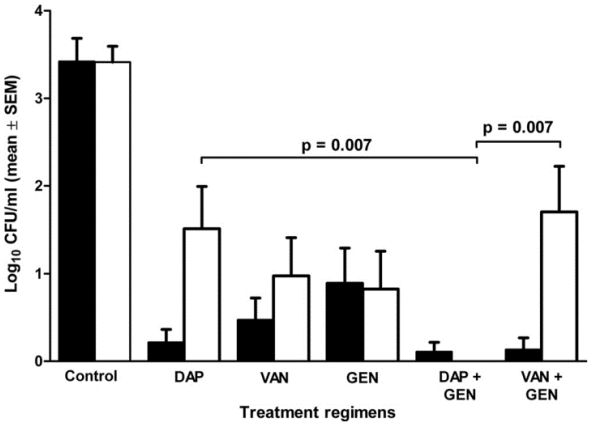

FIG. 5. Bacterial load of planktonic bacteria in cage fluid during treatment (black bars) and 5 days after treatment (white bars). Values are means ± standard errors of the means (SEM). DAP, daptomycin; VAN, vancomycin; GEN, gentamicin.

tion with rifampin, which acts against biofilms (1, 16, 31, 32, 35, 42). In addition, combination regimens may prevent the emergence of resistant strains.

In our animal studies, the injected dose of daptomycin in guinea pigs (40 mg/kg) corresponds to a dose of 8 mg/kg in humans (4, 13, 37), which is higher than that currently recommended for staphylococcal infection in clinical practice (19). The most efficient regimen for killing planktonic *E. faecalis* was the combination of daptomycin and gentamicin, followed by gentamicin alone, as observed in the *in vitro* experiments. When evaluating the activity against adherent bacteria, daptomycin with gentamicin showed the highest cure rate (55%), whereas vancomycin with gentamicin showed a lower cure rate (33%). Gentamicin alone was able to eradicate the biofilm in 50% of the cages. However, gentamicin monotherapy is not recommended in clinical use because of the risk of emergence of resistance. This was shown in a study by Lefort et al. (18), in which gentamicin alone showed a significant reduction of the number of *E. faecalis* bacteria in the vegetation in a rabbit model of endocarditis but selected gentamicin-resistant mutants.

The activity of gentamicin combination against enterococcal biofilms was investigated in several laboratory and clinical studies. *In vitro* enterococcal biofilms were inhibited by high concentrations of ampicillin, vancomycin, and linezolid. In combination with gentamicin, these agents significantly reduced the MIC, MBC, and minimal biofilm inhibition concentration (MBIC) against several tested enterococcal isolates (24). Furthermore, in a rabbit endocarditis model, improved activity of daptomycin was observed against *Enterococcus faecalis* when combined with gentamicin (8). However, in a retrospective clinical study, including enterococcal prosthetic joint infections, no benefit for the treatment outcome of infections of the addition of gentamicin to aminopenicillins or glycopeptides was observed (14). Whether daptomycin in combination with gentamicin is superior to other antimicrobial regimens against adherent enterococci, including vancomycin-resistant enterococci (VRE), and whether the infected implants can be retained with this combination, needs to be investigated in future clinical studies. The optimal regimen against strains with a high level of resistance to gentamicin remains to be determined.

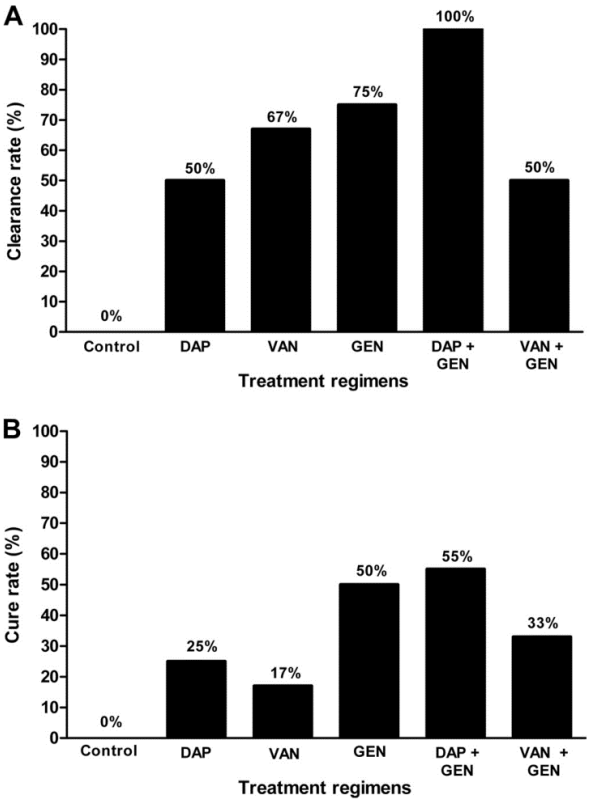

FIG. 6. Clearance rate of planktonic bacteria in cage fluid (A) and cure rate of adherent bacteria from explanted cages (B). DAP, daptomycin; VAN, vancomycin; GEN, gentamicin. The numbers above the columns indicate the clearance and cure rate, respectively.

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Chapter 4.

Role of rifampin against *Propionibacterium acnes* biofilm *in vitro* and in an experimental foreign-body infection model.

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Role of Rifampin against *Propionibacterium acnes* Biofilm *In Vitro* and in an Experimental Foreign-Body Infection Model

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***Propionibacterium acnes* is an important cause of orthopedic-implant-associated infections, for which the optimal treatment has not yet been determined. We investigated the activity of rifampin, alone and in combination, against planktonic and biofilm *P. acnes in vitro* and in a foreign-body infection model. The MIC and the minimal bactericidal concentration (MBC) were 0.007 and 4 μg/ml for rifampin, 1 and 4 μg/ml for daptomycin, 1 and 8 μg/ml for vancomycin, 1 and 2 μg/ml for levofloxacin, 0.03 and 16 μg/ml for penicillin G, 0.125 and 512 μg/ml for clindamycin, and 0.25 and 32 μg/ml for ceftriaxone. The *P. acnes* minimal biofilm eradication concentration (MBEC) was 16 μg/ml for rifampin; 32 μg/ml for penicillin G; 64 μg/ml for daptomycin and ceftriaxone; and ≥128 μg/ml for levofloxacin, vancomycin, and clindamycin. In the animal model, implants were infected by injection of 10^9 CFU *P. acnes* in cages. Antimicrobial activity on *P. acnes* was investigated in the cage fluid (planktonic form) and on explanted cages (biofilm form). The cure rates were 4% for daptomycin, 17% for vancomycin, 0% for levofloxacin, and 36% for rifampin. Rifampin cured 63% of the infected cages in combination with daptomycin, 46% with vancomycin, and 25% with levofloxacin. While all tested antimicrobials showed good activity against planktonic *P. acnes*, for eradication of biofilms, rifampin was needed. In combination with rifampin, daptomycin showed higher cure rates than with vancomycin in this foreign-body infection model.**

Propionibacterium acnes is a facultative anaerobic Gram-positive branching rod physiologically residing in sebaceous glands of the skin (24). It is the major agent of inflammatory acne. In addition, in 2 to 14% of cases, it is identified as the cause of various implant-associated infections, including prosthetic-joint infections, particularly shoulder prosthesis (25, 40, 43); spine implant surgery (4, 14, 19, 29); breast implant surgery (11, 27); electrophysiological cardiac devices (28); and neurosurgery involving ventricular drains and ventriculoperitoneal shunts (10). The role of *P. acnes* in foreign-body infections is probably underestimated due to technical reasons. Detection of anaerobes requires rapid transport to the microbiology laboratory or special transport media and needs incubation for up to 14 days due to slow growth (7, 40). Late growth and/or growth in enrichment media only is often misinterpreted as contamination. Furthermore, although *P. acnes* is usually introduced during surgery, clinical symptoms of low-grade infections often manifest only months to years after implantation. Therefore, the association between implant surgery and infection is not always obvious (14).

Recent studies showed that *P. acnes* forms biofilm on a wide range of materials (2, 26). However, little is known about the mechanisms involved in biofilm formation at the cellular and molecular levels. *P. acnes* is uniformly resistant to metronidazole but susceptible to several other antimicrobials, including penicillin G, ceftriaxone, vancomycin, and clindamycin (13, 16). However, the antimicrobial susceptibility is significantly reduced in biofilms, causing chronic and persistent infections that are difficult to cure without removal of the device. In addition, *P. acnes* can escape the immune response by resisting phagocytosis and surviving inside macrophages (37).

While the role of rifampin in eradication of staphylococcal biofilms was demonstrated in several experimental and clinical studies (1, 18, 33, 35, 39, 45), its role in *P. acnes* biofilm infections is less clear. The aim of this study was to investigate the activity of rifampin alone and in combination with other antimicrobials against *P. acnes* biofilm *in vitro* and in a foreign-body guinea pig infection model. *In vivo* studies were performed to determine the most active treatment regimen for eradication of *P. acnes* biofilms from implants in the clinical setting.

(Parts of the results of the present study were presented at the 21st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Milan, Italy, 7 to 10 May 2011, and at the 2nd European Congress on Microbial Biofilms, Copenhagen, Denmark, 6 to 8 July 2011.)

MATERIALS AND METHODS

Study organism. All experiments were performed with *P. acnes* strain ATCC 11827. The bacteria were stored at −70°C using the cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). For inoculum preparation for *in vitro* studies, one bead was spread on a blood agar plate and incubated for 72 to 96 h at 37°C anaerobically using an Anaerogen system (Oxoid, Basingstoke, Hampshire, England). One distinct colony was resuspended in 10 ml reduced (cooked) brain heart infusion (rBHI) and incubated anaerobically at 37°C. Seventy-two-hour cultures were adjusted to a turbidity of 0.5 McFarland standard (corresponding to $\sim 5 \times 10^7$ CFU/ml). For the inoculum for animal infection, a 72-h culture of *P. acnes* in rBHI was washed twice with sterile 0.9% saline before injection. The exact inoculum size was determined by CFU counting on blood agar plates incubated anaerobically. The ability of our test strain to form biofilm was confirmed by staining using Filmtracer (Invitrogen, Zug, Switzerland) and imaging with confocal microscopy.

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Antimicrobial agents. Daptomycin powder for injection was supplied by Novartis Pharma AG (Bern, Switzerland). A stock solution of 50 mg/ml was prepared in sterile 0.9% saline. Vancomycin was purchased from Teva Pharma AG (Aesch, Switzerland) as 10-mg powder ampoules. The stock solution of 50 mg/ml was prepared in sterile 0.9% saline. Levofloxacin hemihydrate injectable solution (5 mg/ml; Sanofi Aventis Pharma AG, Zurich, Switzerland) and rifampin powder (prepared in sterile water; 60 mg/ml; Sandoz AG, Steinhausen, Switzerland) were purchased from the respective manufacturers. Clindamycin (1 g) powder was purchased from Sigma and dissolved in sterile water (2 g/ml), penicillin G (25 mg/ml) was purchased from Grünenthal Pharma AG (Mitlödi, Switzerland), and ceftriaxone injectable solution (100 mg/ml) was purchased from Roche Pharma AG (Reinach, Switzerland).

Antimicrobial susceptibility of planktonic *P. acnes*. The MIC and minimal bactericidal concentration (MBC) were determined by the broth macrodilution method, as described by Hall et al. (15). An inoculum of $\sim 1 \times 10^6$ CFU/ml was used. Serial 2-fold dilutions of the antimicrobials were prepared in rBHI. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth at 48 h. In addition, the MIC was determined by Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. An inoculum of 1 McFarland standard ($\sim 1 \times 10^8$ CFU/ml) was used, and the plates were read after 48 h of anaerobic incubation at 37°C. The MBC was defined as the lowest antimicrobial concentration that killed ≥99.9% of the initial bacterial count (i.e., ≥3 $\log_{10}$ CFU/ml) in 48 h using rBHI (9). Growth media for daptomycin studies were supplemented with 50 mg/liter Ca^{2+}. All experiments were performed in triplicate.

Biofilm formation on glass beads. *P. acnes* biofilms were investigated using sintered glass beads (Siran carrier; SiKUG 023/02/300/A; Schott-Schleiffer AG, Muttenz, Switzerland) using a protocol adapted from previous studies (22, 42). The diameters of the beads ranged from 2 to 3 mm, and the porosity was 0.2 m^2/g with a pore size of 60 to 300 μm. For biofilm formation, beads were placed in rBHI, inoculated with 2 to 3 CFU of *P. acnes*, and incubated anaerobically at 37°C under static conditions for 3 h, 24 h, or 72 h.

Killing of *P. acnes* biofilm on glass beads. Biofilm was formed for 72 h as described above. Beads were then rinsed thrice with sterile 0.9% saline to remove planktonic bacteria, placed in rBHI containing serial 2-fold dilutions of antimicrobials, and incubated anaerobically for 24 h. After antimicrobial challenge, the beads where rinsed thrice and placed in microcalorimetric ampoules containing 4 ml rBHI. Recovering bacteria were detected by measuring heat production at 37°C for 72 h, allowing the quantification of the remaining biofilm bacteria (see below). The minimal biofilm eradication concentration (MBEC) was defined as the lowest antimicrobial concentration killing biofilm bacteria on beads, leading to absence of regrowth after 72 h of incubation in the microcalorimeter, indicated by the absence of (growth-related) heat flow. All experiments were repeated three times.

Microcalorimetric assay for quantification of biofilm on glass beads. Replicating viable microorganisms produce heat, which can be detected with a microcalorimeter designed for precise real-time measurement (6). An isothermal microcalorimeter (TAM III; TA Instruments, New Castle, DE) was used. This instrument is equipped with 48 microcalorimetry channels, allowing independent parallel measurements. The instrumental detection limit of heat flow is 0.2 μW. The heat production is related to microbial metabolism and increases exponentially with their growth in appropriate medium (36). Microcalorimetry was recently used for investigation of staphylococcal biofilms on bone grafts and bone substitutes (8). Experiments were performed in 4-ml glass ampoules containing growth medium inoculated with sintered glass beads coated with *P. acnes* (with or without previous antimicrobial exposure). The ampoules were sealed and introduced first into the equilibration position for 15 min. In this time, the measuring temperature of 37.0000°C is reached, and heat disturbance by lowering the ampoule in the measuring position is minimized. The heat flow was then recorded at 10-s intervals for 72 h. The detection limit was determined at 10 μW to distinguish microbial heat production from the thermal background (e.g., nonspecific heat flow generated by degradation of the growth medium). The detection time was inversely proportional to the biofilm quantity, which allowed precise quantification of biofilm bacteria on the beads. Sterile beads (containing no bacterial biofilm) served as a negative control.

Animal model. A foreign-body infection model in guinea pigs was used, as previously described by Zimmerli et al. (44). Male albino guinea pigs (Charles River, Sulzfeld, Germany) were housed in the Animal Care Facility at the University Hospital Lausanne, Lausanne, Switzerland. Experiments were performed according to the regulations of Swiss veterinary law. The animals were regularly weighed and observed for behavioral changes to monitor their well-being during the whole experiment. After an adaptation phase of 1 to 2 weeks, four sterile polytetrafluorethylene (Teflon) cages with 130 regularly spaced perforations 1 mm in diameter (Angst-Pfister AG, Zürich, Switzerland) were subcutaneously implanted in the flanks of the guinea pigs (weight range, 450 to 550 g). The surgery was performed under aseptic conditions, and a single dose of vancomycin (25 mg/kg of body weight) was injected intraperitoneally 30 min before skin incision. The wound clips were removed after 7 days. The sterility of the cages was confirmed by cultures of aspirated cage fluid before cage infection. The inoculation of *P. acnes* was performed after complete wound healing (i.e., 10 to 14 days after cage implantation) in sterile cages. The establishment of infection was confirmed by aspiration of cage fluid, followed by quantitative cultures and CFU enumeration on blood agar plates under anaerobic conditions.

Infection profile of planktonic *P. acnes* and persistence of biofilm *P. acnes* in animals. Cages were infected by percutaneous inoculation of 200 μl of *P. acnes* containing 5×10^7 CFU/cage (low inoculum) or 1×10^9 CFU/cage (high inoculum). To determine the infection profile of untreated animals, cage fluid was aspirated from animals every 3 to 5 days. Two animals (one with high inoculum and one with low inoculum) were sacrificed 16 days after infection, and an additional four animals (two each with high and low inocula) were sacrificed 50 days after infection. At sacrifice, the cages were removed under aseptic conditions, placed in 5 ml rBHI, and incubated anaerobically for 10 to 14 days. After incubation, 100 μl of the medium was spread on a blood agar plate and incubated anaerobically at 37°C. A positive culture with *P. acnes* was defined as persistent infection.

Antimicrobial treatment of animals. For treatment studies, infection was established with a high inoculum (1×10^9 CFU/cage). Three days after infection, quantitative cultures of aspirated cage fluid were performed, followed by starting the antimicrobial treatment. For each treatment regimen, at least three animals, each holding 4 cages, were randomized (i.e., 12 cages per treatment regimen): a control group (no antibiotic treatment), 40 mg/kg daptomycin, 10 mg/kg levofloxacin, 15 mg/kg vancomycin, 12.5 mg/kg rifampin, and the combination of rifampin with either daptomycin, levofloxacin, or vancomycin at the same doses mentioned above. All antimicrobials were injected intraperitoneally every 12 h, except daptomycin, which was given every 24 h. The duration of antimicrobial treatment was 4 days. The antimicrobial dose was determined based on pharmacokinetic studies in serum and cage fluid performed in previous studies in the same guinea pig model, mimicking antimicrobial concentrations achieved in humans (1, 5, 18, 31, 38).

Activity on planktonic and biofilm *P. acnes* in animals. To determine the activity against planktonic *P. acnes*, cage fluid was aspirated before the start of treatment, during treatment (before administration of the last dose), and 5 days after completion of treatment. The bacterial counts were expressed as $\log_{10}$ CFU/ml cage fluid. To determine the activity against biofilm *P. acnes*, animals were sacrificed 5 days after completion of treatment, and the cages were explanted under aseptic conditions and incubated in 5 ml rBHI. After 10 days of incubation of the cages in BHI, aliquots of 100 μl were spread on a blood agar plate and incubated at 37°C for an additional 72 h. The treatment efficacy against adherent bacteria was expressed as the cure rate (as a percentage) defined as the num-

TABLE 1 Antimicrobial susceptibility of planktonic and biofilm *P. acnes*

Parameter	Value (μg/ml)[a]						
	Rifampin	Daptomycin	Levofloxacin	Vancomycin	Clindamycin	Penicillin G	Ceftriaxone
MIC	0.007	1	1	1	0.125	0.03	0.25
MBC	4	4	2	8	512	16	32
MBC/MIC ratio	571	4	2	8	4,096	5,333	128
MBEC	16	64	512	512	128	32	64

[a] The values are medians of triplicates. The MBC was determined by broth macrodilution at 48 h. The MBEC was determined by microcalorimetry.

ber of cages without *P. acnes* growth divided by the total number of cages in the individual treatment group.

P. acnes isolates recovered from animals receiving rifampin (alone or in combination) were tested for emergence of rifampin resistance using an Etest (AB Biodisk), as described above.

Statistics. Comparisons were performed by the Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables, as appropriate. For all tests, differences were considered significant when P values were <0.05. The graphs in the figures were plotted with Prism (version 5.04) software (GraphPad Software, La Jolla, CA).

RESULTS

Antimicrobial susceptibility of planktonic *P. acnes*. Table 1 summarizes the antimicrobial susceptibility of planktonic *P. acnes*. The MIC values were lowest for rifampin, clindamycin, and β-lactam antibiotics (<0.2 μg/ml), whereas other antimicrobials all had an MIC of 1 μg/ml. MIC values obtained by the broth macrodilution method were congruent with results obtained with the Etest assay (differences within one dilution). The MBCs were lower for levofloxacin, rifampin, and daptomycin (≤4 μg/ml) than for vancomycin, penicillin G, and ceftriaxone (8 to 32 μg/ml). Clindamycin was only bacteriostatic. The MBC/MIC ratio was ≤4 for daptomycin and levofloxacin, indicating their bactericidal activity. Rifampin had a higher MBC/MIC ratio, due to the extremely low MIC (0.007 μg/ml) and not to a high MBC (2 μg/ml). The bactericidal concentration is achievable *in vivo*.

Antimicrobial susceptibility of biofilm *P. acnes*. Figure 1 shows heat production by *P. acnes* biofilms of different ages. Young biofilms on glass beads (3 h old) were detected (e.g., heat flow exceeding 10 μW) in ~30 h, whereas mature biofilms (72 h old) were detected in ~8 h. Beads without biofilm did not produce any detectable heat flow. Figure 2 shows heat produced by bacteria remaining on beads after a 24-h exposure to individual antimicrobials. The MBEC was considered the concentration inhibiting regrowth after 72 h of recovery, indicated by absence of detectable heat in the microcalorimeter. Rifampin (Fig. 2A) was the most active drug for eradicating biofilm *P. acnes in vitro* (MBEC, 16 μg/ml), followed by daptomycin (Fig. 2B), penicillin G (Fig. 2F), and ceftriaxone (Fig. 2G), with an MBEC of ≥32 μg/ml. Clindamycin (Fig. 2E) showed activity only at 128 μg/ml. Vancomycin (Fig. 2D) and levofloxacin (Fig. 2C) were even less active, inhibiting biofilm *P. acnes* only at 512 μg/ml. Concentration-dependent antibiofilm activity was particularly observed with rifampin, daptomycin, and clindamycin.

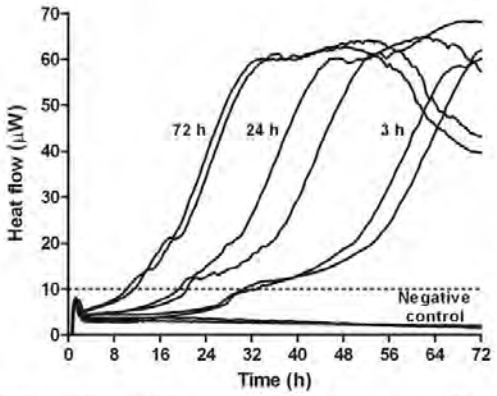

FIG 1 Heat production of *P. acnes* in biofilm. Heat was produced by biofilm bacteria on sintered glass beads after 3 h, 24 h, and 72 h of biofilm formation (in duplicate). The dashed line marks the detection limit of 10 μW. A sterile bead served as a negative control.

Infection profile of planktonic *P. acnes* in animals. When both inocula were used, planktonic bacteria in the cage fluid decreased over time (Fig. 3). When the cages were explanted 16 days after infection, 25% of the implant-associated infections spontaneously healed after infection with the low inoculum. In contrast, after infection with the high inoculum, all infections persisted. When the infection was prolonged to 50 days, infection persisted in all 8 of the cages (100%) infected with the high inoculum, whereas 7 of the 8 cages (87.5%) infected with the low inoculum spontaneously cleared all bacteria.

***In vitro* activity on planktonic *P. acnes*.** Figure 4 shows the bacterial density in aspirated cage fluid throughout the experiment. Three days after infection, before the start of treatment, the bacterial density in the cage fluid was ~6 $\log_{10}$ CFU/ml. Spontaneous reduction of planktonic bacteria (−1.9 $\log_{10}$ CFU/ml) was observed in the cage fluid of untreated control animals during the observation period (12 days). Compared to untreated animals (during and after therapy), a significantly greater reduction in planktonic bacteria was observed with all treatment regimens, except levofloxacin alone and levofloxacin plus rifampin.

***In vivo* activity on biofilm *P. acnes*.** Figure 5 shows the cure rates of different antimicrobial regimens against biofilm *P. acnes*. No cure was observed in untreated animals (controls) and those receiving levofloxacin. Among single-drug therapies, rifampin was the most active antimicrobial, eradicating biofilms in 36% of infected cages, whereas daptomycin cured 4% and vancomycin 17% of cage infections. When combined with rifampin, the cure rate increased to 63% with daptomycin, 25% with levofloxacin, and 46% with vancomycin. No emergence of rifampin resistance was detected in animals receiving rifampin, either as a single drug or in combination treatment.

DISCUSSION

P. acnes is an emerging pathogen in implant-associated infections. The growing prevalence is at least partly an artifact. It can be

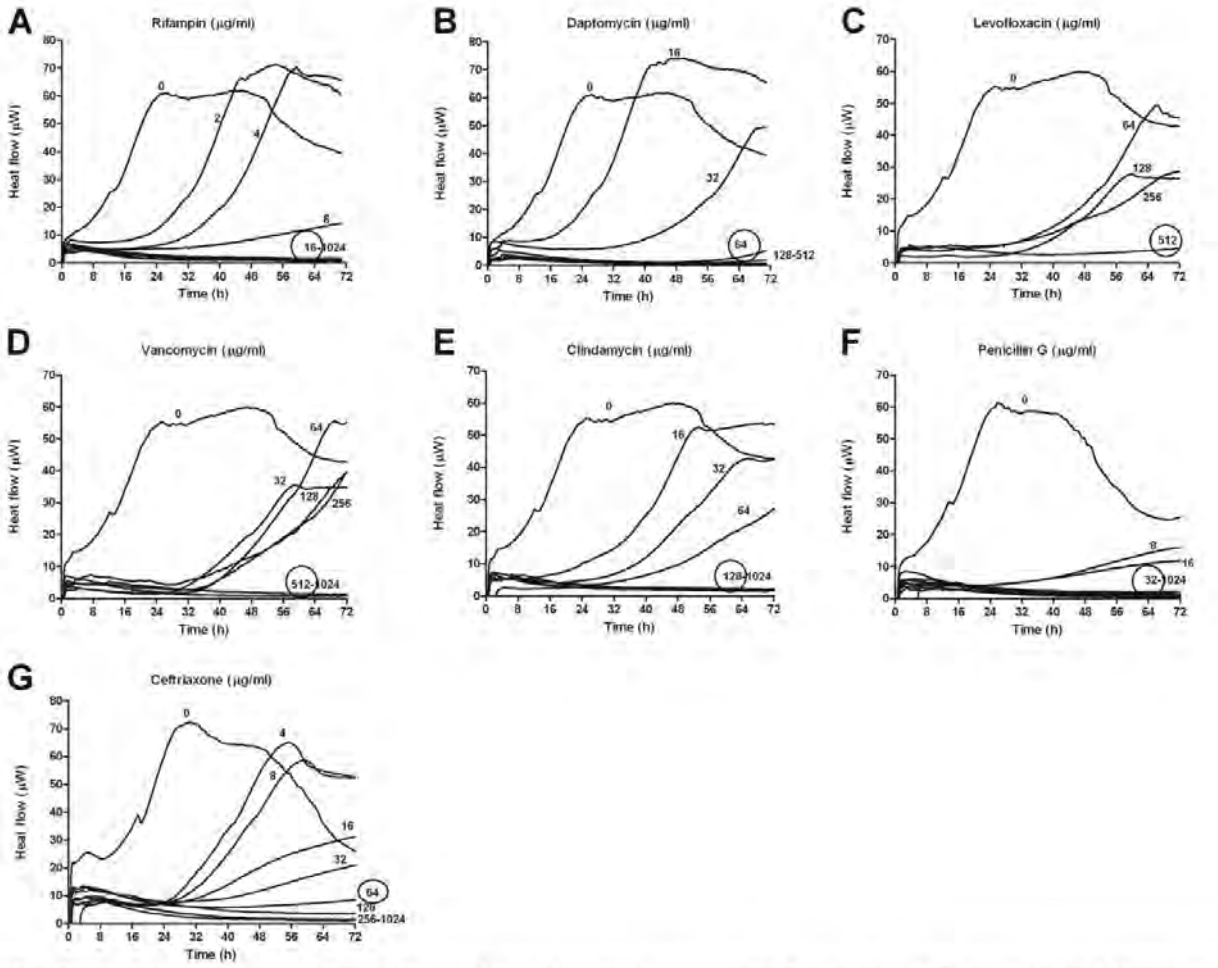

FIG 2 MBECs determined by microcalorimetry. Shown is heat produced by recovering biofilm *P. acnes* after a 24-h exposure to serial dilutions of rifampin (A), daptomycin (B), levofloxacin (C), vancomycin (D), clindamycin (E), penicillin G (F), and ceftriaxone (G). The MBECs are circled. The detection limit was 10 μW.

explained by improved diagnostic tools, such as sonication of explanted material (25, 29, 32), optimized culture conditions for anaerobes (7), and implementation of various molecular assays (34). The optimal antimicrobial treatment for *P. acnes* infections associated with implants has not yet been determined. It is especially unknown whether rifampin plays a favorable role similar to that in implant-associated staphylococcal infection (1, 18, 33, 35, 39, 45). Therefore, we investigated the activity of antimicrobials against biofilms *in vitro* and *in vivo*, modifying a previously established animal model (44). Conventional antimicrobials commonly used against *P. acnes* (β-lactams, vancomycin, and clindamycin), antibiotics with bactericidal activity on planktonic bacteria (levofloxacin), and those showing antibiofilm activity (rifampin and daptomycin) (18) were tested.

A laboratory strain of *P. acnes* (ATCC 11827) was chosen, exhibiting a susceptibility pattern typically observed in clinical isolates (16). The MIC values of all tested drugs for this strain were low. In contrast, the MBCs of commonly used antimicrobials, such as penicillin G (16 μg/ml), ceftriaxone (32 μg/ml), and clindamycin (512 μg/ml), were high for *P. acnes* infections. Interestingly, rifampin, daptomycin, and levofloxacin demonstrated low MBCs (≤4 μg/ml), suggesting superior killing of planktonic *P. acnes*.

In order to investigate the activities of antimicrobials against *P. acnes* biofilms *in vitro*, a microcalorimetry assay with glass beads was used. This assay allowed quantification of biofilm remaining on beads after previous exposure to the antimicrobial drug. Microcalorimetry was recently evaluated for testing antifungal activity on *Candida* sp. biofilms (E. Maiolo, U. Furustrand, D. Sanglard, and A. Trampuz, presented at the 51st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 17 to 20 September 2011). In this study, the microcalorimetric assay (as shown by the MBEC values in Table 1) demonstrated the highest activity against *P. acnes* biofilm by rifampin, followed by penicillin G. Levofloxacin was the least active antimicrobial against *P. acnes* biofilms, despite bactericidal activity

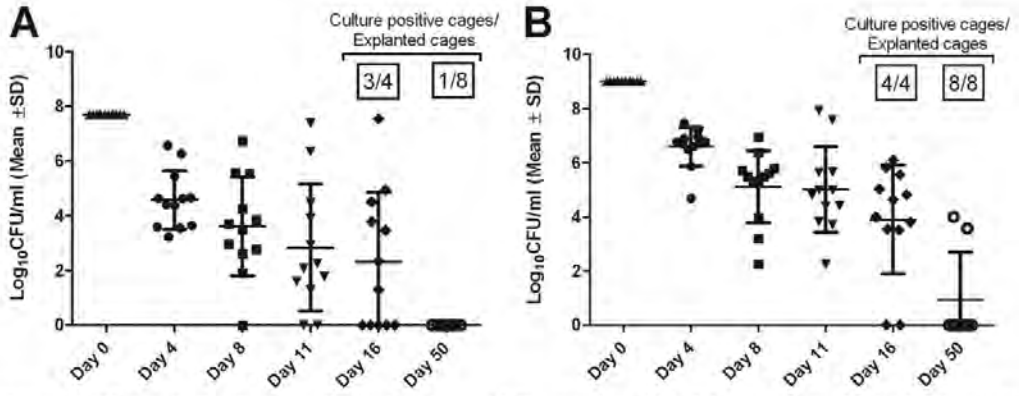

FIG 3 Infection profile of *P. acnes* in a foreign-body guinea pig infection model. Shown are bacterial loads in aspirated cage fluid during 50 days of infection and the percentages of culture-positive explanted cages after 16 days and 50 days of infection using a low infection inoculum of 5×10^7 CFU/cage (A) and a high infection inoculum of 1×10^9 CFU/cage (B). The values are means ± standard deviation.

against planktonic *P. acnes*. Since guinea pigs do not tolerate β-lactams and clindamycin (showing gastrointestinal disturbance), these antimicrobials (and their combinations) could not be tested *in vivo* (reference 44 and unpublished observations).

When a high infection inoculum (10^9 CFU/cage) was injected into the tissue cage fluid of guinea pigs, *P. acnes* persisted on implanted cages for 50 days, despite spontaneous clearance of planktonic *P. acnes* from aspirated cage fluid. This finding highlights the great ability of *P. acnes* to adhere to the implant surface and its change from the planktonic to the biofilm phenotype. Indeed, it is a clinical observation that *P. acnes* is not often detected in cerebrospinal fluid or synovial fluid aspirated from prosthetic joints.

For treatment studies, a high inoculum (10^9 CFU/cage) was chosen, and antimicrobial treatment was started 3 days after infection. These conditions were modified from previous studies using methicillin-resistant *Staphylococcus aureus* (1, 18) and *Enterococcus faecalis* (12) in order to mimic a delayed, low-grade infection by *P. acnes*. In the untreated group, the number of planktonic *P. acnes* cells decreased over time. All treatment regimens reduced planktonic *P. acnes* significantly more than the spontaneous reduction in the untreated group, except levofloxacin and levofloxacin plus rifampin. The most efficient regimen against *P. acnes* biofilms *in vivo* was the combination of daptomycin and rifampin, achieving a cure rate of 63%. In our study, rifampin was the most efficient single drug, with a cure rate of 36%.

Limited data exist about treatment outcomes in a clinical setting. Rifampin in various combinations has been used in the treatment of complicated *P. acnes* infections, often involving implants (17, 20, 21, 30, 41). Penicillin G, linezolid, and linezolid plus rifampin were investigated against *in vitro P. acnes* biofilms after 14 days of exposure (3); no regrowth was detected with penicillin G and linezolid plus rifampin. A case report described a successful treatment of *Propionbacterium* sp. skull osteomyelitis with daptomycin (13), suggesting that the antimicrobial may be used for *P. acnes* bone infections.

To our knowledge, emergence of rifampin resistance has not yet been reported in *Propionibacterium* species. Resistance was, however, described for antimicrobials used for treatment of acne

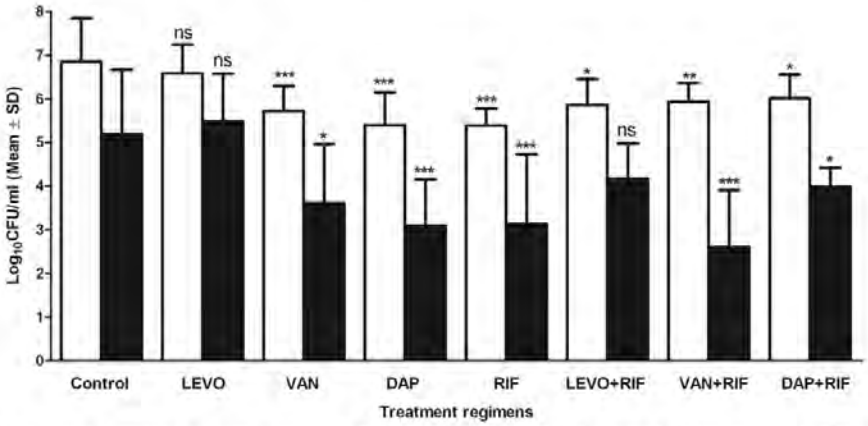

FIG 4 Treatment efficacy against planktonic *P. acnes*. Shown are the bacterial loads in cage fluid aspirated during treatment (white bars) and 5 days after treatment (black bars). The values are means and standard deviations (SD). DAP, daptomycin; VAN, vancomycin; LEVO, levofloxacin; RIF, rifampin. The treatment groups are compared to the control during treatment and after treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ns, not significant.

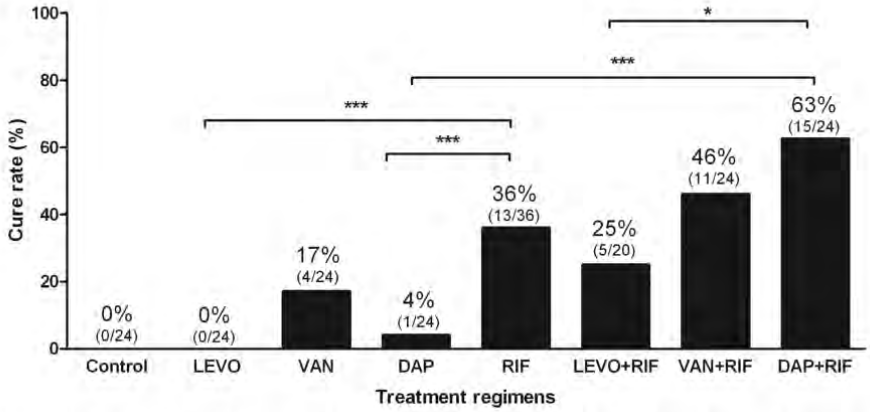

FIG 5 Treatment activity against biofilm *P. acnes*. Shown are the cure rates of adherent bacteria from explanted cages. The percentages above the columns indicate the cure rates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

vulgaris, including MLS antibiotics (macrolides, lincosamides, and streptogramins), such as clarithromycin and clindamycin (23). No rifampin-resistant *P. acnes* isolate was observed in rifampin treatment failures in our animal model.

In summary, rifampin showed the highest activity against *P. acnes* biofilms as a single drug, both *in vitro* and *in vivo*. The combination of rifampin and daptomycin was the most active regimen against experimental *P. acnes* biofilms. Based on *in vitro* biofilm studies, the combination of rifampin and penicillin G or ceftriaxone may represent alternative options, but we were not able to investigate this in the animal model. The present study has important clinical implications, since it may initiate clinical studies with the above-mentioned antimicrobial regimens. This topic is also important because *P. acnes* implant-associated infections are expected to continue to increase in the future, and an optimal regimen needs to be defined.

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Chapter 5.

In vitro emergence of rifampicin resistance in *Propionibacterium acnes* and molecular characterization of mutations in the *rpoB* gene.

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In vitro emergence of rifampicin resistance in *Propionibacterium acnes* and molecular characterization of mutations in the *rpoB* gene

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Objectives: Activity of rifampicin against *Propionibacterium acnes* biofilms was recently demonstrated, but rifampicin resistance has not yet been described in this organism. We investigated the *in vitro* emergence of rifampicin resistance in *P. acnes* and characterized its molecular background.

Methods: *P. acnes* ATCC 11827 was used (MIC 0.007 mg/L). The mutation rate was determined by inoculation of 10^9 cfu of *P. acnes* on rifampicin-containing agar plates incubated anaerobically for 7 days. Progressive emergence of resistance was studied by serial exposure to increasing concentrations of rifampicin in 72 h cycles using a low (10^6 cfu/mL) and high (10^8 cfu/mL) inoculum. The stability of resistance was determined after three subcultures of rifampicin-resistant isolates on rifampicin-free agar. For resistant mutants, the whole *rpoB* gene was amplified, sequenced and compared with a *P. acnes* reference sequence (NC006085).

Results: *P. acnes* growth was observed on rifampicin-containing plates with mutation rates of 2 ± 1 cfu$\times 10^{-9}$ (4096× MIC) and 12 ± 5 cfu$\times 10^{-9}$ (4× MIC). High-level rifampicin resistance emerged progressively after 4 (high inoculum) and 13 (low inoculum) cycles. In rifampicin-resistant isolates, the MIC remained >32 mg/L after three subcultures. Mutations were detected in clusters I (amino acids 418–444) and II (amino acids 471–486) of the *rpoB* gene after sequence alignment with a *Staphylococcus aureus* reference sequence (CAA45512). The five following substitutions were found: His-437→Tyr, Ser-442→Leu, Leu-444→Ser, Ile-483→Val and Ser-485→Leu.

Conclusion: The rifampicin MIC increased from highly susceptible to highly resistant values. The resistance remained stable and was associated with mutations in the *rpoB* gene. To our knowledge, this is the first report of the emergence of rifampicin resistance in *P. acnes*.

Keywords: biofilm, implant-related infections, combinations

Introduction

Propionibacterium acnes is increasingly recognized as the cause of foreign-body infections, including those involving prosthetic joints, spine hardware and ventriculo-peritoneal shunts.[1–3] *P. acnes* is highly susceptible to a wide range of antimicrobials, including clindamycin, β-lactams and quinolones.[4] However, the optimal treatment regimen of *P. acnes* biofilm infections has not been defined. The efficiency of rifampicin for the eradication of *P. acnes* biofilms has been demonstrated *in vitro*[5] and, recently, *in vivo* in an animal model of foreign-body infection.[6]

Rifampicin acts by interacting with the β-subunit of the bacterial RNA polymerase encoded by the *rpoB* gene.[7] Alignment of the *rpoB* gene sequence from different species has confirmed conserved domains among the sequences.[8] Resistance to rifampicin has been described in several bacterial species, such as *Staphylococcus aureus*,[9] *Escherichia coli*,[10] *Streptococcus pyogenes*[11] and *Mycobacterium tuberculosis*.[12] Resistance is generally due to point mutations in the *rpoB* gene leading to a reduced binding between the antibiotic and the enzyme. Mutations particularly occur in the conserved domains of cluster I (amino acids 507–533), cluster II (amino acids 563–572) and cluster III (amino acids 684–690), according to *E. coli* numbering.[13]

The aim of this study was to investigate the emergence of rifampicin resistance in *P. acnes in vitro* and whether combination with an additional antimicrobial agent can prevent the emergence of rifampicin resistance. In addition, the molecular background of rifampicin resistance in *P. acnes* isolates was characterized.

Materials and methods

Study organism

All experiments were performed with *P. acnes* strain ATCC 11827. The MICs of rifampicin, clindamycin, penicillin G, daptomycin and levofloxacin were 0.007, 0.125, 0.03, 1 and 1 mg/L, respectively.[6] Bacteria were stored at −70°C by using the cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). For inoculum preparation, one bead was spread on a blood agar plate and incubated for 72 h. One distinct colony was resuspended in 10 mL of reduced brain heart infusion (BHI) and incubated at 37°C for another 72 h. All incubations were performed in anaerobic conditions using an AnaeroGen™ system (Oxoid, Basingstoke, Hampshire, UK) at 37°C.

Antimicrobial agents

Rifampicin powder (prepared in sterile water, 60 mg/mL; Sandoz AG, Steinhausen, Switzerland), clindamycin powder (prepared in sterile water 200 mg/mL; Sigma), levofloxacin solution (5 mg/mL; Sanofi Aventis Pharma AG, Zurich, Switzerland) and penicillin G (25 mg/mL; Grünenthal Pharma AG, Mitlödi, Switzerland) were purchased from the respective manufacturers. Daptomycin powder was supplied by Novartis Pharma AG (Bern, Switzerland). A stock solution of 50 mg/mL was prepared in sterile 0.9% saline.

Rifampicin resistance studies

For the analysis of spontaneous rifampicin resistance, agar-based single-step mutation studies were performed, as previously described.[14] *Brucella* agar supplemented with vitamin K, haemin and horse blood prepared according to CLSI M11-A7 guidelines was used.[15] Briefly, a large inoculum (10^9 cfu) of the bacteria was spread on *Brucella* agar plates containing rifampicin concentrations of 4× and 4096× MIC, corresponding to 0.03 and 32 mg/L, respectively. The lower concentration corresponded to an increased MIC that is still in the susceptible range (0.03 mg/L) and the higher concentration corresponded to full resistance (32 mg/L). The plates were incubated for 5–7 days before the colonies were enumerated. The spontaneous resistance rate was calculated from the number of colonies that grew on plates containing drug versus the number of colonies that grew on drug-free agar.

Table 1. Primers designed to amplify and sequence the *rpoB* gene of *P. acnes*

Primer	Primer sequence (5′→3′)	Location[a]
PARI-1	CCATAGCGTTGTCGGCAC	+3472 to 3455
PARI-2	GGTGTCAACGAGCATCTCG	+2975 to 2958
PARI-3	CCGGTTTGCTGCAGTACG	+2912 to 2930
PARI-4	CCTTCGGAGTGACCTTGC	+2386 to 2369
PARI-5	GGCATCGTGCGTATCGGT	+2320 to +2337
PARI-6	GCTGCATATTCGCGCCCA	+1805 to +1788
PARI-7	CCATTCCTCGAGCACGAC	+1751 to 1770
PARI-8	GGCCTCAATGTCCTGCGT	+1191 to +1174
PARI-9	CCAGAACCAGTTGCGTACC	+1113 to +1132
PARI-10	CGACGGGATCACCTTACAG	+582 to 564
PARI-11	TGGTGTCCCAGTTGGTGC	+484 to +502
PARI-12	GCGTACCGCGTCGAAGAA	+12 to +29

[a]According to the *P. acnes rpoB* gene reference sequence.

For progressive rifampicin resistance selection, a protocol adapted from Enenza *et al.*[16,17] was used. Briefly, bacteria were exposed to serial 2-fold increasing concentrations of rifampicin in BHI for a total of 10 cycles, each cycle being 72 h. A series of tubes containing 2-fold increasing concentrations of rifampicin were inoculated with either 10^6 or 10^8 cfu/mL (final concentration). Following incubation, the MIC of rifampicin was read and 1 mL (for 10^8 cfu/mL) or 0.1 mL (for 10^6 cfu/mL) samples from the tubes containing the highest antibiotic concentration, and still showing turbidity, were used to inoculate a new series of tubes containing antibiotic dilutions. The experiments were performed twice.

To investigate whether a secondary agent could prevent the emergence of rifampicin resistance, experiments were performed as described above in the presence of 0.25× MIC of daptomycin, clindamycin, levofloxacin or penicillin G, for a total of 10 cycles using an inoculum of 10^6 cfu/mL. A subinhibitory concentration of the second drug was used to allow bacterial growth. The MIC of rifampicin was read after each cycle. After the last cycle, the MICs of the combination drugs were determined by macrobroth dilution, as previously described.[6] For combination studies including daptomycin, growth media were supplemented with 50 mg/L Ca^{2+}.

The stability of all rifampicin-resistant isolates was confirmed by subculture on rifampicin-free agar three times. The MIC of rifampicin was then retested by Etest (bioMérieux SA, Marcy l'Etoile, France) using an inoculum of 1 McFarland and anaerobic incubation for 48 h. All resistant isolates were identified by biochemical tests and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with a Bruker Daltonic mass spectrometer.

Detection of mutations in the rpoB gene in rifampicin-resistant P. acnes isolates

Total DNA from *P. acnes* ATCC 11827 and isogenic resistant isolates was extracted using the InstaGene Matrix method (Bio-Rad Laboratories, Hercules, CA, USA). The procedure was performed according to the manufacturer's instructions. After centrifugation, the supernatant was used as DNA template for PCR analysis.

The whole *rpoB* gene, including the rifampicin resistance-determining region in the *rpoB* gene of *E. coli*[10] and *S. aureus*,[18] was amplified by PCR. Different sets of primers were designed according to the sequence alignment of four strains (GenBank accession numbers CP002815, CP002409, NC006085 and CP001977) and are presented in Table 1. Six overlapping regions of the *rpoB* gene from *P. acnes* were amplified: a 571 bp fragment from nucleotide positions +12 to +582; a 708 bp fragment from nucleotide positions +484 to +1191; a 693 bp fragment from nucleotide positions +1113 to +1805; a 636 bp fragment from nucleotide positions +1751 to +2386; a 656 bp fragment from nucleotide positions +2320 to +2975; and a 561 bp fragment from nucleotide positions +2912 to +3472, corresponding to the whole genome (*P. acnes* coordinates using *P. acnes* KPA171202; GenBank accession number NC006085).[19] PCR was performed in a final volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each nucleotide, 0.5 μM of each primer and 2.5 U of *Taq* DNA polymerase (Phusion® High-Fidelity DNA Polymerase; Finnzymes, Illkirch, France). The PCR conditions were as follows: a 90 s first step of denaturation at 94°C, 30 cycles with 60 s of denaturation at 94°C, 60 s of hybridization at 55°C and 60 s of extension at 72°C, with a final extension step of 7 min at 72°C.

The PCR fragments were purified and sequenced using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Courtaboeuf, France). Sequence analysis was performed on a 3130XL Genetic Analyzer DNA sequencer (Applied Biosystems, Courtaboeuf, France). The sequence of the *rpoB* gene was compared with that of the *rpoB* gene of the *P. acnes* reference strain (GenBank accession number NC006085) using different free software available on the

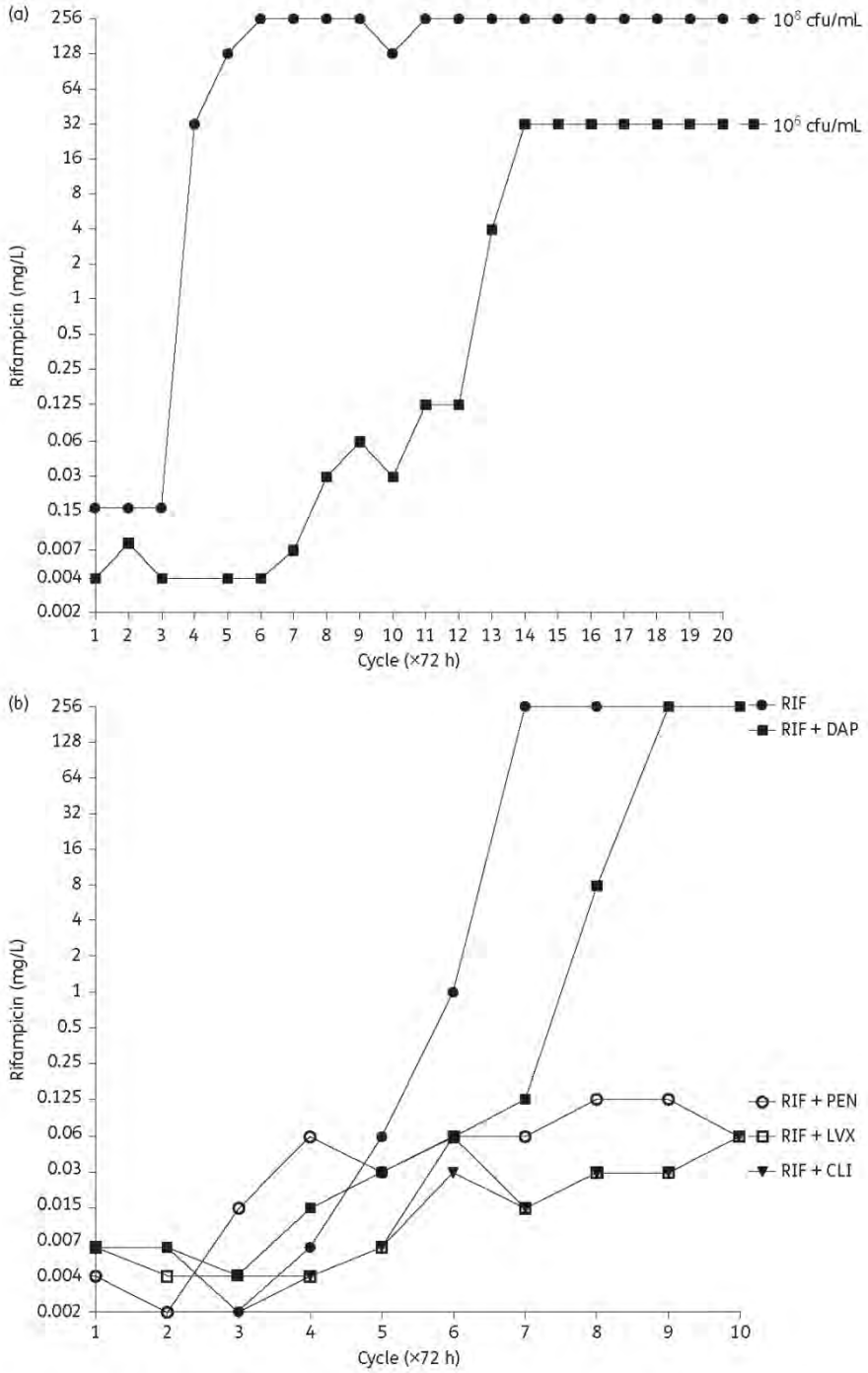

Figure 1. Progressive emergence of rifampicin (RIF) resistance in *P. acnes* using two different inocula (a) and in combination with 0.25× MIC of daptomycin (DAP), levofloxacin (LVX), clindamycin (CLI) or penicillin G (PEN) using an inoculum of 10^6 cfu/mL (b).

Internet (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.genome.jp/tools/clustalw/ and http://web.expasy.org/translate/).

Results

Resistance studies

To determine the rate of spontaneous emergence of resistance, bacteria were exposed to rifampicin concentrations equal to 4× and 4096× MIC (0.03 and 32 mg/L). The mutation rate was $12 \pm 5 \times 10^{-9}$ and $2 \pm 1 \times 10^{-9}$ cfu for the low and high rifampicin concentrations, respectively. After three subcultures on rifampicin-free agar plates, the MIC remained 0.03 and >32 mg/L, as determined by Etest for the *P. acnes* derived from plates containing 4× and 4096× MIC of rifampicin, respectively.

Figure 1(a) demonstrates the results of rifampicin resistance selection during continuous exposure of bacteria to 2-fold increasing concentrations of rifampicin using two different inocula, 10^8 and 10^6 cfu/mL. An increase in the MIC was rapidly observed when using a high bacterial concentration, with an MIC of 32 mg/L being observed after four cycles. With a lower inoculum, the MIC increased gradually, with full resistance (MIC 32 mg/L) being observed after 13 cycles.

The addition of a secondary agent did not prevent resistance development when using a high inoculum (data not shown). Figure 1(b) shows the selection of rifampicin resistance, alone and in combination with 0.25× MIC of clindamycin, penicillin G, levofloxacin and daptomycin, using an inoculum of 10^6 cfu/mL. The addition of clindamycin, levofloxacin and penicillin G to the cultures prevented the selection of highly resistant isolates during 10 cycles and the MIC remained <0.06 mg/L. Daptomycin delayed the increase in the MIC of rifampicin, but did not prevent the emergence of resistance. No increase in the MICs of the secondary antimicrobials used in the combination studies was observed.

Molecular characterization of the rpoB gene in rifampicin-resistant isolates

The *rpoB* gene of rifampicin-resistant *P. acnes* isolates, deriving from plates containing 0.03 mg/L (named PARif1–2, exhibiting reduced susceptibility) and 32 mg/L (named PARif3–5, expressing resistance), and from cycle 10 in the cycling experiment using a high inoculum (named PARif6, expressing resistance), was sequenced. The five different amino substitutions detected in the isolates are summarized in Table 2. Figure 2 shows the alignment of the *rpoB* gene sequences, including clusters I and II, of *E. coli* (GenBank accession number EG10894),[20] *M. tuberculosis* (GenBank accession number L27989),[21] *S. aureus* (GenBank accession number X64172)[22,23] and *P. acnes* (GenBank accession number NC006085)[19] reference strains. The amino acid substitutions found in *P. acnes* are indicated by arrows, and previously described mutations in other species are underlined and in bold.

Sequencing of isolates deriving from previous cycles (cycles 3–10) in the cycling experiment revealed that the amino acid substitutions in PARif6 Ser-442→Leu (cluster I) and Ile-483→Val (cluster II) had occurred after four and nine cycles of exposure, respectively.

Table 2. Amino acid substitutions found in rifampicin-resistant *P. acnes* isolates

Isolate	Rifampicin MIC (mg/L)[a]	Amino acid substitution	GenBank accession number
PARif1	0.03	Ser-485→Leu	JX501524
PARif2	0.03	Leu-444→Ser	JX501525
PARif3	>32	His-437→Tyr	JX501526
PARif4	>32	His-437→Tyr	JX501526
PARif5	>32	Ser-442→Leu	JX501527
PARif6	>256	Ser-442→Leu Ile-483→Val	JX501528

[a]The MIC was determined by Etest, except for PARif6, where microbroth dilution was used.

Discussion

We describe, for the first time to our knowledge, the amino acid substitutions conferring rifampicin resistance in *P. acnes*. Three substitutions were detected in cluster I of the *rpoB* gene associated with either high- or low-level resistance. Interestingly, the position of the amino acid change His-437→Tyr found in PARif3 and PARif4 (MIC 32 mg/L) corresponded to the position of the His-481→Tyr substitution conferring high-level rifampicin resistance in *S. aureus*.[9,18] In *M. tuberculosis*, mutations conferring rifampicin resistance are mainly located in an 81 bp hot-spot region of cluster I.[1,2] Sequence alignment of the *rpoB* gene of *M. tuberculosis* and *P. acnes* revealed that the substitutions Ser-442→Leu (PARif6) and Leu-444→Ser (PARif2), associated with high and low resistance, respectively, were located within this conserved region. Two amino acid changes were detected in cluster II: Ile-483→Val and Ser-485→Leu, of which the first was a secondary mutation in the already resistant *P. acnes* isolate, PARif6, and the second was detected in an isolate exhibiting only low-level resistance. These codons (483 and 485) correspond to codons previously described to confer rifampicin resistance in *S. aureus* (527 and 529)[18] and *E. coli* (572 and 574).[10]

During progressive exposure to rifampicin, a double-mutant was obtained and sequencing demonstrated that the first mutation (Ser-442→Leu) occurred in cluster I after four cycles of exposure, leading to a major increase in the rifampicin MIC, whereas the second mutation (Ile-483→Val) occurred in cluster II after nine cycles of exposure and did not increase the MIC further. The frequency of the Ile-483→Val mutation is presumably lower and could imply there is a fitness cost associated with the drug resistance.[24]

We observed that rifampicin resistance emerged rapidly, if the bacterial load was high, expressing a mutation rate of $2 \pm 1 \times 10^{-9}$. Compared with rifampicin resistance mutation rates of 10^{-7}–10^{-8} in *S. aureus* and *E. coli*,[9,25] the low mutation rate in *P. acnes* may reflect its slow growth rate. Mutation rates in high-density *P. acnes* biofilms have not yet been investigated. The emergence of rifampicin resistance in *P. acnes* biofilms needs further investigation, since this risk has important consequences in the treatment of implant-associated infections, such as prosthetic valve endocarditis, neurosurgical shunt and prosthetic joint infections.

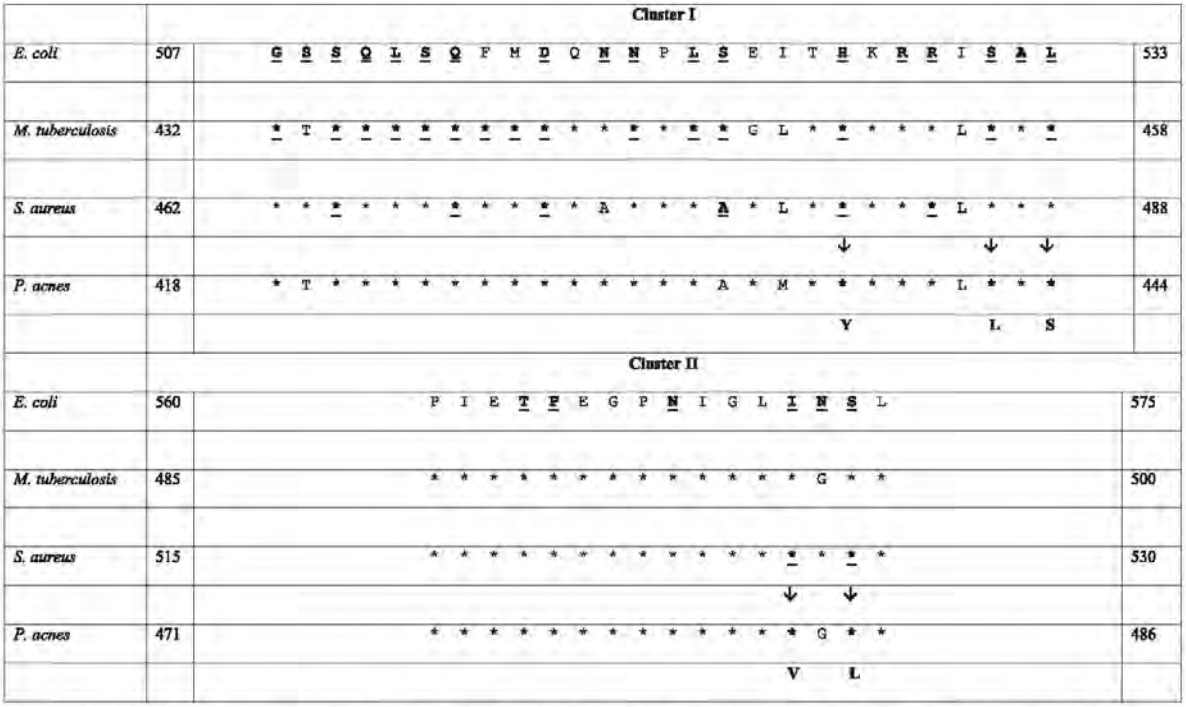

Figure 2. Amino acid sequence comparison of clusters I and II of the *rpoB* gene of *E. coli*, *M. tuberculosis*, *S. aureus* and *P. acnes*. Identical amino acids are indicated by an asterisk. Downward-pointing arrows indicate amino acid substitutions detected in rifampicin-resistant *P. acnes* isolates. Positions involved in rifampicin resistance in *E. coli*, *M. tuberculosis* and *S. aureus* are underlined and in bold.

To prevent the emergence of resistance, rifampicin is administered in combination with another antimicrobial agent. In an animal model of foreign-body infection, the emergence of rifampicin resistance in methicillin-resistant *S. aureus* was prevented when the drug was administered in combination with levofloxacin or daptomycin.[26] In this study, we investigated the potential of levofloxacin and daptomycin, as well as two other antimicrobials commonly used in the treatment of *P. acnes* infections (clindamycin and penicillin G), for preventing the emergence of rifampicin resistance *in vitro*. High-level rifampicin resistance was prevented by the addition of clindamycin, levofloxacin and penicillin G when the bacterial inoculum was in the range of 10^6 cfu/mL, whereas none of the antimicrobials tested was able to prevent resistance if the bacterial concentration was elevated (10^8 cfu/mL). Daptomycin was not able to completely prevent rifampicin resistance; however, no increase in the MIC of daptomycin was observed despite continuous exposure to a subinhibitory concentration of the drug.

The impact of rifampicin resistance in *P. acnes* in clinical practice is unknown. Importantly, the rifampicin resistance in our experimental setting was stable when the antibiotic pressure was removed, both for low-level and high-level resistance. In future studies, testing of the stable isolates in a foreign-body infection animal model, which was adapted for *P. acnes*,[9] will allow investigation of the influence of low- and high-level rifampicin resistance on the treatment outcome. In addition, the use of rifampicin combinations, especially with clindamycin, levofloxacin and penicillin G, needs to be evaluated *in vivo* to determine the potential of these antimicrobials for the prevention of resistance.

In conclusion, we demonstrated that rifampicin resistance in *P. acnes* can easily be selected *in vitro* and can be prevented by combination with levofloxacin, clindamycin and penicillin G. Rifampicin resistance was associated with point mutations concentrated in clusters I and II of the *rpoB* gene and occurred in codons conferring rifampicin resistance in other bacterial species.

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Transparency declaration

None to declare.

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Chapter 6. General conclusions and outlook.

Implants are increasingly used in modern medicine to improve the quality of life of a continuously ageing population. Infections of implants rarely occur but the treatment is challenging, and includes intensive antimicrobial therapy for eradication of the biofilm growing on the implant surface. Staphylococci cause majority of infections and antimicrobial treatment concepts have been optimized with the help of animal and clinical studies. However, less prevalent organisms, such as enterococci and fungi, are currently considered as difficult to treat, and the optimal treatment is to be defined. Additionally, thanks to improved diagnostic tools, microorganisms previously difficult to detect, such as *P. acnes*, are more frequently confirmed as the cause of infection, for which the optimal antimicrobial therapy neither not yet has been determined.

Conventional susceptibility tests may be misleading for guiding the treatment of biofilm infections, as they are using metabolically active bacteria for determination of antimicrobial susceptibility. In chapter 3, we applied a previously described method using bacteria in stationary growth phase, for determination of antimicrobial susceptibility of non-dividing, metabolically inactive *E. faecalis*. We demonstrated an excellent *in vitro* activity of gentamicin against stationary-phase *E. faecalis*, with an MBC_{stat} of 4 μg/ml. The addition of gentamicin at subinhibitory concentrations improved the *in vitro* activity of vancomycin and daptomycin against *E. faecalis*, both in stationary and logarithmic growth phase. In chapter 4, we established an *in vitro* assay for the assessment of anti-biofilm activity of different antimicrobial agents by microcalorimetry. The assay allowed an indirect quantification of biofilm on porous glass beads, based on the growth-related heat produced by the dispersing biofilm bacteria. We demonstrated a superior activity of rifampin for eradication of *P. acnes* biofilm (MBEC 16 μg/ml) in comparison with other antimicrobials tested, as was previously

shown for staphylococci. The MBECs of penicillin G, daptomycin and ceftriaxone were between 32 and 64 μg/ml, whereas clindamycin, levofloxacin and vancomycin only showed limited activity against *P. acnes* biofilms *in vitro*.

Based on *in vitro* results, we investigated the different treatment regimens in a guinea-pig model for foreign-body infections. Before initiating treatment studies, the experimental conditions were optimized for two organisms of interest. A low infective dose and a short duration (3 h) of infection were used for *E. faecalis*, as treatment failure occurred with all treatment regimens when the duration of infection exceeded 24 h. This observation highlights the difficulty in treating enterococcal infections and the ability of these bacteria to rapidly form persistent biofilms. In contrast to other bacteria, a high infective dose for *P. acnes* was needed to induce a persistent infection without spontaneous decrease in planktonic bacteria in cage fluid, possibly due to the effect of immune system and a switch from the planktonic into a biofilm growth mode. Despite culture-negative tissue-cage fluid, bacteria were present in biofilm on the explanted tissue-cages. This finding was in agreement with the fact that *P. acnes* is often undetectable by culture of cerebrospinal or synovial fluid, despite infection.

We were able to confirm our *in vitro* findings *in vivo* by performing treatment studies. Gentamicin alone showed good activity against *in vivo* biofilm of *E. faecalis* (cure rate 50 %) and improved the cure rate of vancomycin and daptomycin, from 17 % to 33%, and from 25% to 55%, respectively. However, the overall cure rates were low, in particular when considering the short duration of infection. Despite using a penicillin-susceptible strain, we were unfortunately not able to compare our results with regimens containing β-lactams, such as penicillin G, ampicillin and amoxicillin, due to restricted antimicrobial tolerance of the guinea pigs. The daptomycin combination was the most active antimicrobial regimen and could be a potential treatment option for biofilm infections caused by penicillin-resistant and vancomycin-resistant enterococci, since there is no daptomycin cross-resistance observed

with glycopeptides or β-lactams. Considering the reported emergence of daptomycin resistance in enterococci, it should be preferably given at high-dose (8-12 mg/kg in humans) and in combination with an aminoglycoside. The dose of 40 mg/kg used in our study corresponds to a dose of 8 mg/kg in humans.

Moreover, we were able to prove the anti-biofilm activity of rifampin against *P. acnes in vivo* (cure rate 36%), and also in this study, a combination with daptomycin showed the highest cure rate (63%). As predicted based on *in vitro* studies, vancomycin and levofloxacin showed lower cure rates, both alone (17% and 0%) and in combination with rifampin (46% and 25%). We were not able to test penicillin G and ceftriaxone in the animal model, but based on their *in vitro* anti-biofilm activity, these antimicrobials could present potential treatment options in combination with rifampin. A rapid emergence of rifampin resistance has been observed in several bacterial species, especially if the antimicrobial is administered as monotherapy. In our animal studies, rifampin resistant *P. acnes* isolates were not detected, or proven as the cause for treatment failure. If prolonging the duration of treatment, resistance could potentially have emerged in the slow-growing *P. acnes*, but due to limited antimicrobial tolerance of the guinea pigs, we were only able to perform a short-term therapy. For long-term therapy, a tissue-cage rat model could be used. However, whether a stable *P. acnes* infection could be established in this animal is not certain, as a high infection inoculum already was required in the guinea pigs. Nevertheless, in chapter 5, we demonstrated that *in vitro* rifampin resistance can emerge in *P. acnes* during progressive exposure to the drug, or spontaneously if the bacterial concentration is high with a mutation frequency of 10^{-9}. Mutations in the coding region for the rifampin target, rpoB, were associated with an increase in MIC, as previously described in other bacterial species. In total five different mutations were found, in cluster I and II of the *rpoB* gene. Interestingly, none of the mutations detected in cluster II conferred to high-level resistance, whereas of the mutations found in cluster I, two mutations were

associated with high-level resistance and one with low-level resistance. By combination with clindamycin, penicillin G or levofloxacin, an emergence of high-level resistance could be prevented *in vitro*. The impact of rifampin resistance in a clinical practice is currently clear, but could be predicted by animal studies using our already established model.

In view of the growing number of implant-associated infections, in combination with an increasing prevalence of antimicrobial resistance, new alternative treatment strategies for biofilm infections are needed. One approach is to prevent the bacterial attachment to the surface by rendering the implant surface antimicrobial but without influencing the host biocompatibility. Promising preventive strategies include antimicrobial coating of the device, and the use of surface coatings favoring the adhesion of host cells while preventing the adhesion of bacterial cells. Another approach, already widely used, is the addition of antimicrobial substances within cement used for fixation of prosthetic joints. However, there are still unsolved problems regarding the release kinetics of the antimicrobials *in vivo*, in particular the impact of the local and systemic presence of sub-inhibitory drug concentrations for the emergence of antimicrobial resistance. When a mature biofilm already is established, interference with the cell-to-cell communication by quorum-sensing inhibitors, or degradation of the extracellular matrix by biofilm-dispersing enzymes, may improve the eradication of the infection.

PART II.

Antifungal susceptibility of molds by isothermal microcalorimetry.

Chapter 7. General introduction.

Invasive mold infections.

The innate immune system protects effectively against pathogenic fungi and infections are in general mild and non-invasive. However, when mold spores are present in large quantities, they constitute a health risk, causing allergic reactions and respiratory problems [1]. In contrast, immunocompromised patients are at high risk for life-threatening infection caused by growth of opportunistic molds. The usual patients are neutropenic cancer patients, solid organ or hematopoetic stem cell transplant recipients, and patients receiving immunosuppressive therapy [2]. Invasive fungal infections are increasingly reported in these patient groups, and are associated with high morbidity and mortality rates often above 50 %, depending on disease and pathogen [3]. Invasive mold infections occur when fungal conidia (figure 1) are inhaled and, in the absence of an immune response, invade the pulmonary tissue or sinus through infiltration of hyphae (figure 1), causing an often aggressive disease course. Through dissemination of fungal conidia from the initial infection site or through hyphal invasion, the mold can spread via blood vessels, eventually causing hemorrhage, necrotic skin lesions and brain abscess [2].

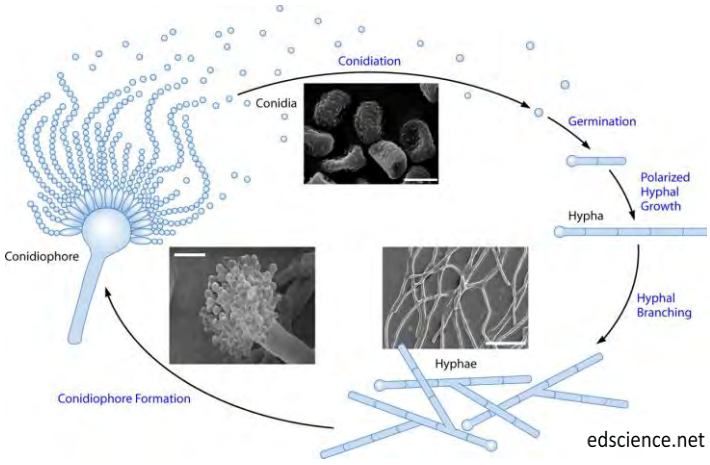

Figure 1. Asexual life cycle of *Aspergillus* spp. including conidia formation, germination, hyphal formation and branching, and formation of a conidiophore.
From: edscience.net

Microbiology.

While *Candida* and *Aspergillus* species are the predominant pathogens accounting for approximately 80% and 15% of infections, respectively [4], the incidence of non-*Aspergillus* molds is continuously rising [5, 6]. The changing epidemiology may reflect the increased use of antifungals for prophylaxis and the introduction of new antifungal agents in clinical use [7]. In addition, progress in the field of fungal species identification by the use of molecular tools and nucleic-acid sequencing, has allowed identification of species belonging to the same class or genus, but exhibiting distinctive susceptibility patterns [8, 9].

A. fumigatus is the most prevalent species, isolated in up to 90% of patients with invasive aspergillosis[10], followed by *A. flavus* and *A. niger* [11]. Nevertheless, increased prevalence of rare Aspergilli exhibiting antifungal resistance, such as *A. terreus* and *A. lentulus* [12] challenges the already difficult treatment. Infections due to non-*Aspergillus* molds, such as species from the Mucorales order, *Fusarium* spp. and *Scedosporium* spp., are particularly difficult to treat, because of rapid tissue invasion and their intrinsic resistance to most first-line antifungal agents [13, 14]. The most prevalent species in the order of Mucorales include *Rhizopus arrhizus*, *Rhizomucor pusillus* and *Lichtheimia corymbifera* [15], whereas *F. solani* is the principal pathogen among *Fusarium* species [16]. The genus *Scedosporium* includes at least two medically important members *Pseudallescheria apiosperma* and *S. prolificans* [17], which may colonize the respiratory tract and disseminate locally or systemically. *S. prolificans*, in particular, is highly resistant to most antifungal agents [9].

Diagnosis.

Due to the high mortality associated with invasive mold infections, a rapid diagnosis is crucial for the treatment outcome and an immediate start of appropriate antifungal therapy[18]. Conventional diagnostic methods include tissue culture and histology, bronchoalveolar lavage

(BAL), chest X-ray and computed tomography scan. These methods are invasive, not sufficiently sensitive or specific, and results are often not available in time to be clinically useful [18]. During the past decades, newer less invasive and non-culture-based diagnostic assays, such as the galactomannan test and the 1,3-β-D-glucan assay, have been developed. Galactomannan is a polysaccharide component, specific to the cell wall *Aspergillus* spp., which is released in the serum during hyphal formation and growth [11]. 1,3-β-D-glucan is another component of the fungal cell wall present in a wide range of species, except the Mucorales, and can thus be used as a panfungal marker [11]. Molecular-based diagnostics can detect fungi with high sensitivity providing results rapidly. Nucleic-acid based diagnostic techniques, such as polymerase chain reaction (PCR), represent the currently fastest growing diagnostic segment, but are not yet commercial due to lack of standardization [18].

Antifungals.

The three major classes of antifungals currently used for treatment of invasive mold infections, summarized in figure 2 [19], owe their antifungal activities to a direct interaction with, or synthesis inhibition of, ergosterol (amphotericin B and triazoles) or 1,3-β-D-glucan (echinocandins). Ergosterol is the main component of the fungal cell membranes, equivalent to cholesterol in mammalian cells. 1,3-β-D-glucan, on the other hand, composes together with α-glucan, mannan and chitin the fungal cell wall [20].

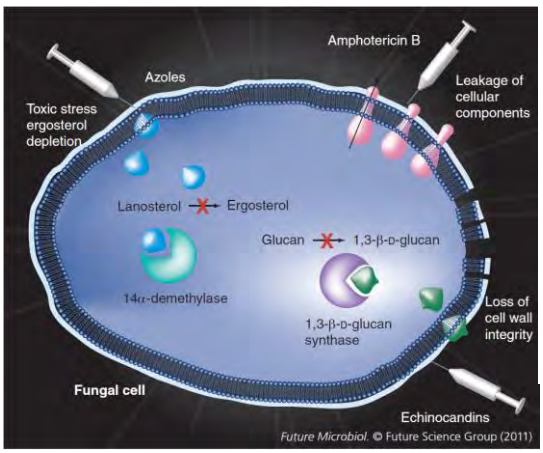

Figure 2. Overview of the mechanisms of action of the three major antifungal classes, amphotericin B, the azoles, including the triazoles, and the echinocandins, used for treatment of invasive mold infections. From (52).

Amphotericin B.

The polyene amphotericin B was for a long time considered as the "gold standard" for antifungal therapy. The compound was originally extracted from *Streptomyces nodosus* and was used as an antifungal agent already in 1959 [21]. The polyenes act by binding to ergosterol in the fungal cell membrane, leading to formation of membrane-spanning channels that cause leakage of cellular components and osmotic cellular lysis, and eventual cell death [20]. The second mechanism of action known, primarly for killing of *Candida* spp., involves oxidative damage of the cell [20].

The antifungal spectrum of amphotericin B is particularly broad and several fungal species are susceptible to low concentrations of the drug. Amphotericin B is commonly used in patients with suspected invasive fungal disease, but without documentation of *Aspergillus* as the causative agent [10], and as the first choice for treatment of mucormycosis [22]. Due to the toxicity of conventional amphotericin B [11], lipid formulations, including liposomal amphotericin B, amphotericin B lipid complex and amphotericin B colloid dispersion, were developed in the late 1990s [21]. Several studies have demonstrated that the lipid formulations of amphotericin B are consistently less toxic and may be even more effective than the conventional drug [21].

Triazoles.

The triazoles are the largest class of antifungal compounds and include fluconazole, itraconazole, voriconazole and posaconazole, as well as isavuconazole which is still in development. All compounds, except fluconazole, show activity against molds and are relatively well tolerated [23]. Voriconazole is a synthetic azole derivative of fluconazole, which has been shown to be superior to amphotericin B for the primary treatment of invasive aspergillosis [24], and is additionally active against *Fusarium* and *Scedosporium* spp. [14].

Posaconazole is the most recent azole in clinical use with a wide antifungal spectrum, also showing activity against Mucorales spp. [23]. Posaconazole is primarly used for salvage therapy in patients with invasive aspergillosis, and as prophylaxis for neutropenic patients or hematopoietic stem cell transplant recipients [23]. The oral bioavailability of posaconzole is highly variable and therapeutic drug monitoring is recommended [25].

The triazoles inhibit the synthesis of ergosterol from lanosterol, targeting the the cytochrome P450 dependent 14-α-demethylase (CYP51), which catalyses the reaction [23]. Consequently, it leads to the substitution of methylated sterols and ergosterol depletion in the fungal membrane, as well as accumulation of toxic sterol intermediates finally causing inhibition of fungal growth [19]. Triazoles are in general fungistatic, with the exception of voriconazole and itraconazole exhibiting fungicidal activity against *Aspergillus* spp. [23].

Azole resistance.

Besides intrinsic antifungal resistance, acquired resistance in molds is rare. Nevertheless, clinical and laboratory studies have revealed that *A. fumigatus* can develop azole resistance, generally due to mutations in the *cyp51A* gene, encoding the target CYP51[19]. In *A. fumigatus*, different substitutions at codons 54, 98, 138, 220 and 448, and a 34-bp tandem repeat in the promoter region, are associated to resistance to one or more azoles [19]. Azole resistance can develop during azole therapy and clinical data suggests that reduced *in vitro* susceptibility is associated with increased probability of failure to azole therapy [19]. Additionally, a possible fungicide-driven route of resistance has been described proving a link between the use of azole fungicides in the environment and resistance development to medical azoles [26].

Echinocandins.

The newest class of antifungal agents is the echinocandins, including caspofungin, anidulafungin and micafungin. The echinocandins are large lipopeptides that act by inhibiting the fungal 1,3-β-D-glucan synthase, responsible for the biosynthesis of 1,3-β-D-glucan. The precise interaction between the drug and its target enzyme on a molecular level is not yet elucidated [27]. The echinocandins show concentration-dependent fungicidal activity against *Candida* spp. but only fungistatic activity against *Aspergillus* spp., where they introduce abnormal hyphal morphology (figure 3), lysis of rapidly growing bud tips and a reduced growth rate.

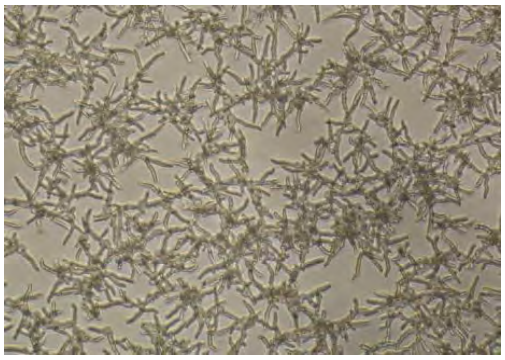

Figure 3. Abnormal hyphal growth of *A. fumigatus*, characterized by short abundant branching, in the presence of anidulafungin at a concentration of 0.03 μg/ml. Magnification x 40.

Echinocandins are not recommended as initial treatment of aspergillosis, due to their fungistatic activity but can be used in combination with amphotericin B and voriconazole [28]. Against other non-*Aspergillus* molds echinocandins show limited or no activity [29].

Antifungal combinations.

The availability of new antifungal agents, with novel modes of action, has raised the interest for combination therapy, when investigating new treatment options. Synergistic drug interactions could increase antifungal efficacy, prevent the emergence of resistance, and provide broader-spectrum antifungal activity than monotherapy regimens [30]. On the other hand, in case of antagonistic interactions, combination therapy may decrease the antifungal efficacy and increase toxicity [30]. In addition, if two or more antifungals are administered

simultaneously, the cost of the therapy will be greater and the risk for drug interactions increases [31]. Several antifungal combinations appear to have an improved activity *in vitro* and in animal models, but no appropriate clinical trials have so far been conducted [31]. Based on *in vitro* assays, potential antifungal combinations against *Aspergillus* spp include amphotericin B plus caspofungin or voriconazole, and voriconazole plus caspofungin, micafungin or itraconazole [31].

Antifungal susceptibility testing.

Different methods, including broth- and agar-based assays, exist for determination of *in vitro* antifungal susceptibility of molds. Both the Clinical Laboratory Standards Institute (CLSI) [32] and the Antifungal Susceptibility Testing Subcommittee of EUCAST (EUCAST-AFST) [33] guidelines recommend a microbroth dilution assay for the determination of the minimal inhibitory concentration (MIC). The guidelines recommend RPMI 1640 (CLSI) or RPMI 1640 supplemented with 2% glucose (EUCAST) as test culture media. Moreover, CLSI recommend an inoculum of 0.4 - 5 x 10^4 conidia/ml adjusted by spectrophotometry, whereas EUCAST use an inoculum of 1 - 2.5 x 10^5conidia/ml determined by counting using a hemocytometer chamber. Both tests are interpreted by visual examination at 24 h for Mucorales species and at 48 h for other species and the MIC is defined as the lowest concentration causing 100% of growth inhibition.

Due to the different mechanism of action of the echinocandins, the minimal effective concentration (MEC) was introduced to evaluate the antifungal activity, and is proposed as endpoint by both guidelines. The MEC is defined as the lowest concentration of drug causing abnormal growth, characterized by short abundant branching as observed by microscopy (figure 3) [34]. CLSI and EUCAST define the MEC as the lowest concentration resulting in macroscopic small compact rounded hyphal forms or microcolonies. Determination of the

MEC represents a subjective assessment of the appearance of growth, requiring experienced personnel for accurate interpretation.

Microbroth dilution assays are labor-intensive and need experienced personnel for interpretation. Therefore, several commercial test assays have been developed to facilitate the antifungal susceptibility testing. Sensititre Yeast One is a colorimetric microdilution method based on the CLSI guidelines for susceptibility testing of yeast [35]. The assay includes Alamar-blue that converts into pink in the presence of growth, which facilitates the test interpretation. Sensititre Yeast One showed good correlation with standard susceptibility testing of molds for amphotericin B and triazoles, whereas further evaluation is needed for echinocandins [36-38]. Agar-based methods, including disk diffusion testing and Etest , are easy-to-perform and cost efficient. Currently, there are no standardized conditions for the use of disk diffusion testing of molds but test parameters have been evaluated for different mold species, including optimization of culture conditions and correlation of the inhibition zone with MIC and MEC values obtained by the conventional method [39]. However, the disk diffusion method does not allow differentiation between susceptible, intermediate and resistant values for all species and antifungals [39]. Etest has shown good correlation with the microbroth dilution method for testing amphotericin B and triazoles against molds, but its utility for testing echinocandins is not fully evaluated [40, 41].

Susceptibility assays for evaluating antifungal combinations.

Microdilution broth checkerboard is the most commonly technique used to study antifungal combinations *in vitro*. The interaction is assessed based on the fractional inhibitory concentration (FIC) index [42]. The FIC is determined for each drug by dividing the MIC of the drug in combination by the MIC of the drug alone. A FIC index of < 0.5 indicates synergy and an index above 4 indicates antagonism. Majority of *in vitro* combination studies report results with FIC indices within the range of 0.5 to 4 concluding indifference or additivity [31].

The validity of this FIC range has been questioned, as it was chosen without *in vivo* or clinical correlation studies [43]. There are only few studies that have been using other methods, such as time-kill studies, for assessing antifungal combinations against molds. However, the utility of conidia in time-kill studies is not clear as conidia generally are absent in infected tissues and the fungicidal activity against actively growing hyphae would be more predictable for the treatment outcome [44, 45].

Clinical relevance of antifungal susceptibility testing .

Due to the often complex status of the host, it is difficult to confirm a correlation between *in vitro* susceptibility and treatment outcome, and establishing clinical breakpoints for molds is challenging. In addition, *in vitro* tests do not consider the dynamic biology of the molds *in vivo*, such as hyphal formation and infiltration, the pharmacokinetics at the site of infection and the host immune response [46]. Most often a poor outcome is related to the status of the host, a late diagnosis or a lack of appropriate antifungal therapy. The disagreement between *in vitro* and *in vivo* data is described by the "90-60 rule", according to which infections caused by susceptible strains respond to treatment in 90% of the cases, whereas in the case of resistant strains, the treatment response is 60 % [47].

The two standard methods for antifungal susceptibility testing (CLSI and EUCAST) differ in inoculum size and culture media, which influence the MIC and MEC values obtained. Breakpoints suggested by the CLSI can thus not be extrapolated to the EUCAST method and vice versa [48]. In addition, invasive mold isolates are often not identified at species level, despite that variability in susceptibility within genus has been reported [9, 12]. Nevertheless, the antifungal susceptibility subcommittee of EUCAST recently published breakpoints for amphotericin B, itraconazole and posaconazole for *Aspergillus* spp. based on epidemiological cut-off MIC values and clinical experience, but without a direct correlation between MIC and

clinical outcome [25]. For posaconazole, pharmacokinetic and pharmacodynamic data were also included. For *A. fumigatus* breakpoints of ≤ 1 μg/ml and > 2 μg/ml, corresponding to susceptible and resistant, respectively, were determined for amphotericin B and itraconazole. MIC values of ≤ 0.12 μg/ml and > 0.25 μg/ml, were the breakpoints for posaconazole. There are currently not sufficient data for assigning breakpoints for other *Aspergillus* spp.. Besides for optimization of antifungal therapy, performance of antifungal susceptibility testing is highly important for a continued surveillance of resistance as the current number of antifungal agents is limited.

Use of isothermal microcalorimetry in microbiology.

Microcalorimetry is a highly sensitive method, which enables measurement of microbial heat production in the range of microwatt. Replicating microbes produce heat proportionally to their metabolism and growth rate [49] . By isothermal microcalorimetry, variations in heat are measured under constant temperature and pressure [50]. A bacterial or fungal culture constitutes a close thermodynamic system, which will exchange heat with its surrounding proportionally to the number of replicating organisms. The heat produced can be recorded in real time, and plotted as heat flow (Watt) versus time (figure 4). The slope of the heat flow curve at each time point depends on the replication rate, whereas the total heat (Joule) (the area under the heat flow curve) is proportional to the final number of cells (figure 4) [49].
Currently, most of the isothermal microcalorimeters used for microbiological measurements are multi-channel batch calorimeters of heat-conduction type, such as “The Thermal Activity Monitor” (TAM 48, TA Instruments, New Castle, DE, USA), shown in figure 5A [50].

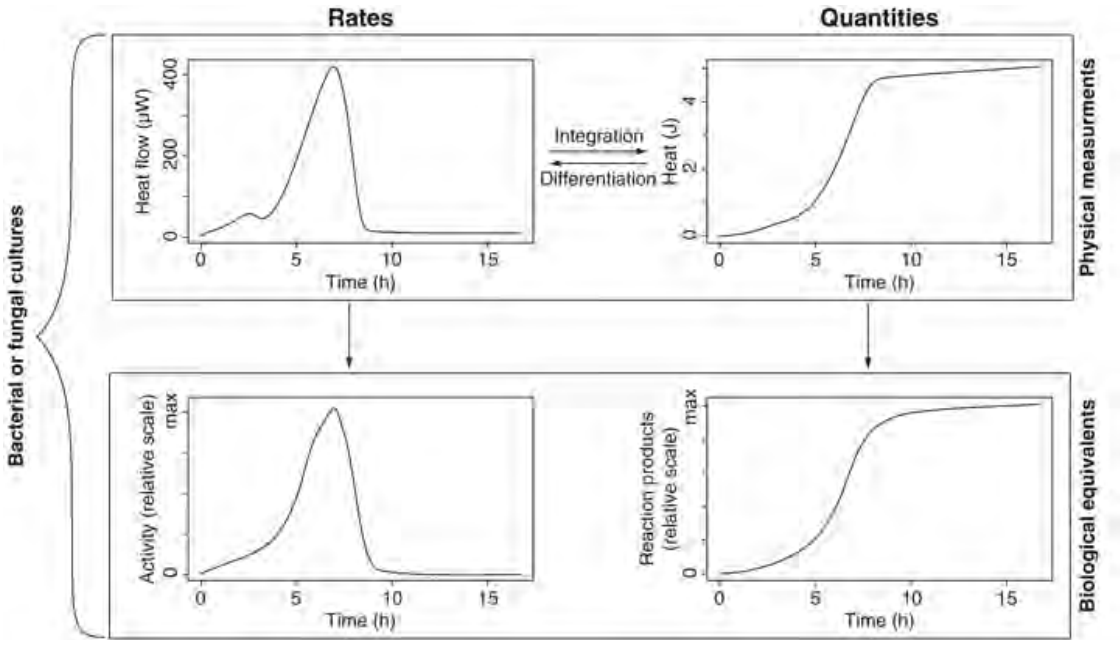

Figure 4. Relation between the microcalorimetric measurements and their biological equivalents. The heat flow represents the activity (growth rate) of a microbial culture. The area under the heat-flow curve gives the total amount of heat produced, representing the products resulting from microbial activity (total number of cells, biomass). Adapted from [49].

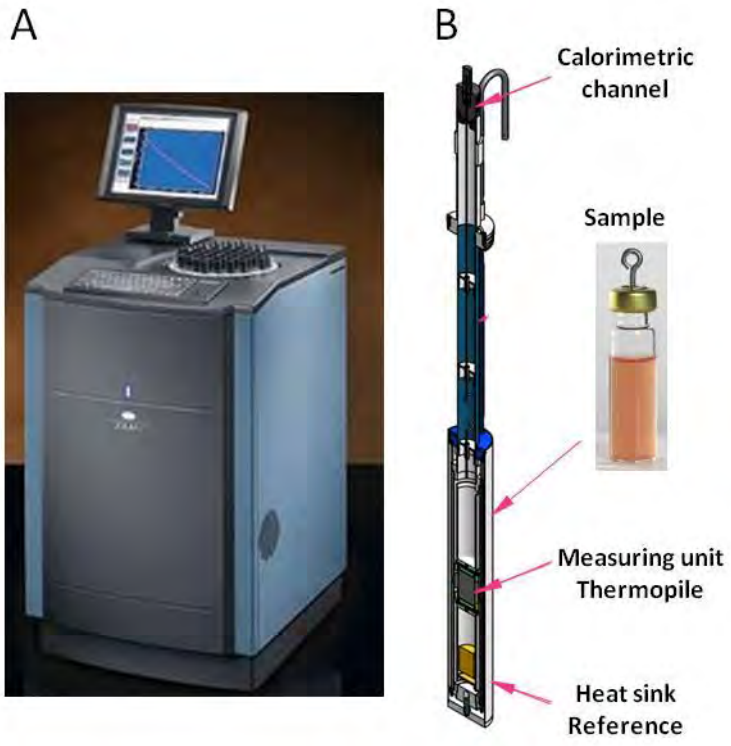

Figure 5.

A.The isothermal micrcocalorimeter TAM 48 (TA Instruments) with 48 independent channels.

B A schematic picture of a calorimetric channel including the heat sink, functioning like an inert reference and the sample (hermetically closed, 4 ml glass ampoule). The thermopile measures the difference in heat between the sample and the reference under a constant temperature.

From: TA Instruments (personal communication).

Heat-conduction calorimeters continuously measure difference in temperature between the sample and the heat sink (figure 5B). The heat sink functions like a thermally inert reference, and is generally made of aluminium [49]. The heat between the sample and the heat sink is transferred through a thermopile, which allows monitoring of consumed or produced heat by converting minor temperature differences into electrical signals [51]. The sensitivity of the TAM 48 is 0.2μW, which corresponds to approximately 100 000 bacteria assuming that a bacterial cell produces ~2pW [49]. The reaction vessel, including the sample and the heat sink, is positioned in a liquid (water or oil) thermostat, ensuring a temperature stability of 10^{-5}°C [50]. The temperature of the thermostat is adjustable in the range of 15-150 °C, and is in a microbiological setting normally set at 37°C.

An advantage of microcalorimetry for a microbiological assay, besides the sensitive growth detection, is that samples do not require specific preparation and they can still be used for further analysis after the microcalorimetric measurement. On the other hand, a major drawback is the measurement of non-specific signals, related to all chemical and physical reactions taking place in the sample [49]. Moreover, to perform an isothermal measurement a closed system is required, thus the sample is placed in a hermetically sealed ampoule (figure 5B). Chemical factors, such as oxygen depletion and accumulation of metabolic waste products, might influence the microbial growth and need to be taken into account [51].

Potential applications of isothermal microcalorimetry in clinical microbiology include primarily early growth detection and determination of antimicrobial susceptibility. A microcalorimeter measures all the growth-related heat originating from the sample and identification down to species level may be difficult, particularly in the case of mixed cultures. However, the use of selective growth media could allow the recovery and detection of specific microbes. During the last years, there have been several reports on the utility of isothermal microcalorimetry for growth detection of different pathogens, including urinary

tract pathogens [52] and mycobacteria [53], and detection of bacterial growth in donated blood platelets [54] and in cerebrospinal fluid from a rat model of bacterial meningitis [55].

Determination of antimicrobial susceptibility can be performed by incubation of the isolated pathogen with the antimicrobial of interest. Growth of susceptible microorganisms is inhibited and heat will not be produced, whereas resistant microbes are not inhibited and heat is produced also in the presence of the antimicrobial agent. The use of isothermal microcalorimetry for this application was reported for a rapid (5 h) differentiation between methicillin-susceptible and methicillin-resistant *S. aureus* [56], and determination of antimicrobial susceptibility of *Escherichia coli* , *S. aureus* [57], mycobacteria [58] and *Borrelia burgdorferi* (Achermann Y, Steinhuber A, Seiler E, Vogt M, and Trampuz A, presented at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in Washington, DC, 12-15 September 2009, poster Nr. 198). Isothermal microcalorimetry was also shown to be a promising tool for evalutation of anti-parastic drugs against *Schistosoma mansoni* [59], *Trypanosoma brucei* and *Plasmodium falciparum* [60] .

In order to render the currently available microcalorimeters suitable for routine microbiologic procedures, an input from the technical side is needed to make the instrument more user-friendly and enable automated high-throughput screening. In addition, the current cost of multichannel calorimeters is too high for a cost efficient clinical laboratory, taking into account the hands-on time and consumables. However, for experimental microbiology, an isothermal multichannel microcalorimeter is a valuable tool for evaluation of antimicrobial inhibitory profiles, including dose and time dependency and evaluation of drug-drug interactions.

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Chapter 8. Aim of the study.

The general aim of the study was to investigate the growth-related heat production of medically important molds and the effect of antifungal agents on the heat production by isothermal microcalorimetry. The microcalorimetric results were compared with conventional methods for susceptibility testing.

The first specific aim (chapter 9) was to evaluate microcalorimetry for antifungal susceptibility testing of *Aspergillus* species, including non-*fumigatus* species.

The second specific aim (chapter 10) was to validate the first study by testing other molds, such as species from the Mucorales order, *Fusarium* and *Scedosporium* species.

Finally (chapter 11), we used microcalorimetry for evalutation of antifungal combinations against *A. fumigatus* and *A. terreus*.

Chapter 9.

Isothermal microcalorimetry: a novel method for real-time determination of antifungal susceptibility of *Aspergillus* species.

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Isothermal microcalorimetry: a novel method for real-time determination of antifungal susceptibility of *Aspergillus* species

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Abstract

We evaluated microcalorimetry for real-time susceptibility testing of *Aspergillus* spp. based on growth-related heat production. The minimal heat inhibitory concentration (MHIC) for *A. fumigatus* ATCC 204305 was 1 mg/L for amphotericin B, 0.25 mg/L for voriconazole, 0.06 mg/L for posaconazole, 0.125 mg/L for caspofungin and 0.03 mg/L for anidulafungin. Agreement within two 2-fold dilutions between MHIC (determined by microcalorimetry) and MIC or MEC (determined by CLSI M38A) was 90% for amphotericin B, 100% for voriconazole, 90% for posaconazole and 70% for caspofungin. This proof-of-concept study demonstrated the potential of isothermal microcalorimetry for growth evaluation of *Aspergillus* spp. and real-time antifungal susceptibility testing.

Keywords: Antifungals, *Aspergillus* spp., heat production, microcalorimetry, susceptibility testing

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The prevalence of less susceptible *Aspergillus* strains is continuously rising, including non-*fumigatus* species [1], highlighting the need for antifungal susceptibility testing. Conditions for susceptibility testing of moulds by microbroth dilution have been defined by the CLSI [2] and the Antifungal Susceptibility Testing Subcommittee of EUCAST [3]. Broth-dilution assays are labour-intensive, variably reproducible and require experienced personnel. Sensititre Yeast One is a colorimetric microdilution method adapted from the susceptibility testing of yeast by the CLSI guidelines [4]. While Sensititre Yeast One showed good correlation with standard susceptibility of moulds for amphotericin B and triazoles, further evaluation is needed for echinocandins [5,6].

The principle of microcalorimetry relies on the measure of microbial heat production based on their growth and metabolism [7]. Recently, the potential of isothermal microcalorimetry was studied in the microbiology setting, including the differentiation between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* within 5 h [8], the susceptibility testing of *Escherichia coli* and *S. aureus* [9] and the evaluation of drugs against *Schistosoma mansoni* [10]. In this study, we investigated the potential of microcalorimetry for antifungal susceptibility testing of medically important *Aspergillus* species, including defined resistant mutants.

Ten *Aspergillus* strains were investigated (Table 1). They were subcultured on Sabouraud dextrose agar and identified on the basis of macroscopic and microscopic morphological features. Amphotericin B (Sigma, St Louis, MO, USA), voriconazole and posaconazole (TRC, North York, ON, Canada), caspofungin (Merck & Co., Inc., Whitehouse Station, NJ, USA) and anidulafungin (Pfizer Pharma AG, Zürich, Switzerland) were tested. Microbroth dilution was performed as described in the CLSI document M38-A2 [2]. The minimal effective concentration (MEC) was determined only for caspofungin, defined as the lowest drug concentration at which short and branched hyphae were observed [11]. Antifungal susceptibility testing was additionally performed with the commercial Sensititre YeastOne panel (Trek Diagnostic System Ltd, East Grinstead, UK) according to the manufacturer's instructions. Colorimetric MEC was defined as the lowest concentration visibly reducing growth (despite presence of red or purple colour). We added the colorimetric Sensititre YeastOne assay for comparison of susceptibility testing because several routine microbiology laboratories are using this method rather than the CLSI-reference microbroth dilution.

For microcalorimetric evaluation of growth characteristics, Sabouraud dextrose broth (SDB) (Oxoid CM0147; Basingstoke, Hampshire, UK) was used (3 mL medium and 1 mL air in the headspace of the ampoule). An inoculum of c. 2.5×10^4 conidia/mL was used, determined by microscopic

TABLE 1. Susceptibility testing of *Aspergillus* spp. (in mg/L), expressed as MICs (determined by microbroth dilution according to CLSI guidelines or by colorimetric Sensititre YeastOne assay) and MHICs (determined by isothermal microcalorimetry). For echinocandins, the minimal effective concentrations (MECs) are reported instead of MICs

Species	Strain description	Amphotericin B			Voriconazole			Posaconazole			Caspofungin			Anidulafungin		
		MIC-CLSI	MIC-Color	MHIC	MIC-CLSI	MIC-Color	MHIC	MIC-CLSI	MIC-Color	MHIC	MEC-CLSI	MEC-Color	MHIC	MEC-CLSI	MEC-Color	MHIC
A. fumigatus	ATCC 204305	0.5	2	1	0.5	0.25	0.25	0.06	0.125	0.06	0.5	0.125	0.125	–	–	0.03
A. fumigatus	Wild-type CM-237	0.5	1	1	0.5	0.25	0.5	0.06	0.125	0.25	0.5	0.06	0.5	–	–	0.03
A. fumigatus	Mutant CM-2097	0.5	1	1	0.5	0.125	0.25	16	2	>8	0.5	0.016	0.06	–	–	2
A. fumigatus	Mutant CM-796	0.5	1	1	1	0.25	0.5	16	1	2	0.5	0.03	0.03	–	–	0.03
A. flavus	ATCC 204305	1	2	2	0.5	0.5	0.25	0.25	0.25	0.5	1	0.125	0.25	–	–	0.03
A. terreus	ATCC 10690	1	1	4	0.5	0.25	0.25	0.125	0.125	0.03	0.5	0.03	0.5	–	–	0.03
A. lentulus	CBS117.885	4	4	2	4	2	2	0.25	0.5	0.25	8	>16	8	–	–	1
A. oryzae	Clinical isolate	1	1	1	1	0.5	0.5	0.25	0.25	0.25	0.5	0.016	0.06	–	–	0.03
A. niger	Clinical isolate	0.06	1	0.5	1	0.5	1	0.125	0.25	0.125	0.25	0.03	0.25	–	–	0.125
A. nidulans	Clinical isolate	1	1	0.5	0.25	0.25	0.5	0.25	0.25	0.125	2	4	0.5	–	–	0.03

MIC-CLSI, MIC determined by microbroth dilution according to CLSI document M38-A2 [2]; MIC-color, MIC determined by colorimetric Sensititre YeastOne assay; MHIC, minimal heat inhibition concentration determined by microcalorimetry. For echinocandins, the minimal effective concentrations (MECs) are reported.

enumeration using a Neubauer haematocytometer. Air-tightly sealed ampoules were introduced into the isothermal microcalorimeter (TAM III; TA Instruments, Newcastle, DE, USA) and measurements were performed at 37°C every 10 s. The detection threshold was determined at 5 μW to distinguish fungal heat production from the thermal background noise (i.e. growth media without moulds). The detection time (in h), heat-flow peak (in μW), the time to peak (h) and total heat produced (in Joules, represented by the area under the heat-flow curve) at 24, 48 and 72 h were determined. For testing antifungal agents, two-fold dilutions of antifungal agents were added to SDB. The minimal heat inhibitory concentration (MHIC) of amphotericin B and triazoles was defined as the lowest antifungal concentration inhibiting 50% of the total heat produced by the growth control at 48 h. For echinocandins, the MHIC was defined as the lowest concentration reducing the heat-flow peak by 50%. Susceptibility testing experiments were performed in duplicate. Data analysis was carried out with the manufacturer's software (TAM Assistant; TA Instruments) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

The thermokinetic characteristics of the *Aspergillus* strains tested in the absence of antifungals are presented in Table S1. The median heat detection time was <5 h, except for *A. fumigatus* CM-2097 and *A. niger*, which required 14 and 7.7 h, respectively. The total heat produced ranged between 4.4–8.3 J after 48 h and 4.7–10 J after 72 h. The median time to reach the heat-flow peak was 22 h. The heat production stopped completely after the peak was reached (i.e. the heat-flow curve returned to baseline), except for *A. flavus* and *A. oryzae*, which continuously produced heat of *c.* 20 μW up to 72 h. Among different growth media tested (data not shown), SDB showed a larger heat production (and shorter detection time) than RPMI 1640. Therefore, SDB was used in subsequent antifungal susceptibility experiments. Similar observations using growth curves were reported by Meletiadis *et al.* [12].

Table 1 summarizes the MHIC values obtained by microcalorimetry, and the MIC and MEC values obtained by the CLSI microbroth dilution and by Sensititre YeastOne. Fig. 1 shows the effect of antifungals tested on the heat production of *A. fumigatus* ATCC 204305. Amphotericin B (Fig. 1a) delayed the heat production in a concentration-dependent manner without significant change of the heat-flow curve profile, indicating its fungicidal activity. A similar killing effect of bactericidal antibiotics was previously described for bacteria [13]. Voriconazole and posaconazole (Fig. 1b,c) showed a fungistatic effect at low concentrations, reflected by a reduced initial heat-flow slope, but delayed the fungal heat production at higher concentrations (fungicidal activity). A similar fungistatic effect of various alcohols was previously reported on yeasts [14].

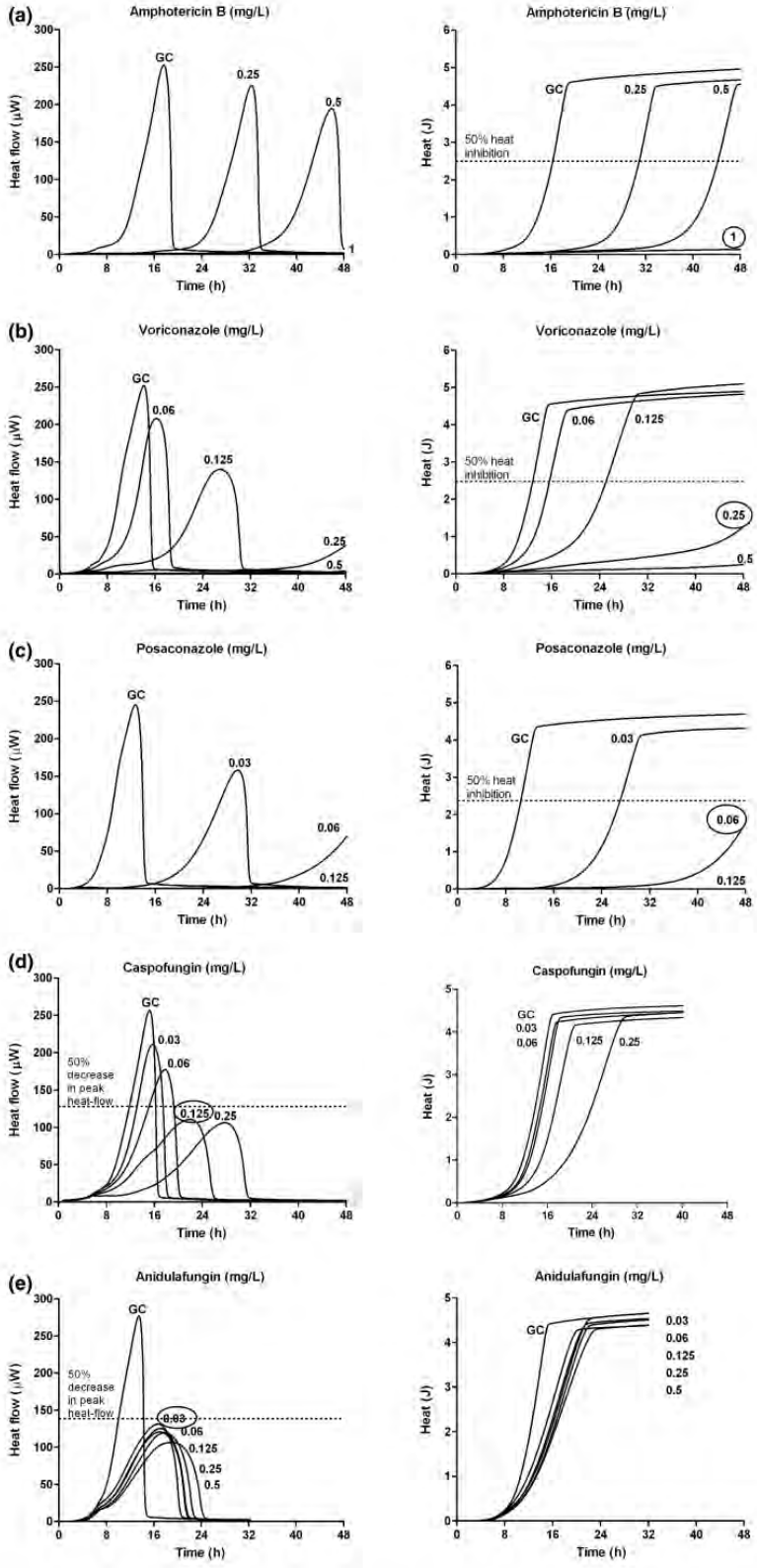

FIG. 1. Determination of the minimal heat inhibitory concentration (MHIC) by microcalorimetry for *A. fumigatus* ATCC 204305. Heat flow (left panel) and total heat (right panel) of *A. fumigatus* in the presence of amphotericin B (a), voriconazole (b), posaconazole (c), caspofungin (d) and anidulafungin (e) in SDB medium using an inoculum of 2.5×10^4 conidia/mL. Numbers indicate the antifungal concentration (in mg/L); GC denotes growth control without antifungals. The dotted horizontal line indicates 50% of inhibition of total heat produced by the growth control after 48 h (for amphotericin B and triazoles) or a 50% decrease in the heat-flow peak (for echinocandins). The MHIC value is circled.

Triazoles in addition decreased the heat-flow peak, whereas this effect was not observed with amphotericin B. While echinocandins are fungicidal against *Candida* species, they are only fungistatic against *Aspergillus* species [15]. This difference was also observed by microcalorimetry, where caspofungin and anidulafungin (Fig. 1d,e) changed the initial slope of the heat-flow and lowered the peak, but were not able to completely inhibit heat production. This effect could be explained by the interference of antifungals in *Aspergillus* metabolic activity, changing their growth mode from filamentous to granular. With the conventional susceptibility method, the change of growth mode is determined by visual inspection; the determination of MEC requires experienced personnel and the interpretation can be subjective. A concentration-dependent activity of echinocandins on *Aspergillus* spp. was observed by microcalorimetry, which is in agreement with other reports [16]. In order to systematically evaluate the antifungal activity on non-*fumigatus Aspergillus* species, one drug from each antifungal class was investigated on each test strain (Figs S1–S6).

We challenged the microcalorimetry susceptibility assay by testing azole-resistant mutants of *A. fumigatus* [17,18] (Table 1 and Fig. S7). While no breakpoints have been determined for triazoles, an epidemiological cut-off value for posaconazole in *A. fumigatus* was determined at 0.25 mg/L and a resistance breakpoint of >0.5 mg/L was proposed [19]. Using this breakpoint value, all three methods were able to detect resistance, although variability in MIC/MHIC values was observed.

The percentage of agreement (within two 2-fold dilutions) between the MHIC and MIC (or MEC) by CLSI was 90%, 100%, 90% and 70% for amphotericin B, voriconazole, posaconazole and caspofungin, respectively. Of note, the Sensititre YeastOne assay has not been fully evaluated for echinocandins and the interpretation of MEC by this colorimetric method is challenging. For anidulafungin, lower MEC values against *Aspergillus* species were reported than for caspofungin [20], as was observed also in our microcalorimetry assay.

In summary, this proof-of-concept study demonstrated the potential of isothermal microcalorimetry for growth evaluation of *Aspergillus* spp. and the determination of its antifungal susceptibility in real time. This method might be used for testing new antifungal agents, including investigation of their mode-of-action, growth-phase specific activity and interactions between antifungals.

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Some clinical strains were kindly provided by Reno Frei from the University Hospital Basel, Switzerland. *A. fumigatus* CM-237 and its isogenic mutants (CM-2097-AF1237 Δcyp51 and CM-796 Δcyp51) were kindly provided by Dr Emilia Mellado from Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain. We thank Doris Hohler for laboratory assistance and practical advice and Sophie Chevalley-Richard for performing the Sensititre YeastOne. Part of the results was presented at the 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Vienna, Austria, 10–13 April 2010, at the 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in Boston, USA, 12–15 September 2010 and at the 51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in Chicago, USA, 16–21 September 2011.

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Transparency Declaration

J.F.M. received grants from Astellas, Merck, Basilea and Schering-Plough. He has been a consultant to Basilea and Merck, and received speaker's fees from Merck, Pfizer, Schering-Plough, Gilead and Janssen Pharmaceutica. A.T. received grants from Pfizer and Gilead. All other authors: no potential conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Heat flow (left panel) and total heat (right panel) of *Aspergillus terreus* in the presence of amphotericin B (a), voriconazole (b) and anidulafungin (c) in SDB medium using an inoculum of *c.* 10^4 conidia/mL.

Figure S2. Heat flow (left panel) and total heat (right panel) of *Aspergillus flavus* in the presence of amphotericin B (a), voriconazole (b) and caspofungin (c).

Figure S3. Heat flow (left panel) and total heat (right panel) of *Aspergillus niger* in the presence of amphotericin B (a), posaconazole (b) and caspofungin (c).

Figure S4. Heat flow (left panel) and total heat (right panel) of *Aspergillus oryzae* in the presence of amphotericin B (a), posaconazole (b) and anidulafungin (c).

Figure S5. Heat flow (left panel) and total heat (right panel) of *Aspergillus lentulus* in the presence of amphotericin B (a), voriconazole (b) and anidulafungin (c).

Figure S6. Heat flow (left panel) and total heat (right panel) of *Aspergillus nidulans* in the presence of amphotericin B (a), posaconazole (b) and caspofungin (c).

Figure S7. Heat flow (left panel) and total heat (right panel) of *Aspergillus fumigatus* CM-237 (wild type) MIC 0.06 (a), CM-796 (*Δ*cyp51a/b) MIC 16 (b) and CM-2097 (*Δ*cyp51a) MIC 16 (c) in the presence of posaconazole.

Table S1. Calorimetric characteristics of *Aspergillus* species in SDB at 37°C. The inoculum was *c.* 10^4 conidia/mL.

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Supporting information

Figure S1. Heat flow (left panel) and total heat (right panel) of *Aspergillus terreus* in the presence of amphotericin B (A), voriconazole (B) and anidulafungin (C) in SDB medium using an inoculum of ~10^4 conidia/ml. Numbers indicate the antifungal concentration (in mg/L); GC denotes growth control without antifungals.

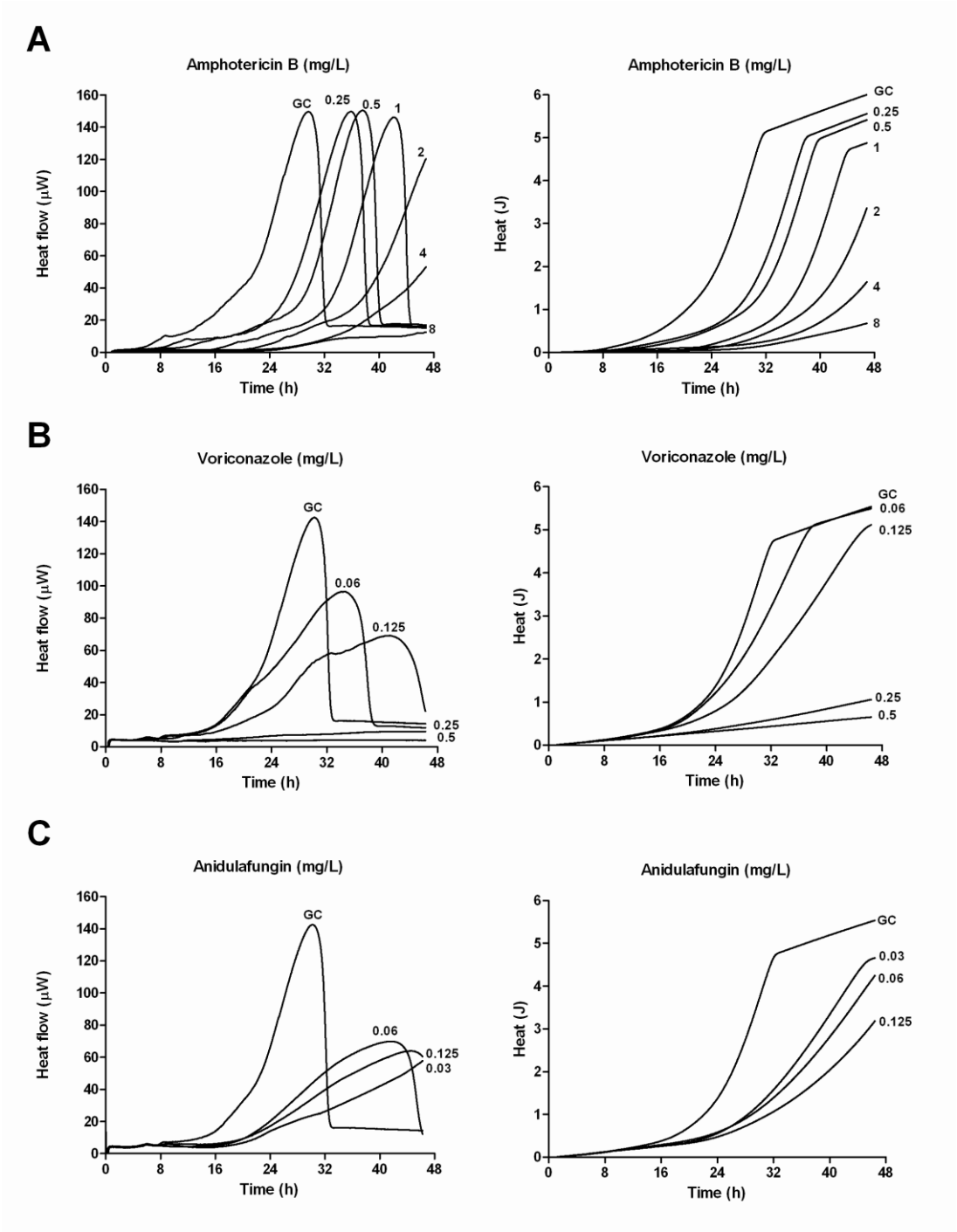

Figure S2. Heat flow (left panel) and total heat (right panel) of *Aspergillus flavus* in the presence of amphotericin B (A), voriconazole (B) and caspofungin (C). For details see legend to Figure S1.

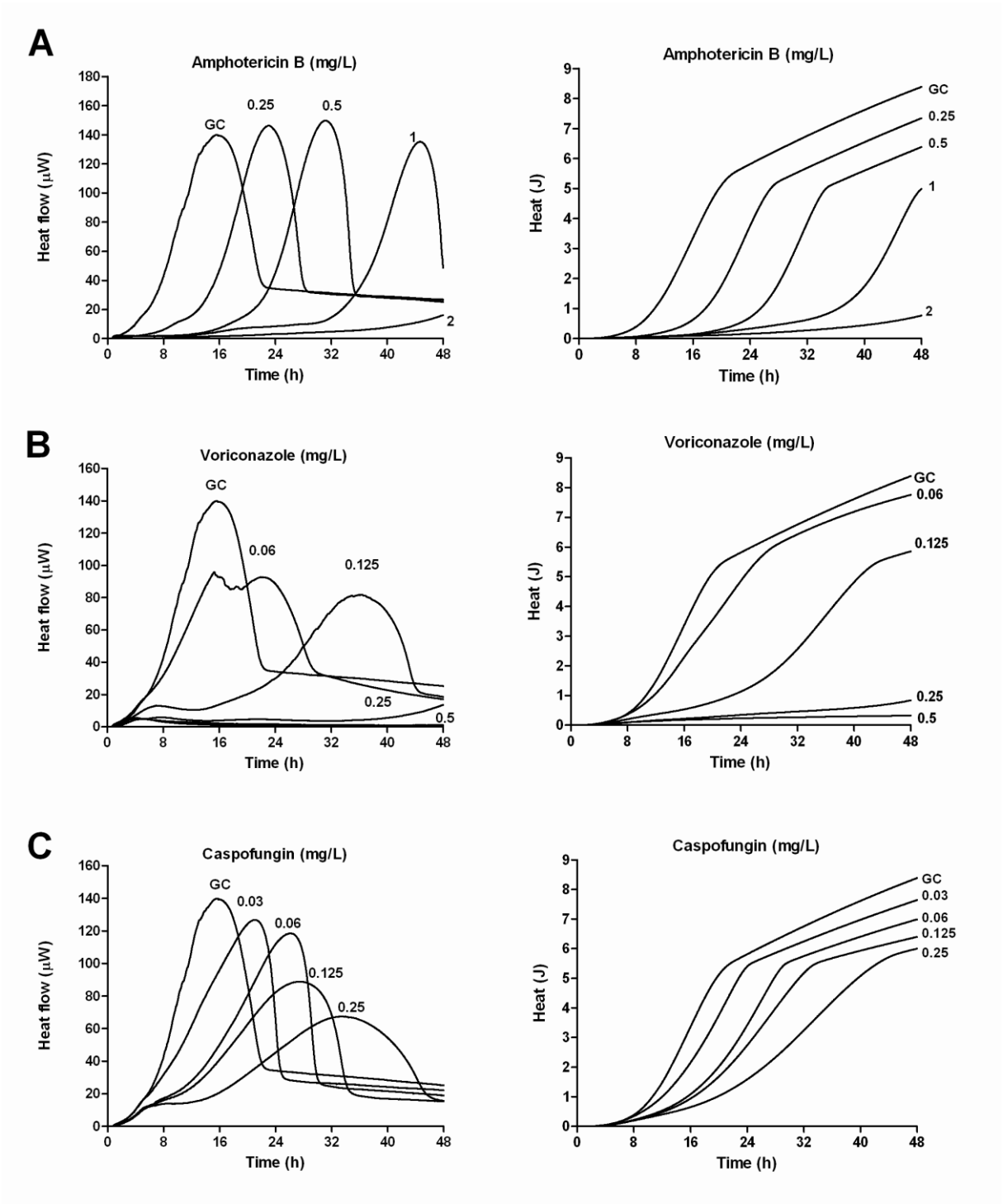

Figure S3. Heat flow(left panel) and total heat (right panel) of *Aspergillus niger* in the presence of amphotericin B (A) posaconazole (B) and caspofungin (C). For details see legend to Figure S1.

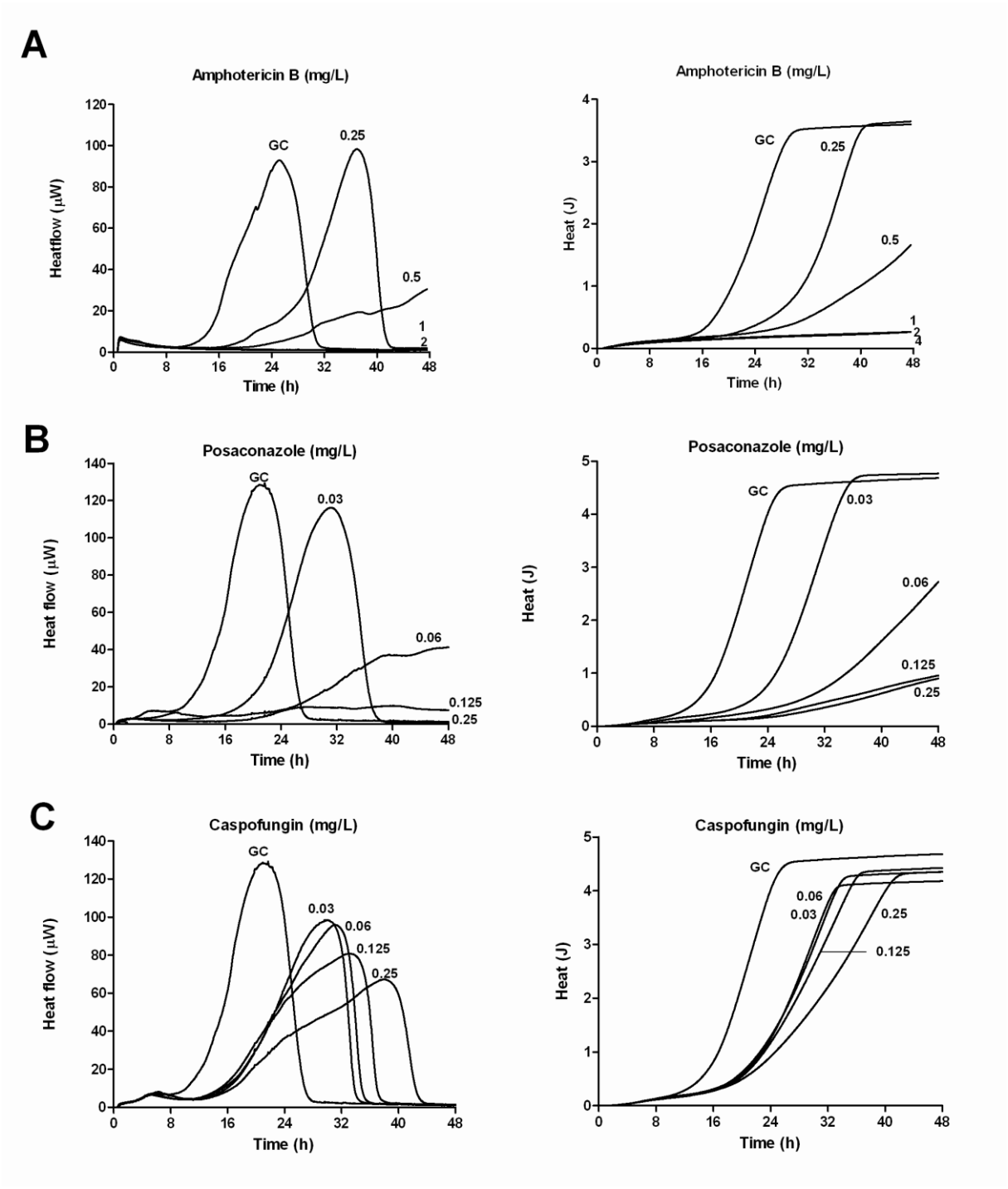

Figure S4. Heat flow (left panel) and total heat (right panel) of *Aspergillus oryzae* in the presence of amphotericin B (A) posaconazole (B) and anidulafungin (C). For details see legend to Figure S1.

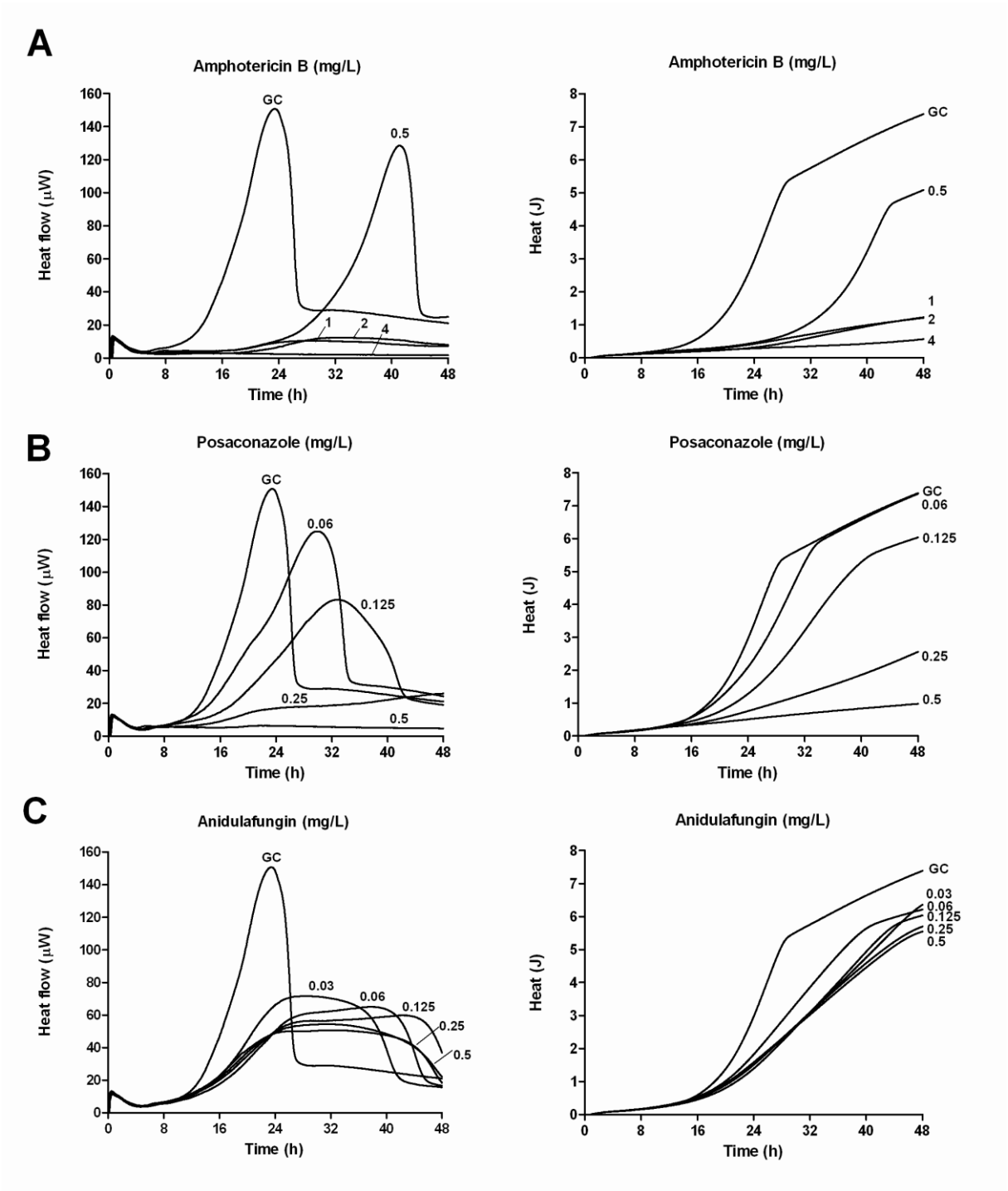

Figure S5. Heat flow (left panel) and total heat (right panel) of *Aspergillus lentulus* in the presence of amphotericin B (A) voriconazole (B) and anidulafungin (C). For details see legend to Figure S1.

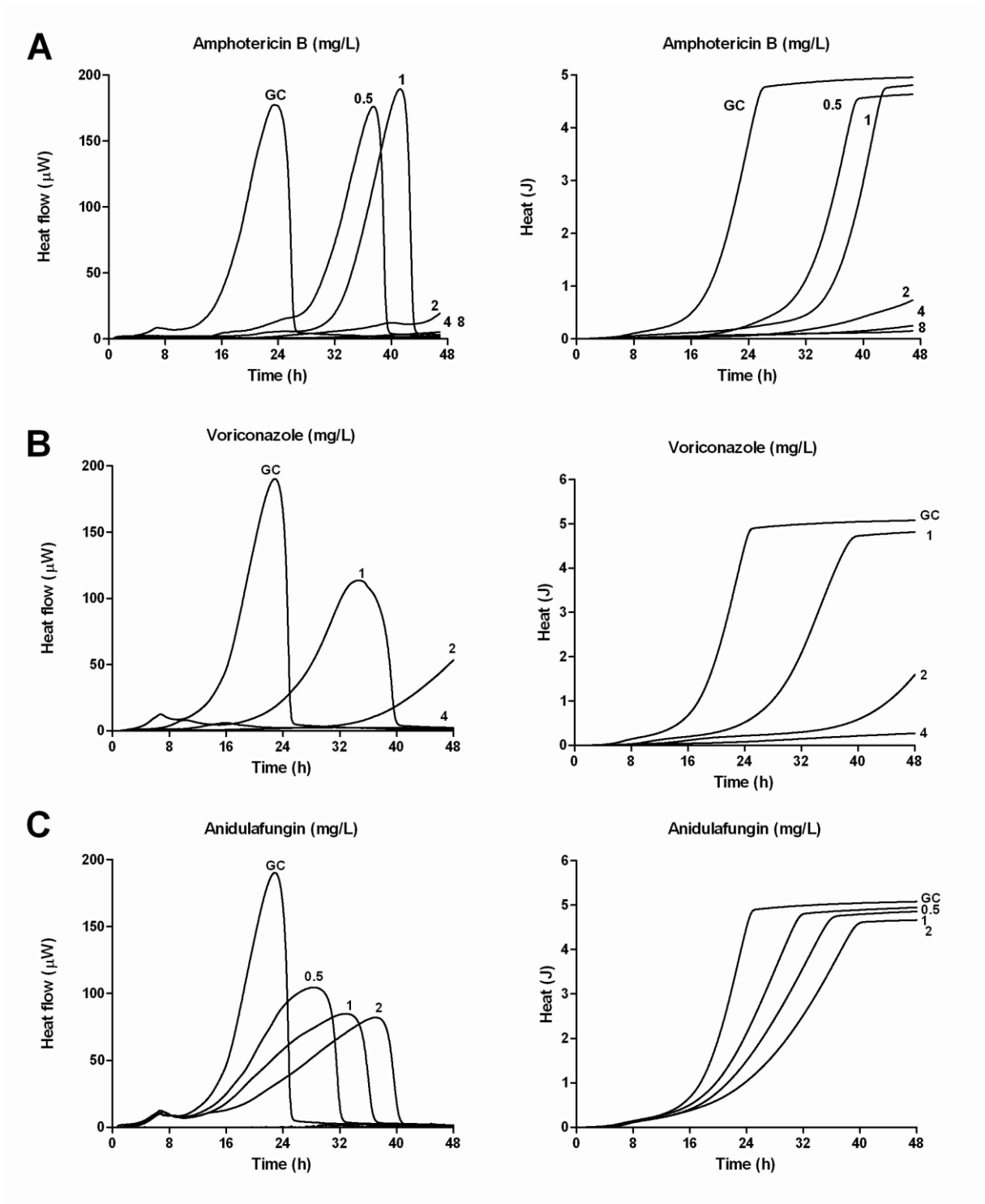

Figure S6. Heat flow (left panel) and total heat (right panel) of *Aspergillus nidulans* in the presence of amphotericin B (A) posaconazole (B) caspofungin(C). For details see legend to Figure S1.

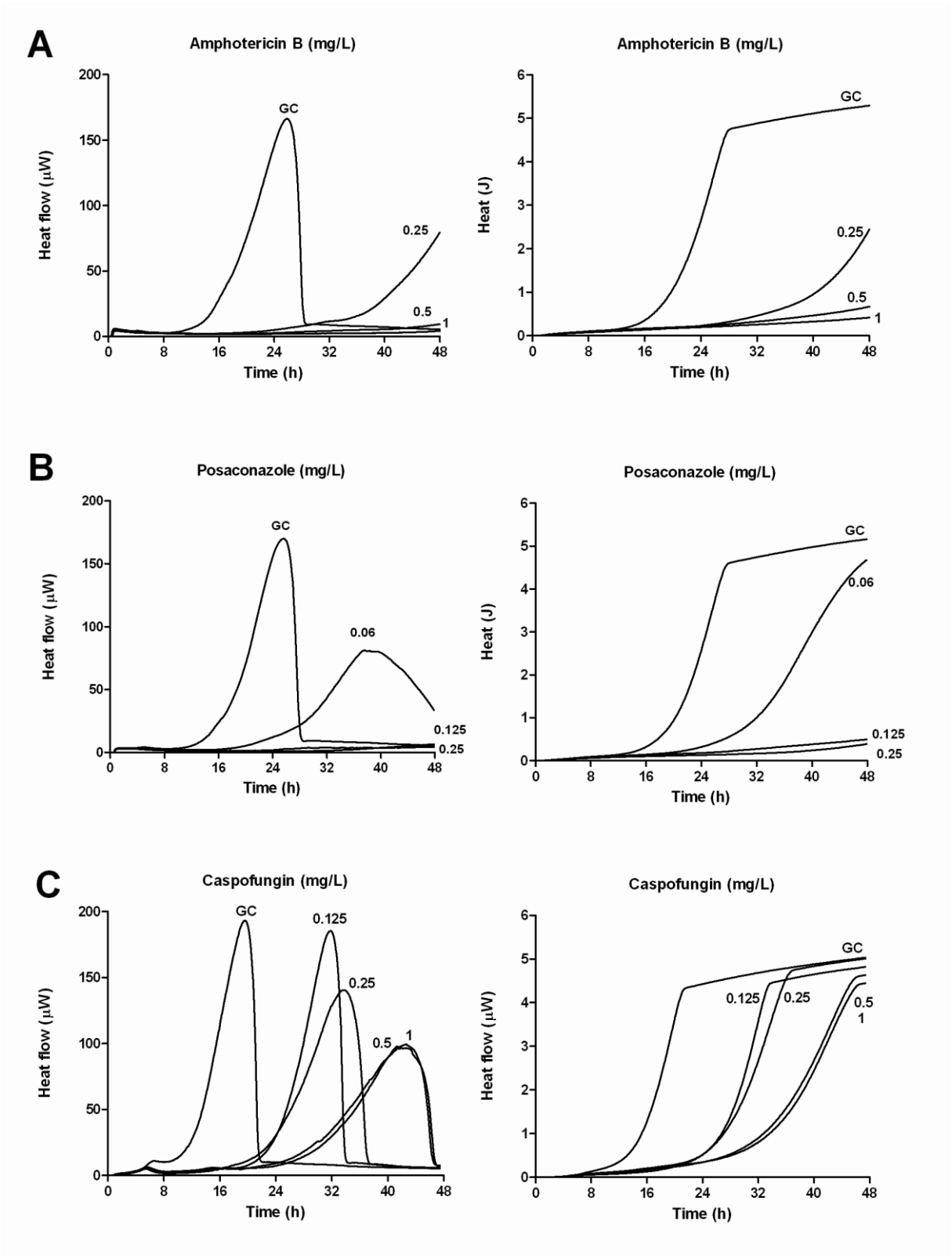

Figure S7. Heat flow (left panel) and total heat (right panel) of *Aspergillus* fumigatus CM-237 (wild type) MIC 0.06 (A), CM-796 (Δcyp51a/b) MIC 16 (B) and CM-2097 (Δcyp51a) MIC 16 (C) in the presence of posaconazole. For details see legend to Figure S1.

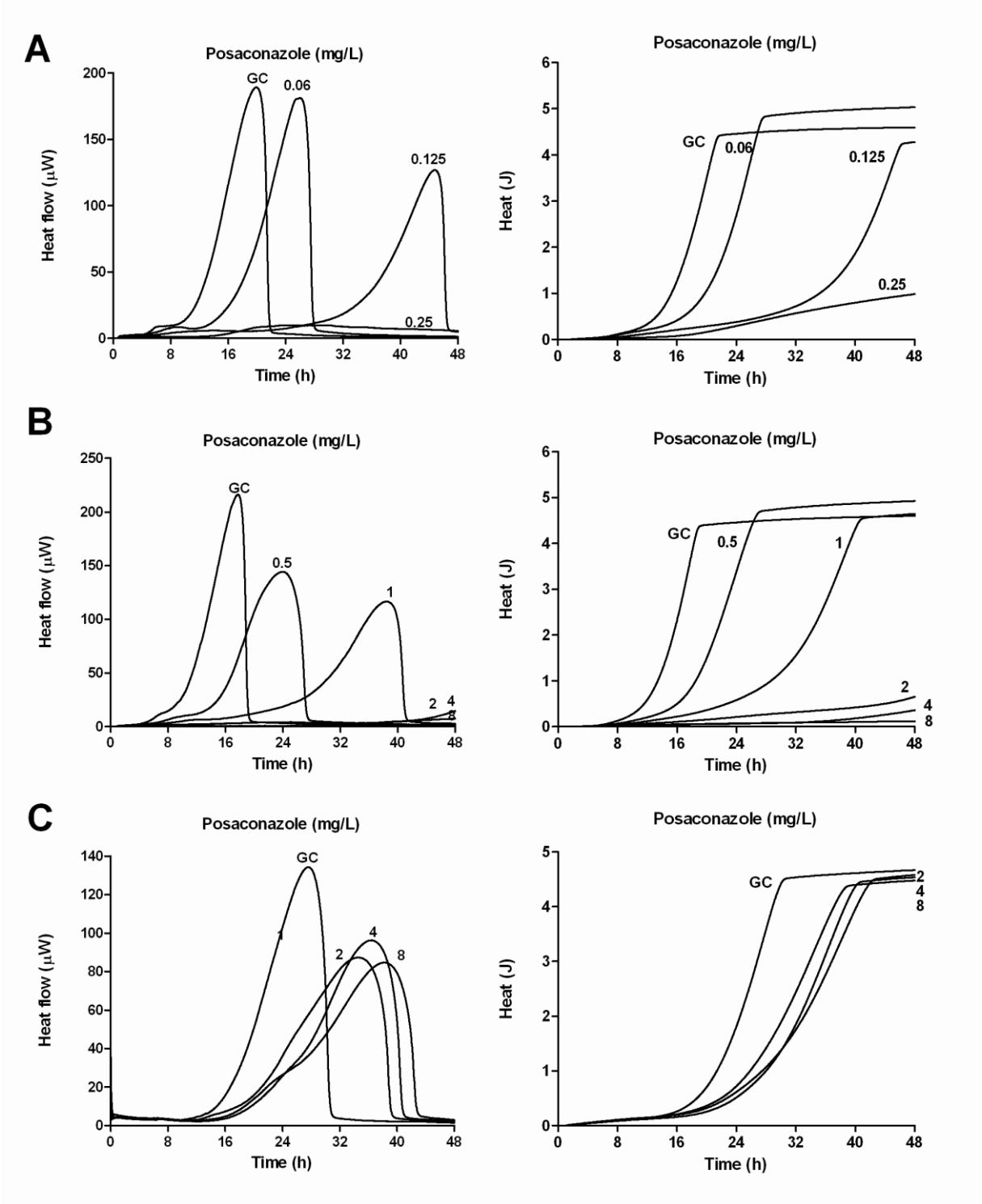

Table S1. Calorimetric characteristics of *Aspergillus* species in SDB at 37°C. The inoculum was ~10^4 conidia/ml.

Species	TTD (h)	Peak (μw)	TTP (h)	TH 24h (J)	TH 48h (J)	TH 72h (J)
A. fumigatus ATCC 204305	4.5 (3.0-5.8)	250 (203-277)	16.4 (11.0-18.6)	4.5 (4.3-4.8)	4.7 (4.3-5.1)	4.8 (4.4-5.2)
A. fumigatus CM-237	3.6 (3.3-3.9)	219 (212-220)	14.5 (14.5-18)	4.5 (4.4-4.7)	4.6 (4.5-4.9)	4.7 (4.6-4.9)
A. fumigatus CM-796	4 (3.7-4.5)	212 (202-232)	19.7 (16-20.9)	4.6 (4.4-4.7)	4.7 (4.5-4.9)	4.7 (4.6-4.8)
A. fumigatus CM-2097	14 (12.5-15.4)	129 (123-134)	30.5 (27.6-33.4)	1.2 (0.65-1.8)	4.8 (4.7-4.9)	4.9 (4.8-5)
A. terreus ATCC 10690	5.5 (5-6.7)	143 (139-150)	30.2 (29.7-30.8)	1.4 (1.4-1.7)	5.6 (5.5-6)	5.6 (5.5-6)
A. flavus ATCC 204304	3.8 (1.7-6.1)	162 (114-164)	25 (15.9-25.5)	5.8 (3.3-5.8)	8.3 (6-8.3)	10 (10-10.1)
A. lentulus CBS117.885	4.7 (4.3-4.7)	180 (170-190)	22.9 (15-22.9)	4.5 (4.4-4.6)	5 (4.7-5.1)	5.1 (4.7-5.2)
A. niger Clinical isolate	7.7 (3.7-12.7)	103 (62-129)	23.3 (21-32.5)	2.9 (1.9-4)	4.4 (3.6-4.7)	4.9 (4.8-4.9)
A. nidulans Clinical isolate	4.4 (4.4-9.6)	176 (165-205)	24.2 (18-24.5)	3.3 (3.1-4.8)	5.2 (5-5.4)	5.5 (5.4-5.6)
A. oryzae Clinical isolate	4.3 (2.1-6.3)	152 (146-157)	18.4 (13.3-26.8)	5 (3-6.6)	7.9 (6.8-8.7)	8.6 (8.1-9)

NOTE. Detection time was defined as exponential increase of the heat flow until 5 μW. TTD; time to detection, TTP; time to peak, TH; total heat.

***** Values are median values from the ≥ 3 replicates tested.

Chapter 10.

Isothermal microcalorimetry for antifungal susceptibility testing of Mucorales, *Fusarium* spp., and *Scedosporium* spp..

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Mycology

Isothermal microcalorimetry for antifungal susceptibility testing of Mucorales, *Fusarium* spp., and *Scedosporium* spp.☆,☆☆,★

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ABSTRACT

We evaluated isothermal microcalorimetry for real-time susceptibility testing of non-*Aspergillus* molds. MIC and minimal effective concentration (MEC) values of Mucorales ($n = 4$), *Fusarium* spp. ($n = 4$), and *Scedosporium* spp. ($n = 4$) were determined by microbroth dilution according to the Clinical Laboratory Standard Institute M38-A2 guidelines. Heat production of molds was measured at 37 °C in Sabouraud dextrose broth inoculated with 2.5×10^4 spores/mL in the presence of amphotericin B, voriconazole, posaconazole, caspofungin, and anidulafungin. As determined by microcalorimetry, amphotericin B was the most active agent against Mucorales (MHIC 0.06–0.125 μg/mL) and *Fusarium* spp. (MHIC 1–4 μg/mL), whereas voriconazole was the most active agent against *Scedosporium* spp. (MHIC 0.25 to 8 μg/mL). The percentage of agreement (within one 2-fold dilution) between the MHIC and MIC (or MEC) was 67%, 92%, 75%, and 83% for amphotericin B, voriconazole, posaconazole, and caspofungin, respectively. Microcalorimetry provides additional information on timing of antifungal activity, enabling further investigation of drug-mold and drug-drug interaction, and optimization of antifungal treatment.

1. Introduction

Invasive mold infections are increasingly reported in the clinical setting, particularly in immunocompromized patients. While *Aspergillus* remains the principal pathogen, other mold genera are emerging (Hsiue et al., 2010; Lass-Florl, 2009; Maschmeyer et al., 2009; Park et al., 2011). Infections due to non-*Aspergillus* species, such as those from the Mucorales order, *Fusarium* spp., and *Scedosporium* spp., are usually difficult to treat because of rapid tissue invasion and their intrinsic resistance to most first-line antifungal agents (Nucci, 2003; Park et al., 2011).

The most common species in the order of Mucorales include *Rhizopus arrhizus*, *Rhizomucor pusillus*, and *Lichtheimia corymbifera* (Chayakulkeeree et al., 2006), whereas *F. solani* is the principal pathogen among *Fusarium* species (Nucci and Anaissie, 2007). In vitro antifungal susceptibility profiles of Mucorales and *Fusarium* spp. (Azor et al., 2009a,b; Venturini et al., 2011) exhibit intermediate or full resistance against several antifungals, including triazoles and echinocandins (Castanheira et al., 2012; Espinel-Ingroff, 2003; Nucci and Anaissie, 2007; Torres-Narbona et al., 2007). However, amphotericin B and posaconazole show activity against most Mucorales with MIC_{50} values of 0.125 and 0.25 μg/mL, respectively (Dannaoui et al., 2003; Vitale et al., 2012). The genus *Scedosporium* includes at least 2 medically important members, *Pseudallesheria apiosperma* and *S. prolificans* (Cortez et al., 2008), which may colonize the respiratory tract and disseminate locally or systemically. Particularly *S. prolificans* is highly resistant to most antifungal agents, with MICs and MECs above 8 μg/mL for most drugs (Lackner et al., 2012). Micafungin, anidulafungin, and voriconazole demonstrated activity against *P. apiosperma* with MEC_{50} and MIC_{50} values of 0.125, 0.5, and 1 μg/mL, respectively (Lackner et al., 2012).

For susceptibility testing of conidia forming fungi, microbroth dilution is recommended by the Clinical Laboratory Standard Institute (CLSI, 2008) and the Antifungal Susceptibility Testing Subcommittee of EUCAST (EUCAST, 2008) as the standard method with defined inoculum preparation and culture conditions. However, with fast-growing Mucorales species it is often difficult to get accurate and

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★ Part of the results was presented at the 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in Boston, USA, 12–15 September 2010 and at the 5th Trends in Medical Mycology (TIMM) in Valencia, Spain, 2–5 October 2011.

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consistent end points (Dannaoui et al., 2003). CLSI recommends 24 h of incubation for *Mucorales* spp., but Dannaoui et al. (2003) demonstrated that most Mucorales strains tested showed sufficient growth to determine MICs after 16 h of incubation. Moreover, interpretive MIC breakpoints have not been defined for non-*Aspergillus* molds (Chayakulkeeree et al., 2006; Nucci and Anaissie, 2007) and the antifungal susceptibility testing is currently recommended only for *Aspergillus* spp. Because of nonavailability of validated susceptibility testing method and missing interpretive MIC breakpoints, the optimal antifungal treatment (either as single or combination regime) is difficult to determine.

Isothermal microcalorimetry enables highly sensitive, precise, and real-time measurement of microbial heat production, related to their growth and metabolism (Braissant et al., 2010a; Wadsö, 2001). The heat flow rate (in watts) is proportional to the growth rate of the microorganism at any time point, whereas the total heat (in joules) represents the cumulative heat produced by microbes until a determined time. The potential application of isothermal microcalorimetry in clinical microbiology was recently demonstrated for susceptibility testing of *Aspergillus* spp. (Furustrand Tafin et al., 2012) and *Escherichia coli* and *Staphylococcus aureus* (von Ah et al., 2009), the differentiation between methicillin-susceptible and methicillin-resistant *S. aureus* within 5 h (Baldoni et al., 2009), and for growth detection and susceptibility testing of mycobacteria (Braissant et al., 2010b; Howell et al., 2012). An advantage of microcalorimetry is measurement of growth-related heat instead of increase in optical quantity (e.g., visual inspection, absorbance, or fluorescence). This facilitates evaluation of growth of heterogeneously growing microorganisms, such as molds. Moreover, data are collected continuously over time allowing evaluation of subinhibitory concentrations, which is particularly interesting for studying concentration dependency and the activity of drug combinations.

In this study, we investigated the potential of isothermal microcalorimetry for determination of susceptibility testing of 3 medically important non-*Aspergillus* mold genera, including those with rapid (Mucorales), intermediate (*Fusarium* spp.), and slow growth characteristics (*Scedosporium* spp.). The microcalorimetry results were compared with conventional microbroth dilution testing. An accurate and reproducible susceptibility testing method for non-*Aspergillus* molds may help in guiding the choice of optimal antifungal treatment (alone or in combination) with potential improved clinical outcome.

2. Materials and methods

2.1. Test organisms

Strains used in this study were obtained from the Centraal Bureau voor Schimmelcultures (CBS), Utrecht, The Netherlands; the German Collection of Microorganisms (DSM); and from strain collections at the clinical microbiology laboratories at University Hospital Lausanne and University Hospital Basel, Switzerland. Among Mucorales, *Rhizopus arrhizus* (DSM 906), *Rhizomucor pusillus* (CBS 294.63), and 2 clinical strains of *Lichtheimia corymbifera* (L09186003, L09304177) were tested. Among *Fusarium* spp., 4 strains of *F. solani* (B1779, L2111, L10291190, L10450902) were tested. Among *Scedosporium* spp., the following strains were tested: *S. prolificans* (L 2121) and 3 strains of *P. apiosperma* (B6211, L09443001, L10361407). For quality control, *Aspergillus fumigatus* (ATCC 204305) and *A. flavus* (ATCC 204304) were used. Molds were subcultured on Sabouraud dextrose agar (SDA) for 3–7 days at 37 °C and identified on the basis of macroscopic and microscopic morphologic features, and by 26S rDNA sequencing. Stocks of each strain were maintained in water at 4 °C for short-time storage and in Sabouraud dextrose broth (SDB) with 20% glycerol at −80 °C for long-term storage.

2.2. Inoculum preparation

The inoculum size of the fungal suspension used for microcalorimetry was determined by microscopic enumeration of spores with a cell-counting haematocytometer (Neubauer chamber; Assistent, Sondheim, Germany). For the microbroth dilution, inoculum was defined according to the CLSI M38A2 guidelines (CLSI, 2008).

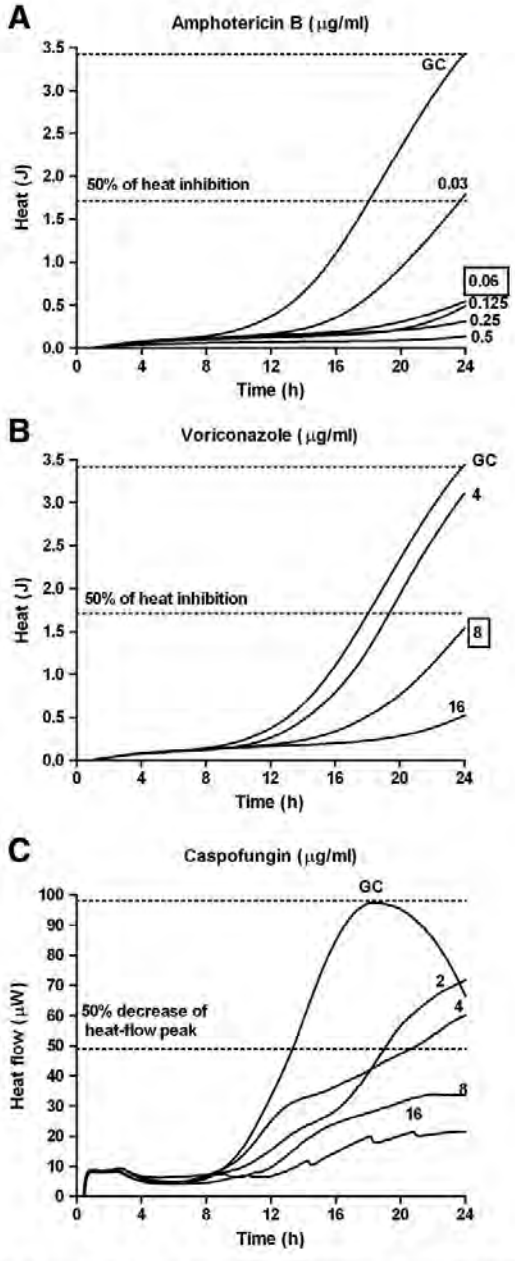

Fig. 1. Determination of the minimal heat inhibitory concentration (MHIC) by microcalorimetry using *L. corymbifera* (L0918 6003) as example and 3 classes of antifungals. The dotted horizontal lines indicate the total heat and a 50% of inhibition of total heat produced by the growth control after 24 h for amphotericin B (A) and voriconazole (B), respectively. In panel C, the dotted line indicates a 50% decrease in the heat-flow peak (for caspofungin). GC denotes growth control without antifungals.

Table 1
Microcalorimetric characteristics of molds in Sabouraud dextrose broth (SDB).

	Detection time (h)*	Heat-flow peak (μW)	Time to peak (h)	Total heat (J)		
				24 h	48 h	72 h
Mucorales	4.1 (1.3–7.3)	235 (79–348)	23 (17–24)	4.6 (2.7–11)	4.8 (4.5–18.5)	4.8 (4.7–20.5)
Fusarium spp.	7.1 (3.1–21.4)	27 (16–56)	64 (59 ->72)	0.6 (0.2–0.9)	1.8 (0.8–3.3)	3.8 (2–6.8)
Scedosporium spp.	17.05 (6.7–26.8)	53 (25–102)	53 (43 ->72)	0.2 (0.2–0.4)	1.2 (0.6–4.4)	3.7 (1.8–4.8)

The inoculum was ≈10^6 conidia/mL, incubated under static conditions without aeration at 37 °C. Numbers represent median (range) values.
* The detection time was defined as the time until heat flow reached 5 μW (for details see Materials and Methods).

2.3. Antifungal agents

Amphotericin B (Sigma, St. Louis, MO, USA), voriconazole, and posaconazole (TRC, North York, ON, Canada) were obtained in powder form and dissolved according to the manufacturer's instruction. Caspofungin was received from Merck (Whitehouse Station, NJ, USA). Anidulafungin was kindly provided by Pfizer Pharma (Zürich, Switzerland).

2.4. Susceptibility testing by microbroth dilution

Microbroth dilution was performed as described in the CLSI M38-A2 document (CLSI, 2008) by using RPMI 1640 medium with 0.2% glucose, round-bottom microdilution trays, inoculum of 0.4×10^4 to 5×10^4 spores/mL, and incubation at 35 °C for 24 h (Mucorales) or 48 h (for other molds); due to slower growth, the MIC for *Scedosporium* spp. was determined in addition after 72 h. The minimal effective concentration (MEC) values were determined for caspofungin and anidulafungin and defined as the lowest drug concentration at which short, stubby, and highly branched hyphae were observed (Kurtz et al., 1994).

2.5. Microcalorimetry measurements

An isothermal microcalorimeter (TAM III, TA Instruments, Newcastle, DE, USA), equipped with 48 calorimeters and a detection limit for heat production of 0.2 μW, was used. The inoculum was adjusted to a final concentration of 2.5×10^4 conidia/mL of growth medium by adding 0.05 mL of fungal suspension to 2.95 mL of medium (without aeration). For evaluation of growth characteristics, 3 mL of SDB (Oxoid CM0147; Basingstoke, Hampshire, UK) was used. In addition to the growth media, 1 mL of air was present in the headspace of the ampoule. The ampoules were air-tightly sealed and introduced into the microcalorimeter, first in the equilibration position for 15 min to reach 37 °C and avoid heat disturbance in the measuring position. Heat flow was recorded with a 10-s interval up to 72 h. The detection limit was determined at 5 μW in order to discard background noise. The negative control never exceeded this limit. The detection time (in hours), peak heat flow (in microwatts, the time to peak (in hours), and total heat produced (in joules, represented by the area under the heat-flow curve) at 24, 48, and 72 h were determined. Growth media without molds served as negative control. Experiments were repeated at least 3 times.

2.6. Susceptibility testing by microcalorimetry

For testing antifungal agents, 2-fold dilutions of antifungal agents were added to 3 mL of SDB. The minimal heat inhibitory concentration (MHIC) for amphotericin B and triazoles was defined as the lowest antifungal concentration inhibiting 50% of the total heat produced by the growth control (containing no antifungals) at 24 h for Mucorales, at 48 h for *Fusarium* spp., and at 48 and 72 h for *Scedosporium* spp. For echinocandins, the MHIC was defined as the lowest concentration reducing the heat-flow peak by 50% accompanied by a change in the initial slope of the heat flow curve. These changes are reflecting the distinct activity of echinocandins on the growth and represent the analogous parameter to the MEC (Kurtz et al., 1994). The parameters of antifungal susceptibility determined by microcalorimetry are illustrated in Fig. 1.

2.7. Data analysis

Data analysis was accomplished using the manufacturer's software (TAM Assistant, TA Instruments). Figures were plotted using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Table 2
Comparison of minimal inhibitory/effective concentration (MIC/MEC) values [obtained by the microbroth dilution assay according to CLSI (2008)] and the minimal heat inhibition concentration (MHIC) values (obtained by microcalorimetry).

Mold species	Strain	Amphotericin B		Voriconazole		Posaconazole		Caspofungin		Anidulafungin	
		MIC	MHIC	MIC	MHIC	MIC	MHIC	MEC	MHIC	MEC	MHIC
Order Mucorales											
R. arrhizus	DSM 906	0.06	0.125	8	8	0.125	0.125	>8	>16		>8
R. pusillus	CBS 294.63	0.06	0.06	16	4	0.5	0.25	0.5	16	4–8*	>8
L. corymbifera	L0918 6003	0.06	0.06	16	8	0.125	0.03	8	8	2–8*	4
L. corymbifera	LI 0930 4177	0.06	0.125	8	8	0.06	0.06	8	8	2–8*	4
Fusarium spp.											
F. solani	B1779	2	4	16	16	>16	>8	>8	8	>8**	>16
F. solani	L 2131	0.5	2	16	>16	>16	2	>8	16	>8**	>16
F. solani	L 10291190	1	2	16	8	>16	>8	>8	>16	>8**	>16
F. solani	L 1045 0902	2	1	16	8	>16	8	>8	>8	>8**	>16
Scedosporium spp.***											
S. prolificans	L2121	4/>16	8/8	16/>16	8/8	>16/>16	8/>8	4/>8	2/2	2–4**	1/1
P. apiosperma	B 6211	4/8	1/4	1/1	0.25/0.5	1/2	0.25/1	4/4	1/1	1–4**	1/1
P. apiosperma	L0944 3001	1/8	1/1	0.5/0.5	0.5/0.5	0.25/2	0.25/0.25	1/2	0.06/0.06	1–4**	0.25/0.25
P. apiosperma	L1036 1407	4/4	4/2	0.5/0.5	1/0.25	1/1	4/1	2/2	4/2	1–4**	4/4

The MIC/MEC and MHIC values were determined at 24 h for Mucorales, at 48 h for *Fusarium* spp., and at 48 and 72 h for *Scedosporium* spp.
* Odabasi et al. (2004).
** Castanheira et al. (2012).
*** The values for *Scedosporium* spp. are obtained after incubation of 48 h/72 h.

3. Results

3.1. Growth characteristics of molds by microcalorimetry

The heat-flow characteristics of the tested mold species are summarized in Table 1. While a similar pattern of heat-flow curves exist within each genus, distinct heat-flow profiles were observed between genera. Different growth characteristics influenced the heat detection time. For example, in fast-growing Mucorales, the detection time was <5 h, whereas the detection time for *Fusarium* spp. and *Scedosporium* spp. ranged between 7 and 17 h. Time to heat-flow peak, reflecting the time to reach the end of the exponential growth phase, was <24 h for Mucorales and ranged from 48 to >72 h for *Fusarium* and *Scedosporium* spp. Among the Mucorales species, *Rhizopus arrhizus* continuously produced heat, despite the heat flow reaching the peak at 20 h; the cumulative

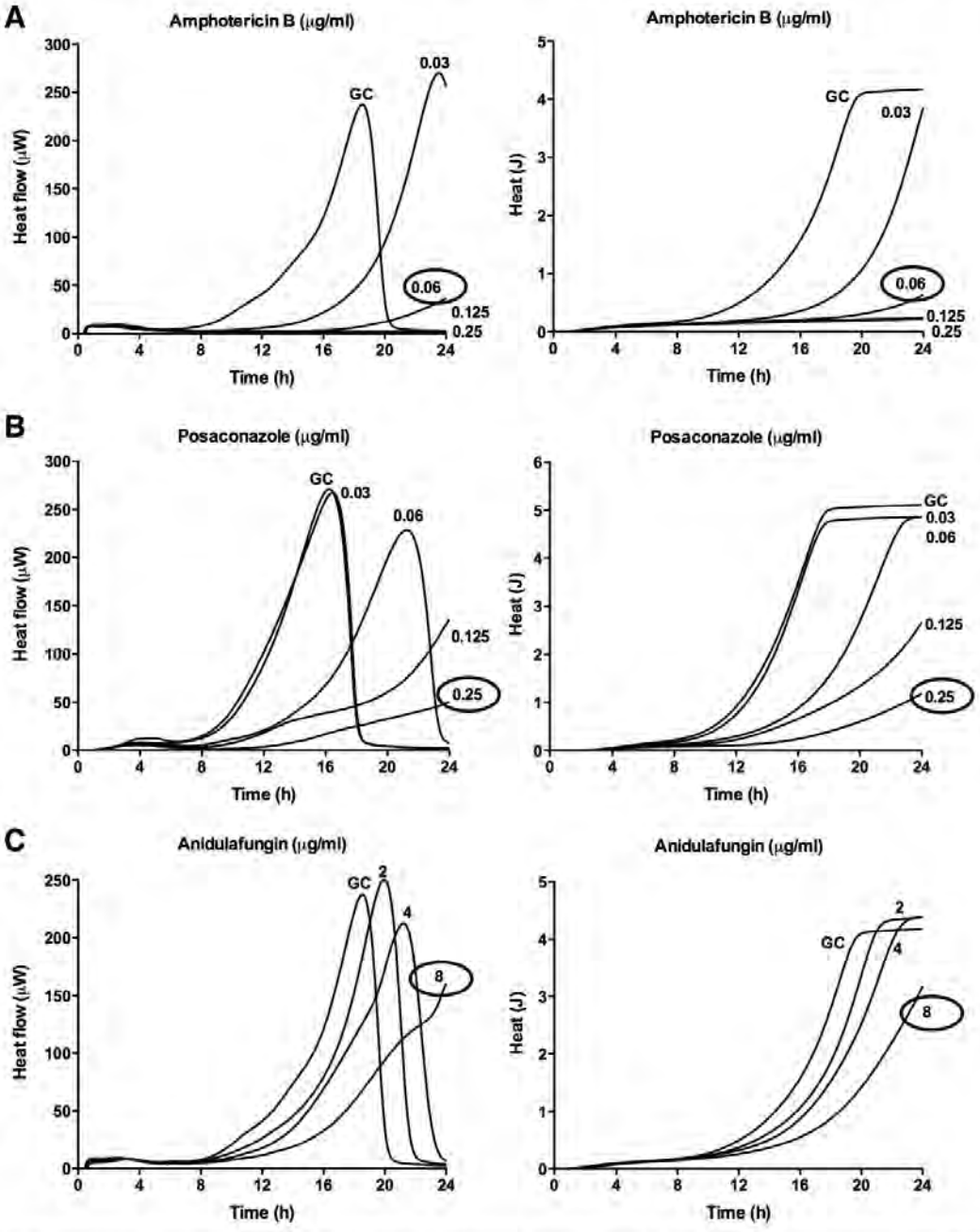

Fig. 2. Heat flow (left panel) and total heat (right panel) of *R. pusillus* (CBS 294.63) in the presence of amphotericin B (A), posaconazole (B), and anidulafungin (C) in SDB medium using an inoculum of ~10^4 conidia/mL. Numbers indicate the antifungal concentration (in micrograms per milliliter); GC denotes growth control without antifungals. The MHIC is circled.

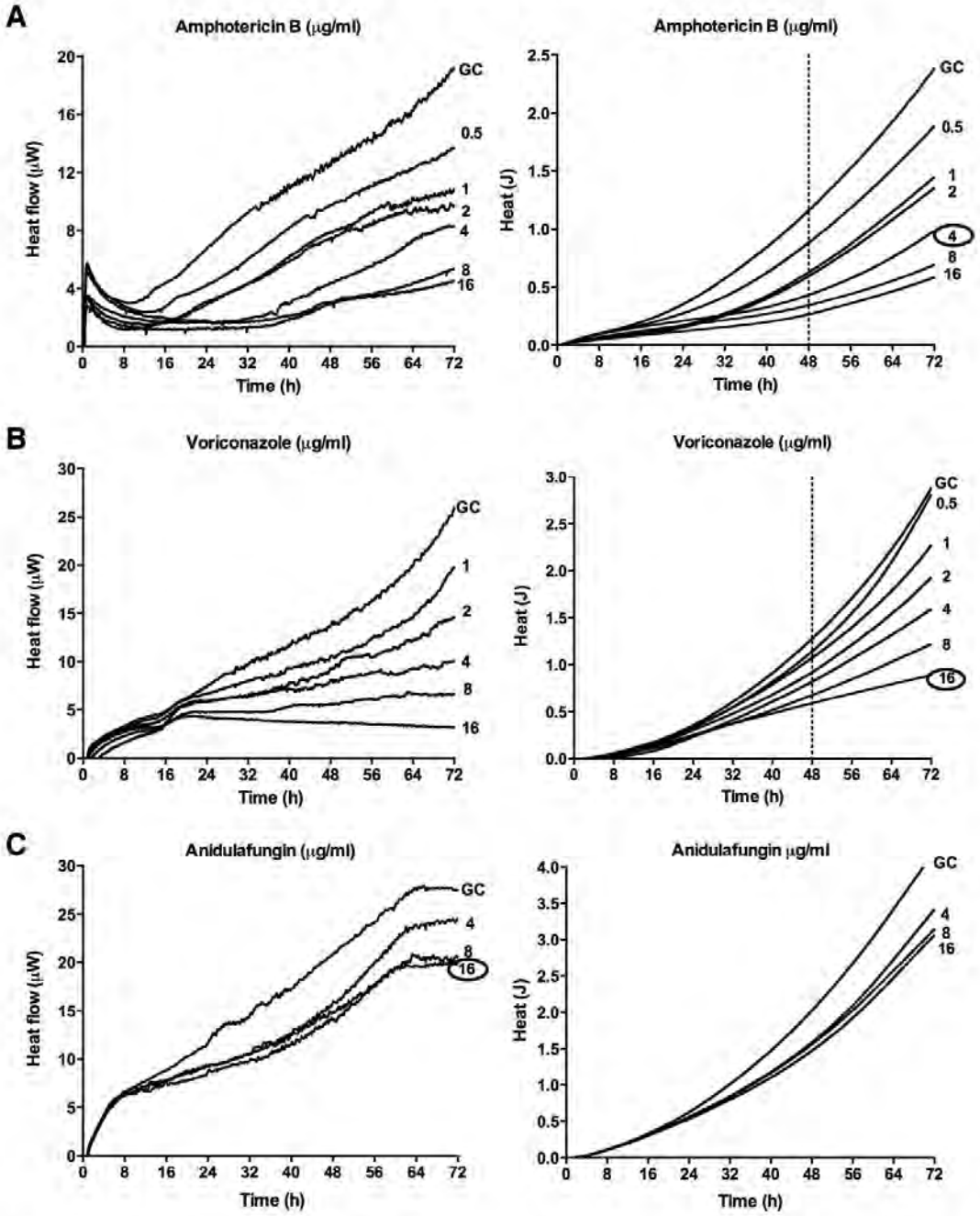

Fig. 3. Heat flow (left panel) and total heat (right panel) of *F. solani* (B1779) in the presence of amphotericin B (A), voriconazole (B), and anidulafungin (C). The dotted vertical line indicates the time point (48 h) at which the MHIC was read. For details see the legend to Fig. 2.

(total) heat increased from 9.3 J (at 24 h) to 16.8 J (at 48 h) and 19.6 J (at 72 h).

3.2. Susceptibility determined by microcalorimetry

Table 2 summarizes the MHIC values for tested molds and antifungals in comparison to MIC values determined according to the CLSI guidelines (CLSI, 2008). In Figs. 2–4, the activity of tested antifungals on 1 representative strain from each genus is shown. Fig. 2 shows the activity of one antifungal from each drug class on the heat production of *R. pusillus*. Amphotericin B (Fig. 2A) was the most active antifungal with an MHIC of 0.06 μg/mL, followed by posaconazole (Fig. 2B) with an MHIC of 0.25 μg/mL. Voriconazole and the echinocandins showed activity only at high concentrations. Fig. 3 shows the heat produced by *F. solani* up to 72 h to illustrate the continuous heat flow, but the end point for the MHIC was set at 48 h.

Amphotericin B was the most active antifungal agent against *F. solani* (Fig. 3A), whereas other antifungals showed no detectable activity against tested *Fusarium* strains (Fig. 3B and C, Table 2). While *S. prolificans* showed high MHICs for all antifungals (Table 2), good activity of triazoles was observed against *P. apiosperma* (Fig. 4B). The effect of anidulafungin (MHIC 1 μg/mL) on the change of the heat-flow curve of *P. apiosperma* is shown in Fig. 4C.

3.3. Correlation between MHIC (microcalorimetry) and MIC (microbroth dilution)

The percentage of agreement (within one 2-fold dilution) between the MHIC and MIC (or MEC) by CLSI was 67%, 92%, 75%, and 83% for amphotericin B, voriconazole, posaconazole, and caspofungin, respectively (incubation of 72 h for *Scedosporium* spp.). Tested Mucorales

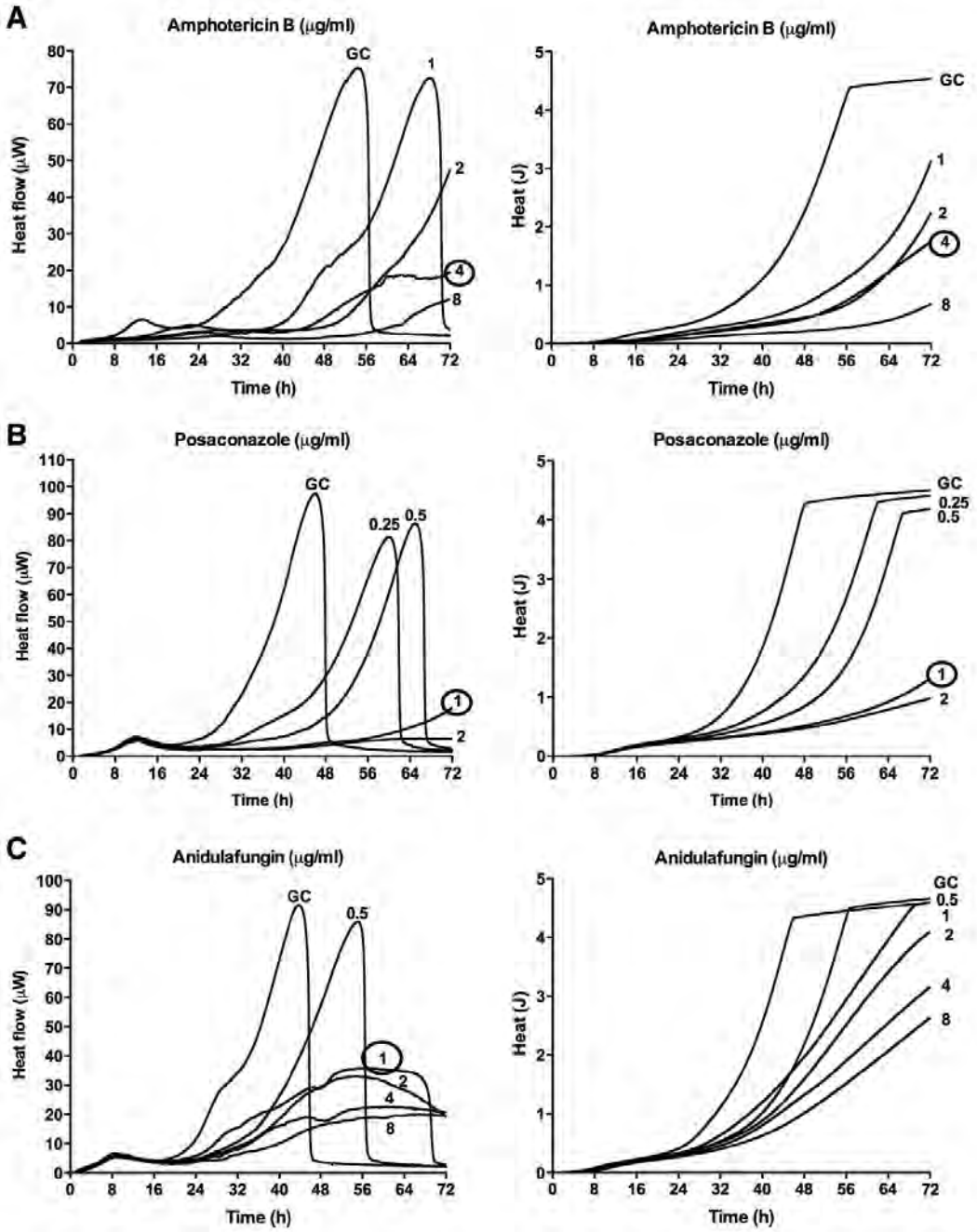

Fig. 4. Heat flow (left panel) and total heat (right panel) of *P. apiosperma* (B 6211) in the presence of amphotericin B (A), posaconazole (B), and caspofungin (C). The MHIC read at 72 h is circled. For details see the legend to Fig. 2.

strains exhibited, by both methods, low MIC/MHIC values for amphotericin B and posaconazole, but reduced susceptibility to voriconazole and echinocandins. Tested *F. solani* strains had MIC/MHIC values for amphotericin B ranging between 0.5 and 4 μg/mL, but poor activity was observed with other tested antifungals. In addition, by both susceptibility testing methods, no antifungal agent was active against *S. prolificans*.

4. Discussion

We evaluated growth characteristics of Mucorales, *Fusarium*, and *Scedosporium* by measuring heat production, related to their growth and metabolism. For tested species, different calorimetric curves (thermokinetic profiles) were observed depending on the metabolic characteristics (metabolic state, replication rate, cell size) of the mold and growth conditions, such as culture medium, incubation temperature, and ambient gas. Further studies are needed to determine whether individual species can be identified based on the shape of the heat-flow curve when using defined media.

Culture media recommended for susceptibility testing by CLSI (2008) and EUCAST (2008) (i.e., RPMI with 0.2% or 2% glucose) did not support well the growth of tested molds in the microcalorimetric assay (data not shown). Since RPMI may not be the most appropriate growth medium for molds (Kuzucu et al., 2004; Meletiadis et al., 2001), SDB was chosen for the microcalorimetric assay. No interaction between the media and the antifungals or reduced antifungal activity was observed.

Susceptibility testing of emerging rare mold species is challenged by their different speed of growth, which requires different end points for reading the susceptibility results depending on the genus. Isothermal microcalorimetry enables real-time measurement of the growth-related heat production, allowing "read-out" throughout the experiment which is an advantage particularly for fast-growing Mucorales, for which a susceptibility testing end point of 16 h was proposed (Dannaoui et al., 2003). Microcalorimetry additionally allows an objective data interpretation not based on visual inspection. Molds tested in this study are considered intrinsically resistant to several antifungals (Miceli and Lee, 2011), but great variability may exist within species belonging to the same genus and species complex, as was recently shown by Vitale et al. (2012) for the order Mucorales and for *Scedosporium* spp.(Lackner et al., 2012). Determination of antifungal susceptibility of these emerging molds is thus important in order to guide accurate treatment.

The definition of the MHIC was adapted to the growth rate of the fungus and in accordance with the end points used for the microbroth dilution. After evaluating the effect of antifungals on different microcalorimetric parameters, such as delay of heat detection and heat-flow peak and inhibition of total heat, a 50% inhibition of the total heat was used for determination of the MHIC for amphotericin B and triazoles. Another approach was taken to assess the activity of the echinocandins because of their different mechanism of action. Echinocandins have little activity against *Fusarium* spp., *Scedosporium* spp., and most Mucorales species (Miceli and Lee, 2011; Vitale et al., 2012). However, for the *P. apiosperma* and *L. corymbifera* strains tested, caspofungin and anidulafungin changed the heat-flow curve shape by lowering the heat-flow peak, which was used for the determination of the MHIC for these drugs. The MHIC is here comparable to the MEC for susceptibility testing of echinocandins. The change in heat-flow curve shape (lower heat flow peak) could reflect the different mode of action interfering with the metabolic activity when the mold changes its growth mode.

A limitation of the study was that the thermostat in the microcalorimeter was set at 37 °C for the current study, which is not the optimal temperature for growth of *Scedosporium* spp. and *Fusarium* spp., which shows optimal growth at 25–35° C (Guarro et al., 2006; Cortez et al., 2008; Palmero Llamas et al., 2008). The detection time for these molds could be shortened by lowering the temperature in the calorimeter. Additionally, anidulafungin was not tested by microdilution and our results were only compared with MEC values found in the literature (Castanheira et al., 2012; Odabasi et al., 2004).

In conclusion, we demonstrated, as a proof of principle, that microcalorimetry has the potential for early growth detection and for susceptibility testing of non-*Aspergillus* molds. However, to be used in a clinical setting the current device needs further technical improvement to automate the testing and enable high-throughput investigation. This method could be further used for testing new antifungal agents, including investigation of their mode of action, growth phase–specific activity, and interactions between antifungal agents.

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Chapter 11.

Evaluation of antifungal combinations against *Aspergillus fumigatus* and *Aspergillus terreus* by isothermal microcalorimetry.

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Abstract

Combining two antifungal agents with synergistic activity may improve the treatment outcome of invasive mold infections. We evaluated a highly sensitive assay for testing the activity of antifungal combinations against *Aspergillus fumigatus* and *A. terreus* based on their growth-related heat production (microcalorimetry). In parallel, microscopical evaluation of antifungal activity was performed by the checkerboard microdilution broth assay. Amphotericin B and voriconazole, alone or in combination with caspofungin or anidulafungin, at concentrations of 0.125 x, 0.25 x, 0.5 x or 1 x MIC (or the MEC for echinocandins), were tested in Sauboraud dextrose broth containing 2.5 x 10^5 conidia/ml. Heat production was measured for 48 h at 37°C. A synergistic effect was defined as an increased delay of fungal heat-production in the presence of an antifungal combination compared to each drug alone at the same concentration. For *A. fumigatus*, the addition of caspofungin to amphotericin B and voriconazole, delayed the heat production with up to 4.1 and 7.4 h, respectively, whereas anidulafungin caused a delay up to 11.2 and 11.8 h, respectively. For *A. terreus*, the addition of caspofungin to amphotericin B and voriconazole, delayed the heat production with up to > 16.2 and 7.6 h, respectively, whereas anidulafungin caused a delay up to 10.9 h and 4.1 h, respectively. Growth reduction and change in hyphal morphology, observed by microscopy were in accordance with the microalorimetric data. Microcalorimetry enables an accurate and real-time evaluation of antifungal combinations against *Aspergillus* species and merits further evaluation for testing of other mold species and antifungal combinations.

Introduction.

In the last decades an increased prevalence of invasive mold infections has been reported (14, 17). The mortality rate associated with invasive infection is high and an early start of efficient antifungal treatment regimen is crucial for an improved outcome (11). The availability of new antifungal agents with different targets and modes of action has raised the interest for investigation of combination therapy to improve the treatment outcome. Drug combinations with synergistic activity could potentially increase the antifungal efficacy, prevent the emergence of resistance, and provide a broader antifungal spectrum for initial empiric therapy (10). However, combination therapy may also decrease the antifungal efficacy in case of antagonistic drug-drug interaction, increase the drug toxicity, promote spread of resistance and considerably rise the healthcare expenses (10). Therefore, investigation of antifungal activity of combination regimens is important and clinically relevant, as it may help planning the design of clinical trials aiming to improve treatment outcome of invasive mold infections.

Only few *in vitro* assays are currently available for testing of the activity of two or more antifungal agents. The checkerboard microdilution broth assay is the most widely used method to study antifungal combinations. The drug-drug interaction is assessed based on the fractional inhibitory concentration (FIC) index, determined by dividing the MIC of each drug in combination by the MIC of the drug alone (19). A FIC index of < 0.5 indicates synergy and an index above 4 indicates antagonism. The majority of combination studies report results with FIC indices ranging between 0.5 and 4, concluding indifference or additivity (22). However, the validity of this FIC range has been questioned, as the correlation with clinical outcome is lacking (15). Few studies have investigated other *in vitro* assays, such as time-kill studies. However, the utility of conidia in time-kill studies is not clear as conidia generally are absent in infected tissues and the fungicidal activity against actively growing hyphae would be more predictable for the treatment outcome (12, 18).

We recently demonstrated the use of isothermal microcalorimetry for real-time antifungal susceptibility testing of *Aspergillus* spp. (8) and non-*Aspergillus* spp. (9). Heat produced by replicating microorganism can be measured by isothermal microcalorimetry in the range of microwatt with high sensitivity (23). In the presence of an antimicrobial agent, the growth-related heat production is suppressed, which can be used to assess the susceptibility of an organism of interest. It was shown that amphotericin B, triazoles and echinocandins affected the heat-flow profiles of *Aspergillus* spp. in different manners, depending on their ability to inhibit or kill molds (i.e. exhibit fungicidal or fungistatic activity). These effects were observed particularly with echinocandins when using Sabouraud dextrose broth (SDB), but not when using RPMI medium, recommended for routine antifungal susceptibility by CLSI (4) and EUCAST (7).

The aim of this study was to evaluate the effect of adding caspofungin or anidulafungin to voriconazole and amphotericin B against *A. fumigatus* and *A. terreus* using isothermal microcalorimetry. Antifungals were chosen based on the clinical guidelines, which generally recommends voriconazole as first choice for the treatment of invasive aspergillosis and lipid formulation of amphotericin B is a valuable alternative (21, 24). For therapy in patients not responding to single-drug therapy adding an echinocandin, such as caspofungin, micafungin or anidulafungin has to be considered (24).

Methods.

Test organisms. *A. fumigatus* ATCC 204305 and *A. terreus* ATCC 10690 were used. Molds were subcultured for 3-5 days prior to testing on Sabouraud dextrose agar (SDA) at 37°C. Stocks of each strain were maintained in water at 4°C for short-term storage and in SDB-20% glycerol at -80°C for long-term storage. An inoculum of ~5 x 10^7 spores/ml was prepared in sterile 0.9% saline. The exact inoculum size was determined by microscopic enumeration of conidia using a hemocytometer (Neubauer chamber; Assistent, Sondheim, Germany).

Antifungals. Amphotericin B (Sigma, St. Louis, MO, USA), caspofungin (Merck & Co., Whitehouse Station, NJ, USA), voriconazole and anidulafungin (Pfizer Pharma AG, Zürich, Switzerland) were tested.

Susceptibility and synergy testing by microbroth dilution. Microdilution broth was performed as described in the EUCAST-AFST E.Def. 9.1 document (7) but SDB was used instead of RPMI medium with 0.2% glucose. After inoculation (2.5 x 10^5 spores/ml), microdilution plates were incubated at 37°C and read after 24 h and 48 h. MIC values for amphotericin B and triazoles were determined visually and by inverted contrast light microscopy(Nikon Eclipse TS100, 40X/0.65). as the lowest concentration of drug that caused complete inhibition of fungal growth compared to the growth control at 24 h and 48 h. The minimum effective concentration values for caspofungin and anidulafungin were determined (at 24 h), defined as the lowest drug concentration at which short, stubby, and highly branched hyphae were observed (13).

Antifungal combinations were tested according the checkerboard methodology (19). The drug dilutions were prepared at four times the strength of the final concentration following the CLSI drug dilution scheme. After 24 h and 48 h of incubation at 37°C plates wells containing following concentrations alone or in combination were examined by inverted contrast light

microscopy; 0.125 x, 0.25 x, 0.5 x and 1x MIC (or MEC for caspofungin and anidulafungin). Experiments were repeated three times.

Susceptibility and synergy testing by microcalorimetry. An isothermal microcalorimeter (TAM III, TA Instruments, Newcastle, DE, USA), equipped with 48 calorimetric channels and a detection limit for heat production of 0.2 μW was used. The inoculum was diluted in SDB (Oxoid CM0147; Basingstoke, Hampshire, UK) to a concentration of 1.5 x 10^7 spores/ml. 0.05 ml of fungal suspension was added to microcalorimetric ampoules containing 2.95 ml of SDB and 2-fold dilutions of antifungals for a final inoculum of 2.5 x 10^5 spores/ml. For combination studies the two antifungals tested were combined at concentrations of 0.125 x, 0.25 x, 0.5 x and 1 x MIC (or MEC for caspofungin and anidulafungin). A synergistic effect was defined as an increased delay of fungal heat-production in the presence of an antifungal combination compared to each drug alone. SDB without antifungals served as growth control and SDB alone as negative control. In addition to the growth media, 1 ml air was present in the headspace of the ampoule. The ampoules were air-tightly sealed and introduced into the microcalorimeter, first in the equilibration position for 15 minutes to reach 37.0000°C and avoid heat disturbance in the measuring position. Heat flow was recorded up to 48 h. The time to reach 20 μW was recorded. Experiments were repeated three times. Data analysis was accomplished using the manufacturer's software (TAM Assistant, TA Instruments, New Castle, DE, USA) and exported for further data analyses and graphic presentation. Figures were plotted using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Results.

Antifungal susceptibility. The MIC and MEC values for *A. fumigatus* and *A. terreus* as determined by visual examination and microscopy at 24 h and 48 h are summarized in table 1. MIC values determined by microscopy were one or two two-fold dilutions higher than MIC values read by visual inspection. The MIC read at 48 h and MEC read at 24 h for by microscopy was used for microcalorimetric and combination studies. Time to heat detection (heat flow above 20 μW) of the growth control was 7.0 ± 1.6 h and 12.6 ± 0.4 h for *A. fumigatus* and *A. terreus*, respectively. Voriconazole (Fig. 1A) and amphotericin B (Fig. 2A) delayed heat production in a concentration-dependent manner with a complete inhibition at MIC after 48 h of incubation. The highest concentration of amphotericin B tested (16 μg/ml) inhibited the heat production of *A. terreus* for 33.7 ± 3.2 h, causing a delay to heat detection of 24.7 ± 4.6 h in comparison with the growth control but did not achieve complete inhibition. The growth-related heat produced was in agreement with the growth observed by microscopy (Fig. 1A and 2A), whereas visual growth not yet was observed at the same time point. Caspofungin and anidulafungin alone did not delay heat production but decreased the peak heat-flow (Fig. 1B and 2B), which could be correlated with the change of hyphal morphology observed by microscopy.

Evaluation of antifungal combinations. The effect of the addition of an echinocandin to amphotericin B or voriconazole was evaluated by calculating the delay of time to heat detection (heat flow above 20 μW) in presence of a combination in comparison to the drug alone at 0.125 x, 0.25 x, 0.5 x and 1 x the MIC or MEC. In parallel the same combinations (according to checkerboard dilution scheme) were examined by microscopy. Microcalorimetric results are summarized in table 2 showing the delay of fungal heat-flow (in h) in presence of an antifungal combination compared to growth control, and the difference (in h) in heat detection of combinations compared to amphotericin B or voriconazole alone.

Table 1. Antifungal susceptibility of *A. fumigatus* and *A. terreus* determined by visual examination and microscopy at 24 and 48h.

		MIC (μg/ml)				MEC (μg/ml)	
Strain	Time	Amphotericin B		Voriconazole		Caspofungin	Anidulafungin
		Visual	Microscopy	Visual	Microscopy	Microscopy	Microscopy
A. fumigatus	24 h	0.25	1	0.25	0.5	0.125-0.25	0.015-0.03
	48 h	1	2	0.25	0.5	0.25	0.03
A. terreus	24 h	2	4	0.25	0.5	0.25-0.5	0.03
	48 h	8	16	0.5	0.5	0.5	0.03

Table 2. Delay of fungal heat-flow (in h) in presence of an antifungal combination compared to growth control and the difference (in h) in heat detection of combinations compared to amphotericin B or voriconazole alone. Experiments were performed three times and values are represents mean and standard deviation.

	0.125 x MIC/MEC		0.25 x MIC/MEC		0.5 x MIC/MEC		1 x MIC/MEC	
	TTD (h)	Delay (h)	TTD (h)	Delay (h)	TTD (h)	Delay (h)	TTD (h)	Delay (h)
A. fumigatus								
AMB + CAS	10.3 ± 1.1	2.2 ± 1.1	15.7 ± 3.0	4.1 ± 1.7	26.5 ± 3.6	3.8 ± 2.4	> 48	0
AMB + ANI	9.6 ± 2.5	1.6 ± 2.1	14.5 ± 3.1	3.3 ± 3.7	31.1 ± 7.1	11.2 ± 4.6	>48	0
VOR + CAS	3.5 ± 0.8	2.7 ± 1.4	9.9 ± 2.2	5.8 ± 3.2	39.7 ± 14.4	7.4 ± 5.4	>48	0
VOR + ANI	6.8 ± 7.1	3.6 ± 3.3	26 ± 15.5	11.8 ± 5.7	45.0 ± 5.2	8.5 ± 9.0	>48	0
A. terreus								
AMB + CAS	31.4 ± 12.5	12.2 ± 0.2	37.5 ± 14.8	>16.2 ± 1.9	>48	>14.7 ± 0.2	>48	>13.7±1.6
AMB + ANI	20.5 ± 0.0	7.8 ± 1.8	24.4 ± 0.4	5.9 ± 2.5	32.4 ± 9.1	10.9 ± 9.9	47.6 ± 0.8	>7.5±1.9
VOR + CAS	5.3 ± 1.8	3.5 ± 0.7	9.1 ± 1.1	2.3 ± 0.4	20.4 ± 3.9	7.6 ± 5.3	>48	0
VOR + ANI	4.8 ± 0.4	3.0 ± 0.7	9.7 ± 6.4	2.9 ± 0.2	17.0 ± 6.5	4.1 ± 3.2	>48	0

Against the *A. fumigatus* strain tested a minor synergistic effect of amphotericin B combined with caspofungin was observed, with a delay of heat detection of 1.6 - 4.1 h, compared to the drug alone. A stronger synergistic effect was achieved when amphotericin B and anidulafungin were combined, which increased the delay to growth detection with up to 11.2 h in comparison to amphotericin B alone at 0.5 x MIC. The improved activity of voriconazole in combination with an echinocandin was more pronounced. Figure 1 shows the activity of voriconazole and caspofungin, alone and in combination, against *A. fumigatus* by microcalorimetry and microscopy at 24 h and 48 h. The most active combination against *A. fumigatus* was voriconazole plus anidulafungin with an increase in heat inhibition of 11.8 ± 5.7 h and 8.5 ± 9.0 h, at 0.25 x and 0.5 x MIC (MEC), respectively, as compared to voriconazole alone.

A. terreus is intrinsically less susceptible to amphotericin B, exhibiting a MIC of >16 µg/ml as determined at 48 h by microscopy. When amphotericin B was combined with caspofungin or anidulafungin, a strong synergistic effect was observed with delay of heat production of 5.9 - >16.2 h. Figure 2 shows the activity of amphotericin B and anidulafungin against *A. terreus* by microcalorimetry and microscopy. A clear reduction in microscopical growth and change in morphology was observed in the presence of the combination at all concentrations tested. A delay of heat detection of up to 10.9 ± 9.9 h was observed in the presence of the combination in comparison to amphotercin B alone. The addition of an echinocandin to voriconazole did only show a minor synergistic effect, with a delay of growth of 2.3 - 4.1 h, except for the combination voriconazole and caspofungin at 0.5 x MIC (MEC) that delayed the growth related heat-production by 7.6 ± 5.3 h.

Figure 1. Growth inhibition of *A. fumigatus* by voriconazole (A) and caspofungin (B) alone or in combination (C), as determined by microcalorimetry (upper panel) and microscopy at 24 h and 48 h (lower panel). The growth control (GC) is presented in duplicate. Antifungals were tested in combination at 0.125 x, 0.25 x, 0.5 x and 1 x MIC or MEC. Magnifications, x40.

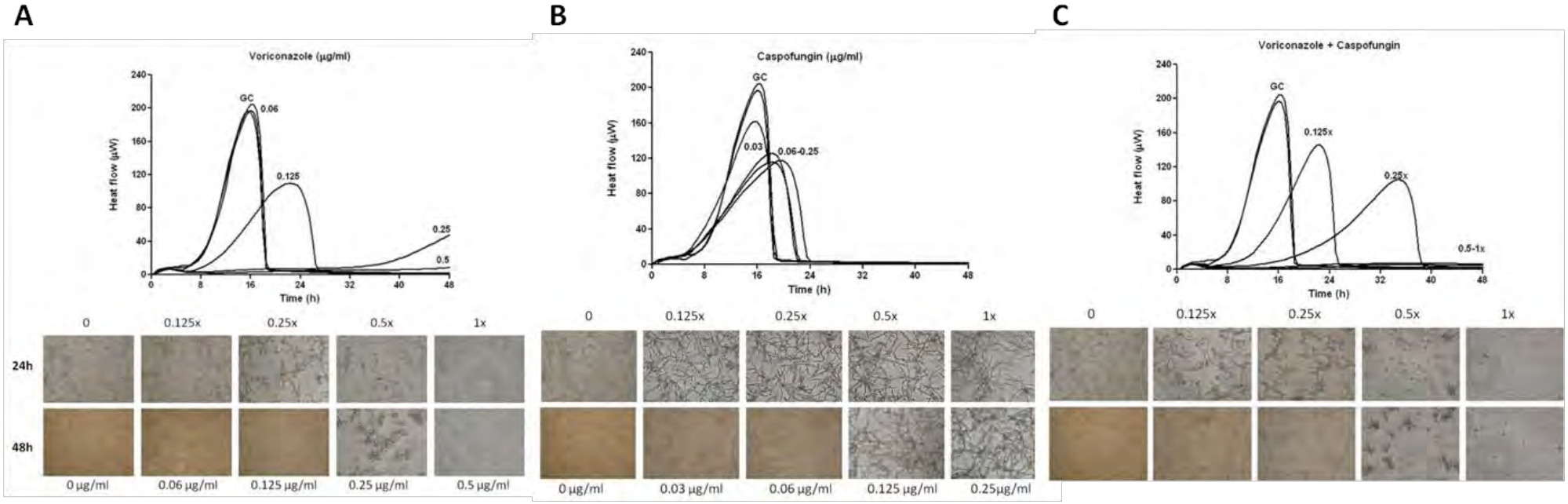
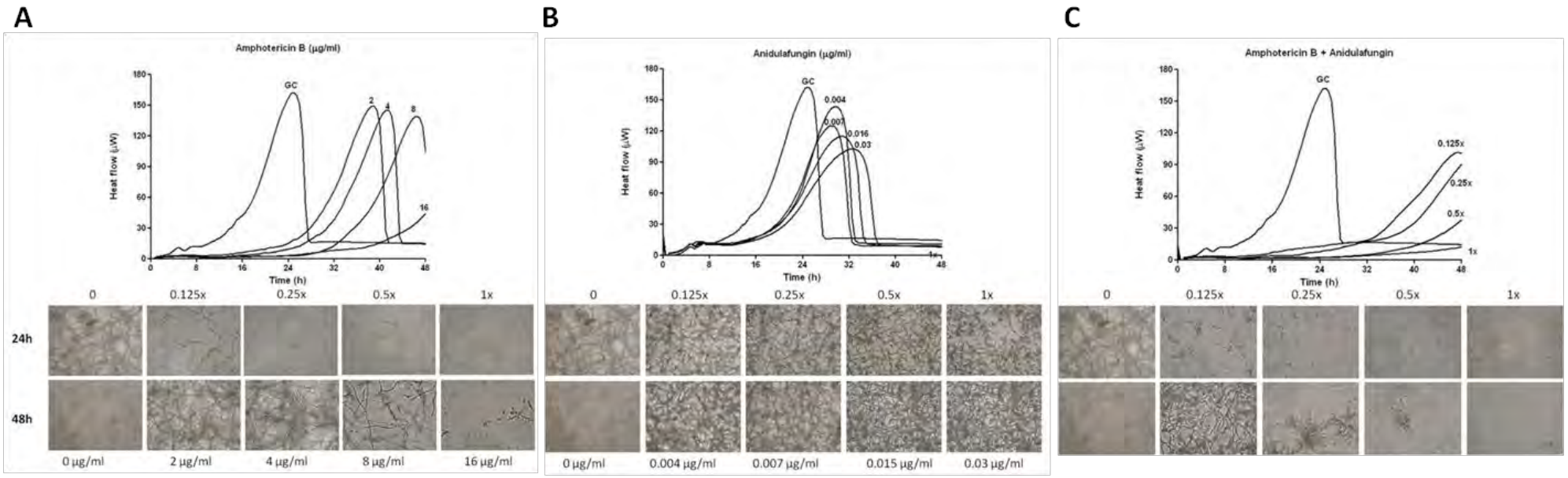


Figure 2. Growth inhibition of *A. terreus* by amphotericin B (A) and anidulafungin (B) alone or in combination (C), as determined by microcalorimetry (upper panel) or microscopy at 24 h and 48 h (lower panel). The growth control (GC) is presented in duplicate. Antifungals were tested in combination at 0.125 x, 0.25 x, 0.5 x and 1 x MIC or MEC. Magnifications, x 40.



Discussion.

In the present study, we demonstrated the potential of isothermal microcalorimetry for evaluation of antifungal combinations against *Aspergillus* spp. In contrast to the standard method for assessing antifungal combinations (i.e. checkerboard microbroth dilution assay), microcalorimetry allows real-time evaluation of antifungal activity. The result is not based on subjective visual examination, rendering the method particularly useful for susceptibility testing of molds, which often show heterogeneous growth characteristics. Due to the incomplete growth inhibition of molds by the echinocandins, two different endpoints are often used by the checkerboard assay. The endpoints, MIC or MIC-0 for optically clear and MEC or MIC-2 for a prominent growth reduction (6, 20), are read by visual inspection, which may be subjective and needs experienced personnel. Furthermore, the choice of the endpoint can influence whether a combination will show synergistic or only additive effect (5). Microcalorimetry has the advantage of testing echinocandins alone or in combination by measuring heat production instead of growth medium turbidity.

In parallel to the microcalorimetric assay, the activity of antifungal combinations was investigated by microscopy and congruent changes in morphology and growth reduction were observed, as shown in Fig. 1 and 2. If similar results are obtained by two different methods, one can suppose that the combination could have a clinical importance. Due to the highly sensitive detection of growth by the microcalorimeter, the MIC determined by visual inspection did not completely inhibit heat-related growth production. Thus, the MIC was additionally determined by microscopy, which better correlated with the microcalorimetric data.

In this proof-of-concept study, only two *Aspergillus* strains were included. Against the tested *A. fumigatus* strain, the addition of an echinocandin improved the activity of voriconazole

more than the activity of amphotericin B. Previous *in vitro* studies have reported both synergy and indifference when combining voriconazole with caspofungin [19] or anidulafungin [18]. An addition of anidulafungin to amphotericin B or voriconazole showed a stronger synergistic effect than the addition of caspofungin, which may be due to the stronger *in vitro* activity against non-germinated conidia observed with anidulafungin in comparison with other echinocandins (1). However, a more recent *in vitro* study only reported minor differences in activity of anidulafungin and caspofungin (16). Even though *A. terreus* is less frequently causing aspergillosis than *A. fumigatus* and *A. flavus*, this species was chosen due to its intrinsically reduced susceptibility to amphotericin B (3). We observed an improved activity of amphotericin B in the presence of an echinocandin, whereas the activity of voriconazole was not considerably improved. A decrease in the MIC of amphotericin B in the presence of caspofungin has been previously described and could be explained by an increased penetration of amphotericin B due to the disturbed cell wall by caspofungin (2). The microcalorimetric curve additionally demonstrates a slower growth of *A. terreus* in comparison with *A. fumigatus*, which may also influence increased susceptibility to amphotericin B. Combinations of echinocandins with azoles or amphotericin B have shown positive results when tested in animal models and their lack of toxicity makes them attractive for combination therapy (10). However, clinical studies are needed to generate solid evidence-based data supporting the use of combination therapy.

In summary, an improved activity of both amphotericin B and voriconazole was observed when combined with an echinocandin, with variations depending on the antifungal and mold combination. This proof-of-concept study demonstrated the potential of isothermal microcalorimetry for real-time evaluation of antifungal combinations against *Aspergillus* spp., in combinations including particularly echinocandins. The assay merits further validation by testing of additional strains, other mold species and antifungal combinations.

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Declaration of interest

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Chapter 12. General conclusions and outlook.

The importance of antifungal susceptibility testing is continuously increasing by the shift in mold epidemiology towards non-*Aspergillus* genera and the emergence and spread of resistant strains. Resistant strains do not always exhibit cross-resistance to all antifungals in the same group, as was shown for itraconazole-resistant *A. fumigatus* strains, remaining susceptible to other triazoles. Progress in the field of nucleic-acid sequencing, allowing fungal species identification, has provided new insights into the molecular taxonomy of molds. Several sibling species to *A. fumigatus,* such as *A. lentulus*, have been identified, exhibiting a reduced susceptibility to many antifungals. Among the emerging molds, such as the Mucorales, species identification revealed variability in susceptibility among the different genera, highlighting that the choice of appropriate antifungal therapy cannot be based on the fungal class but species identification and susceptibility testing is recommended.

The utility of antifungal susceptibility testing is a controversial issue in clinical practice due to lack of well-established clinical breakpoints for molds. The unavailability of these breakpoints is mostly due to great variability of the host status, but may also be due to suboptimal performance of *in vitro* testing methods currently used in routine practice. The lack of appropriate tests is particularly evident for evaluation of antifungal combinations. Susceptibility test methods based on measurement of turbidity of the growth medium are challenged by heterogeneous hyphal growth and variation in growth rate of molds, rendering the visual test interpretation difficult.

Isothermal microcalorimetry is commonly used for analysis of chemical and biochemical reactions, either consuming or producing energy. During the last decade, the utility and advantages of isothermal microcalorimetry for highly sensitive detection of microbial growth

was demonstrated for a range of microorganisms. In chapter 9 and 10, we evaluated the thermokinetic profiles of different mold species, including *Aspergillus* and non-*Aspergillus* spp., and were able to detect fungal growth of *Aspergillus* spp. and Mucorales in <5 h and in 7 h and 17 h for *Fusarium* and *Scedosporium* spp., respectively, under defined conditions. As demonstrated in chapter 11, the sensitivity of the microcalorimeter for growth detection was more comparable to growth detected by microscopy than to macroscopically visual growth. Despite enabling rapid growth detection, microcalorimetry does not allow species identification, as the heat produced is an unspecific signal of all thermal processes taking place in the test ampoule. Nevertheless, the use of a selective media could allow differentiation between genera. In a clinical setting, microcalorimetry could offer a rapid discrimination between culture positivity and negativity of sputum or bronchoalveolar lavage fluid samples.

In chapter 9 and 10, we demonstrated the potential of isothermal microcalorimetry, as a novel method for antifungal susceptibility testing of molds. Due to the complex life cycle of molds, microcalorimetry appears to be a suitable and precise approach for performing antifungal susceptibility testing, as the test interpretation is not based on subjective visual examination and the data is obtained continuously in real-time (as heat-flow curve). We demonstrated that amphotericin B, triazoles and echinocandins affected the growth-related heat production of *Aspergillus* spp. in different manners, depending on their fungistatic or fungicidal properties. The presence of amphotericin B or a triazole delayed the heat production in a concentration-dependent manner and the minimal heat inhibition concentration (MHIC) was determined as the lowest concentration inhibiting 50% of the heat produced at 24 h, 48 h or 72 h, depending on species. Due to the different mechanisms of action echinocandins, the MHIC for this antifungal class was determined as the lowest concentration lowering the heat-flow peak with 50%. For *Aspergillus* spp. (chapter 9), agreement within two 2-fold dilutions between MHIC

and MIC or MEC (determined by CLSI M38A) was 90% for amphotericin B, 100% for voriconazole, 90% for posaconazole and 70% for caspofungin. In order to validate our assay, azole-resistant *A. fumigatus* mutants were included. We further evaluated our assay for antifungal susceptibility testing of non-*Aspergillus* molds (chapter 10). As determined by microcalorimetry, amphotericin B was the most active agent against Mucorales (MHIC 0.06-0.125 μg/mL) and *Fusarium* spp. (MHIC 1-4 μg/mL), whereas voriconazole was the most active agent against *Scedosporium* spp. (MHIC 0.25 to 8 μg/mL). Whereas interpretation of susceptibility data by the conventional microbroth dilution method is rather subjective and requires experienced personnel, microcalorimetry offers an objective approach for data interpretation, based on a delay of heat production or change of the calorimetric curve, related to the inhibition of growth (MIC) or change of growth mode (MEC), respectively.

We compared our data with MIC and MEC values obtained by the standard method for antifungal testing and were able to correlate our results when using the same time of incubation. However, considering the advantage of real-time growth monitoring, the microcalorimetric assay could be further optimized for a rapid detection resistant isolates. Indeed, in a future study we will screen a strain collection of 50 azole-resistant *A. fumigatus* isolates, with the goal to establish a rapid and sensitive assay for detection of azole resistance, as was previously described for the differentiation between methicillin-susceptible and methicillin resistant *S. aureus* using the same methodology.

In chapter 11, we evaluated the activity of four antifungal combinations against *A. fumigatus* and *A. terreus* by microcalorimetry. The standard *in vitro* method used for synergy testing of molds, the checkerboard microdilution method, has been criticized for the FIC index ranges used for interpretation, as a majority of combinations show indifference or additive effect. Based on real-time measurements, we were able to show that the addition of an echinocandin to amphotericin B or voriconazole, delayed the detection of growth-related heat production of

the tested *Aspergillus* spp. in comparison to the drug alone. The improved activity varied between the two echinocandins and fungal species tested. The great potential of microcalorimetry for antifungal synergy testing needs to be further explored by testing of additional strains, other mold species and antifungal combinations.

For a future use of microcalorimetry in a clinical microbiological laboratory, several issues need to be considered. First, the currently used isothermal microcalorimeters are too expensive and need to be simplified and adapted to the test conditions needed for microbiological applications (e.g. a narrow temperature range) in order to lower the cost of the instrument. Second, the instrument needs to allow a semi- or fully automated processing of multiple samples enabling high-throughput testing. In order to meet these criteria, promising development in the field of calorimetric instrument includes the recent market entry of a 48-well plate isothermal microcalorimeter designed for biological assays (CalScreener, SymCel AB) and a high-throughput low-cost chip calorimeter (chipCAL, TTP Labtech Ltd).