CHALLENGES IN LINKING PRECLINICAL ANTI-MICROBIAL RESEARCH STRATEGIES WITH CLINICAL OUTCOMES FOR DEVICE-ASSOCIATED INFECTIONS

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Abstract

Infections related to implanted medical devices have become a significant health care issue in recent decades. Increasing numbers of medical devices are in use, often in an aging population, and these devices are implanted against a background of increasing antibiotic-resistant bacterial populations. Progressively more antibiotic resistant infections, requiring ever more refined treatment options, are therefore predicted to emerge with greater frequency in the coming decades. Improvements in the prevention, diagnosis and treatment of these device-associated infections will remain priority targets both for clinicians and the translational research community charged with addressing these challenges.

Preclinical strategies, predictive of ultimate clinical efficacy, should serve as a control point for effective translation of new technologies to clinical applications. The development of new anti-infective medical devices requires a validated preclinical testing protocol; however, reliable validation of experimental and preclinical antimicrobial methodologies currently suffers from a variety of technical limitations. These include the lack of agreement or standardisation of experimental protocols, a general lack of correlation between *in vitro* and *in vivo* preclinical results and lack of validation between *in vivo* preclinical implant infection models and clinical (human) results. Device-associated infections pose additional challenges to practicing clinicians concerning diagnosis and treatment, both of which are complicated by the biofilms formed on the medical device.

The critical challenges facing both preclinical research and clinical laboratories in improving both diagnosis and treatment of medical device-associated infections are the focus of this review.

Keywords: Medical implants, infection strategy, antibiotic resistance, *in vitro-in vivo* correlations, clinical translational research.

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Introduction and Overview of Device-Associated Infection

Bacterial colonisation of medical devices, leading to infection of the adjacent tissues, is a widely recognised, serious complication for a significant minority of patients receiving such implants (Busscher *et al.*, 2012). Certain aspects of implant-related infections distinguish their cause, effects and possible therapeutic options from normal surgical site infections: low inocula required for infection propagation, wide varieties of pathogens involved, difficulty in eradication using antibiotic therapies alone, and possible immunosuppressive environments induced by implant foreign body reactions. It has been shown numerous times that the mere presence of an implant consistently reduces the number of bacteria required to cause an infection by up to four or more orders of magnitude (Table 1) (James and MacLeod, 1961; Zimmerli *et al.*, 1982; Widmer *et al.*, 1990; Poelstra *et al.*, 2000; Poelstra *et al.*, 2002), a central concept first conclusively shown in human subjects in the 1950s (Elek and Conen, 1957). The importance of the implant is further highlighted by the fact that treatment of these biofilm infections may not be successful unless the implant, and thus the biofilm, is removed (Berkes *et al.*, 2010). Once established, device infections prompt device malfunctions, patient morbidity, substantial risk of mortality, and continuing medical and/ or surgical interventions, often at substantial costs to both patients and payers (Busscher *et al.*, 2012).

It is increasingly evident that, despite sterilisation, nearly all medical devices and/or the surgical field are contaminated peri-operatively prior to or during implantation procedures (Alexander *et al.*, 2013). This is a logical consequence of the fact that the operating suite is not sterile: surgical equipment (*e.g.*, handles, lights, keyboards, floors, walls) is often contaminated (Stone *et al.*, 2002; Pittet *et al.*, 2004; Allo and Tedesco, 2005; Howard and Hanssen, 2007). Similarly, surgical suite doors open and disrupt the laminar air flow in the operating room from 19-50 times per hour across surgical specialties (Lynch *et al.*, 2009) and on average 60 times during a single total hip arthroplasty, or 135 times for a revision arthroplasty (Panahi *et al.*, 2012). Additionally, both the patient and attending surgical staff are not sterile: bacteria/fungal sourcing is common from dust, skin, respiratory particles, hair, and clothing (Trampuz and Widmer, 2004). Furthermore, nearly a third of uncovered surgical trays are contaminated after a few hours of surgery (Dalstrom *et al.*, 2008). Given estimated bacterial seeding rates for a standard operating theatre during a surgical

Table 1. Overview of *in vivo* studies demonstrating reductions of bacterial numbers required to cause infections in the presence of a foreign body *versus* no foreign body.

Study	Host	Foreign Body (FB)	Minimum Infectious dose (CFU)		Pathogen
			No FB	With FB	
Elek 1957 (2)	Human	Sutures	5×10^6	3×10^2	*S. aureus*
James 1961 (3)	Mouse	Sutures	10^6	$< 10^3$	*S. aureus*
Widmer 1988 (5)	Guinea Pig	Cages	$> 10^7$	10^3	*S. epidermidis*
Zimmerli 1982(6)	Guinea Pig	Cages	$> 10^7$	10^2	*S. aureus*
Poelstra 2000 (7)	Rabbit	K-wire	$> 10^4$	500	MRSA
Poelstra 2002 (4)	Mouse	Mesh	$> 10^6$	10^4	*P. aeruginosa*

procedure of ~270 bacteria/cm²/h, (Fitzgerald, 1979) there are plenty of opportunities for contamination in wound sites. Whether modern air filtration and ultra-violet (UV) field sterilisation protocols reduce this pathogen-seeding threat has been recently disputed by studies that show no statistically significant differences in infection rates in ultra-sophisticated *versus* conventional surgical suites (Uckay *et al.*, 2013).

A significant proportion of implant infections are therefore believed to be acquired during normal, modern surgical procedures (Hanssen *et al.*, 1997). This is supported by the consistent success of vigilant infection surveillance programs and infection prevention measures focused on the operating theatre, and by evidence matching pathogenic strains both from surgeons' fingers (Uckay *et al.*, 2010) and from patients nasal cavities (von Eiff *et al.*, 2001; Perl *et al.*, 2002) with infecting organisms. Despite consistent pathogen exposure and high seeding and contamination rates, implant surgeries are actually remarkably successful: infection rates for most medical devices are less than 10 % and sometimes less than 1 % (Uckay *et al.*, 2010). The consistently high contamination rate in comparison with a low, if variable, infection rate, reflect combinations of yet unknown factors that must coincide to successfully propagate infections in certain patient and implant types.

Once established, an infection associated with an implanted medical device presents numerous challenges precluding successful treatment outcomes. A primary reason why implant-associated infections are particularly challenging to treat is the formation of bacterial biofilms on implant surfaces and adjacent tissue sites (Gristina *et al.*, 1988). Once established, these biofilms are generally refractory to antibiotic treatments (Høiby *et al.*, 2010). Hence, expensive surgical intervention is often required to remove the colonised device and debride the surrounding tissue to achieve a successful treatment outcome. The enormous socioeconomic costs and recent refusal by insurance companies to pay for so-called "preventable" complications (www.medicaid.gov in the USA; (Stone *et al.*, 2010)) including common catheter-associated blood stream and urinary tract infections, has driven substantial amounts of research and development recently into diagnosing, preventing and treating medical device-associated infections.

At certain critical points, interventions may significantly influence infection risk. These include: patient pre-operative preparations; stringent infection control procedures in hospital wards; appropriate and timely antibiotic selection and prophylaxis; optimal surgical skill and technique; judicious use of antimicrobial-loaded devices; and careful patient monitoring and follow-up to ensure timely and rapid diagnosis of those relatively rare cases where device infection does develop (Alexander *et al.*, 2013; Uckay *et al.*, 2013). The scientific community, in collaboration with industrial and clinical partners, have the responsibility to provide sufficient basic understanding of the pathological processes occurring when bacteria colonise and eventually cause an infection, and how and when to best intervene in these situations. The challenges and current barriers associated with efficient transformation of this understanding from preclinical laboratories into novel, effective, commercial antimicrobial patient interventions have been comprehensively reviewed recently (Busscher *et al.*, 2012; Grainger *et al.*, 2013).

The introduction of next-generation antibiotic agents has declined in past decades due to limited market incentives, given numerous agents already approved and relatively modest profit incentives for pharmaceutical companies that develop them (Livermore, 2011). Over-reliance on and clinical abuse of the current repertoire of conventional antibiotics has produced a major threat to current medical treatment of infection: the emergence of multiple antibiotic-resistant bacteria such as Methicillin Resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci and multi-resistant *Acinetobacter baumannii*. In contrast to an apparent lack of industrial interest in developing new antimicrobials, development of antimicrobial-loaded medical devices has been increasing (Brooks *et al.*, 2013). This includes increasing antimicrobial additions to medical devices: *e.g.*, multiple manufacturers now provide numerous antimicrobial catheters and endotracheal tubes (Darouiche, 2013; Kollef, 2013). Antimicrobial-releasing devices are now also increasingly available in orthopaedic surgery, currently focusing on high-risk cases such as open tibial fractures (Fuchs *et al.*, 2011), or revision of prosthetic joints (Hardes *et al.*, 2007). As prospective clinical trials for these devices in the current regulatory climate are frequently economically impossible (Busscher *et al.*, 2012; Grainger *et al.*, 2013), the retrospective clinical efficacy of such devices must be followed closely in the coming years as their use becomes more widespread and sufficient clinical follow-up data emerge. Full and careful reporting of the clinical impact of these types of implants will facilitate forecasting of possible roles that this strategy could play

in clinical routine and will ultimately dictate clinical acceptance.

While thousands of "academic" concepts for antimicrobial implants are published annually, most never make it even to human prototyping stages (Grainger *et al.*, 2013). The pathway to product regulatory approval and commercialisation is expensive and time-consuming, requiring commitment and some predictive capabilities of *in vivo* performance for process risk management. The standard product development path for any anti-infective device concept includes extensive preclinical *in vitro* and *in vivo* testing that determines device safety and efficacy prior to further clinical trials in human patients. When applied to addressing medical device infections, however, the pathway lacks clear rationale and scientific rigor. The reality we now face is that critical links between *in vitro* and *in vivo* preclinical testing are neither as correlative nor predictive of clinical efficacy as may have been expected (Grainger *et al.*, 2013). Similarly, preclinical *in vivo* testing does not necessarily correlate with clinical outcomes and performance (Busscher *et al.*, 2012; Proctor, 2012; Grainger *et al.*, 2013). Why these important discrepancies exist is not yet clear. However, their multifactorial basis, including the non-standard designs of *in vitro* assays; the diverse varieties of preclinical animal models chosen; the relative robustness of animal immune anti-infection competence in comparison to human systems; the different microorganisms selected and inability to assay certain key features of actual human infection susceptibility including patient co-morbidities and inter-individual variability, all limit the accuracy, predictability and validity of technology translation beyond preclinical testing (Table 2) (Busscher *et al.*, 2012; Grainger *et al.*, 2013).

Challenges in Studying Device-Associated Infection *in vitro*

In vitro assessment of infection risk is not a required preclinical test for most medical devices. In the case of anti-infective devices, however, preclinical testing of their antimicrobial efficacy in various guises is generally performed to determine the extent and duration of pathogen killing for a number of relevant bacterial species. The exact configuration of the assessment is not usually standardised. However, some International Organization for Standardization (ISO) guidelines exists for testing materials (*e.g.*, ISO 22196). These guidelines are often surpassed in terms of the quantity and variety of tests performed by independent academic laboratories as seen in the published literature (Katsikogianni and Missirlis, 2004; Moriarty *et al.*, 2011). Unfortunately, many controversies surround the (1) performance of these tests, (2) lack of standardisation of many aspects of experimental design and variables, and (3) relevance to the *in vivo* situation, and this lack of consensus is not often addressed. Validated, realistic assessment protocols recognising specific critical assay features, including standards and their predictive utility would represent a significant step forward.

Bacterial adhesion, growth and biofilm formation on biomaterials

The specific affinity of a given bacterial cell for an implant material or surrounding tissue is among the first steps known to influence microbial contamination leading to the development of infection. Bacterial adhesion represents an area of considerable past and current interest in basic understanding of the colonisation process, and also for the development of improved anti-adhesive materials. The process of device colonisation and tissue infection develops in a stage-wise manner: from preconditioning of the biomaterial with host matrix proteins, followed by initial microbe adhesion, growth and ultimately micro-colony and, in select cases, biofilm formation on the surface of the material (Fig. 1). Bacteria react and respond to adhesion within what has recently been described as three different regimes of adhesion forces (Busscher and van der Mei, 2012). Each and all of these regimes and steps in the process are amenable to *in vitro* investigation and much research has been performed on various implant materials and model surfaces (for examples, see references (Hudson *et al.*, 1999; Katsikogianni and Missirlis, 2004;
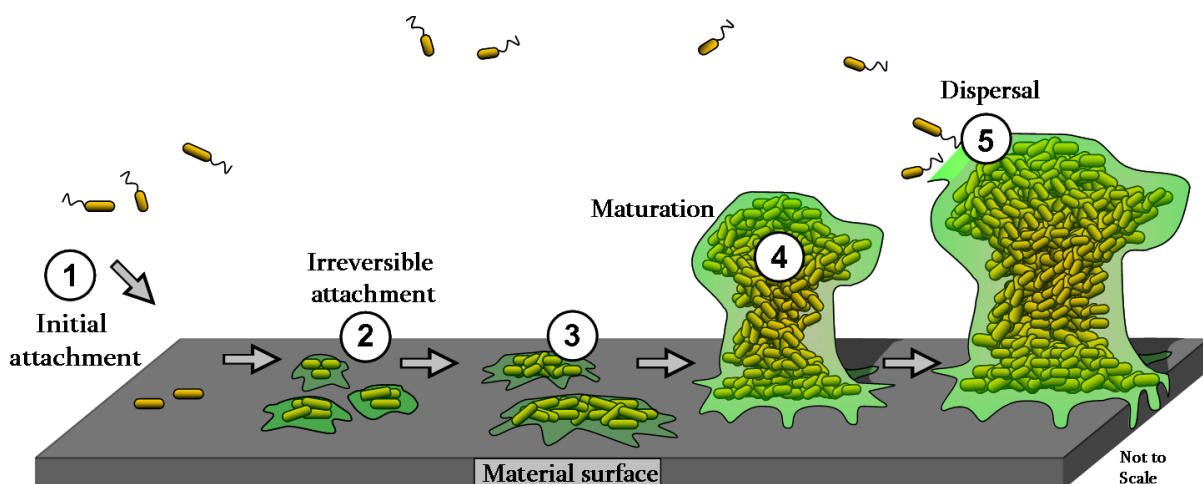
Fig. 1. Overview of bacterial adhesion and biofilm formation (Moriarty *et al.*, 2011). Each step, from initial adhesion to biofilm formation is amenable to *in vitro* evaluation. Generating clinically relevant *in vitro* results remains a challenge. (Figure reproduced by permission).

Table 2. Summary of challenges in preclinical testing related to implant infections.

***In vitro* bacterial adhesion assays:**
Inoculum preparation: Bacteria grow well in complex media (e.g. Tryptic Soy broth, TSB) and other non-physiological media
Proteins in complex media adsorb onto model surfaces as an unrealistic conditioning film.
Buffered saline solutions are often used to suspend bacterial inocula for adhesion assays: Result is the use of media-shocked microbial phenotypes in these assays.
Best available option: Data shows that preconditioning materials and exposing bacteria to human derived fluids will alter bacterial adhesion and gene expression. May be the most representative approach for most purposes.
Quantification methodology: Adherent bacteria are difficult to remove and quantify reliably. Ultrasound and roll-plate do not necessarily correlate with radioactive label assays for bacteria.
Some viable bacteria may be 'non-culturable': PCR can detect genomic signatures for these but cannot distinguish live from dead species.
No standardised (e.g. ISO) method for bacterial adhesion to enable comparisons
Best available option: Confirmation of data with multiple techniques.
Efficacy targets
Log-3 order CFU reduction (1000-fold) is numerically significant (99.9 % decrease in bacterial load) but clinical significance is unclear.
Target: 100 % reduction has been achieved in many preclinical studies and should be a prerequisite for progression to human or animal trials.
***In vivo* infection models:**
Healthy young animals are utilised
Enormous microbial super-dosing required into a healthy wound (10^6-10^9 CFUs/site) to generate reliable infection. Clinically unrealistic.
Low infection rates make acute infection models difficult to power. Highly powered study designs (large CVs = large cohorts = expensive)
Pathogen strain selection can bias antimicrobial outcomes (virulence can be judiciously "selected" to match proposed therapy)
In simpler models, animal tissues are non-equivalent to humans (hard/soft, volume, tissue physiology, mass transport)
Humans are not syngeneic, with intrinsically high variability in infection resistance: not replicated in in-bred laboratory animals

Montanaro *et al.*, 2008; Moriarty *et al.*, 2011). In this regard, bacterial adhesion may in fact be variously represented as an adhesion force, resistance or ease of detachment or a combination of these. In order to generate data that contribute new insights to colonisation, careful attention should be paid to these specific measurements and how they are designed, described and defined. Detailed review of many methods and definitions is outside the scope of this review, however, further information may be found in numerous topical reviews (Gottenbos *et al.*, 1999; Katsikogianni and Missirlis, 2004).

Broadly speaking, bacteria adhesion to biomaterials is mediated in one of two ways: either directly to the bare biomaterial or indirectly by a poorly defined and highly variable protein conditioning film adsorbed on the biomaterial. The adherent protein layer on the biomaterial surface (Kasemo and Lausmaa, 1994) affords contaminating bacteria a more specific adhesion mechanism using highly evolved microbial surface receptors (adhesins) (Chagnot *et al.*, 2013). Specific adhesion may be investigated *in vitro* by preconditioning biomaterial samples in either defined or complex proteinaceous solutions. However, the precise chemical and physical constitution of the conditioning layer on implants *in vivo* is impossible to replicate *in vitro*, or even to validate for accuracy. Furthermore, it is difficult to separate the relative contribution of nonspecific and specific mechanisms of bacterial adhesion to actual infection propagation either *in vitro* or *in vivo*.

Ultimately, the inability of a material to completely and reliably eliminate all bacterial adhesion events is a device performance issue. Material-based reduction of adhesion by 98 % may seem a numerical *in vitro* success. However, the clinical impact must be proven and frequently this *in vitro* microbial adhesion resistance does not translate to clinical infection resistance. The typical claims for *in vitro* efficacy, with a 3 log-order reduction (*i.e.*, 99.9 % reduction) in surface-adherent colony forming units (CFUs) after a 24 h assay, fail to consider many of the dynamics of the *in vivo* situation. Bacterial doubling times of ~20 min in exponential growth phases mean that the remaining few CFUs of adhering bacteria can re-populate any surface with many new colonies in a few hours. They are also capable of rapidly up-regulating genes to remodel their adhesion profiles to produce new armadas of surface attachment mechanisms, and drug efflux pumps to address antimicrobial threats. Additionally, adherent bacteria are intrinsically more resistant to antibiotic assault and cellular phagocytosis, particularly over time as they evolve into biofilms.

A more recent innovation in *in vitro* investigations of bacterial-material interactions has involved co-culture systems whereby bacterial contamination and host (eukaryotic) cell interactions with implanted biomaterials are assessed in the same *in vitro* system. This design has been termed the "race for the surface" (Gristina *et al.*, 1988). By modelling this scenario and including both

prokaryotic pathogens and eukaryotic cells within a single *in vitro* test bed, this process may be a significant step towards more clinically relevant assays (Subbiahdoss *et al.*, 2009). These new systems have shown, for example, that bacterial interactions with fibroblasts, osteoblasts and macrophages may be influenced by the substrate biomaterial *in vitro* (Subbiahdoss *et al.*, 2010a; Subbiahdoss *et al.*, 2010b; Subbiahdoss *et al.*, 2011). One example outcome from these studies has been a realisation that bacterial toxins, such as those produced by *S. aureus* and *Pseudomonas aeruginosa* can effectively and rapidly induce eukaryotic cell death in co-cultures, effectively eliminating eukaryotes in competition for surface adhesion (*i.e.*, bacteria win the "race"). In contrast, less virulent microorganisms, such as *S. epidermidis*, compete with viable host cells for surface adherence. In this system, therefore, host cell responses to local toxin production near biomaterials surfaces and resultant eukaryotic cell death replicate in some ways certain aspects of the clinical situation, whereby *S. epidermidis* causes slower, sub-acute infections, characterised by a more indolent type of infection, in the absence of significant toxin production, and thus less local cell death. One important aspect of these co-culture systems is adapting both the pathogen strain and cell type to a common media supporting growth and correct phenotype for both species. As bacterial growth kinetics greatly outpace mammalian cell division rates, inoculum numbers and media composition can be adjusted to slow bacterial proliferation to level the playing field with the disadvantaged mammalian cells. Identifying optimal growth conditions for both prokaryotes and eukaryotes represents an area that requires development before co-culture assays become a useful addition to the field.

While research into the process of bacterial adhesion to materials, and interference in this process by material design strategies, may potentially provide routes to generating more contamination-resistant, bacterial-repellent biomaterials, few adhesion-resistant innovations have translated to improved antimicrobial clinical performance (Busscher *et al.*, 2012).

Bacterial inoculum preparation for antimicrobial assessments

Most standard *in vitro* assays involve bacteria cultured in a nutrient-rich non-physiological medium, typically a tryptic soy broth (TSB), general nutrient broth, or similar growth media with a complex non-mammalian protein content. In a bacterial adhesion assay, these broth proteins adsorb onto test surfaces, influencing bacterial binding, thereby rendering the results less than fully applicable to any clinical situation. Therefore, in many studies, cultured bacteria are first washed in a buffered salt "minimal" medium generally lacking proteins and then deposited onto the test material in a similar buffered salt solution. This reduces the chance of culture medium protein interference with the result. This scenario however, effectively 'shocks' bacteria in culture without effective media adaptation. The physiological response of non-adapted bacteria is certainly affected in terms of growth rate, transcription profiles (metabolism-, adhesin-, toxin-production related genes, for example) and phenotype. The effect of such

culture media shock on bacterial adhesion assays, or any other *in vitro* assay, has not been fully established to date, though likely to be significant. Similarly, serum-adapted pathogens, capable of surviving serum-based cultures, are rarely used though this would certainly be a step forward toward improved relevance. Numerous examples have shown that growth in *ex vivo* culture conditions can alter gene expression or adhesive ability differently than *in vitro* conditions (Wiltshire and Foster, 2001; Massey *et al.*, 2002; Yarwood *et al.*, 2002). For example, growth in used peritoneal dialysate effectively saturated *S. aureus* adhesins and reduced bacterial adhesion to fibronectin and fibrinogen *versus* controls grown in a conventional Todd-Hewitt broth (Massey *et al.*, 2002).

Bacterial species and strain selection

Biomaterial-associated infections may be caused by a wide range of bacteria, either singly or in polymicrobial infections. However, the majority of isolates from a given device class are from comparatively few species. For example, within orthopaedic devices, the staphylococci account for a majority of isolates cultured using conventional means (Tunney *et al.*, 1999; Trampuz *et al.*, 2007; Schafer *et al.*, 2008), and in catheter-associated urinary tract infections, the staphylococci are again prevalent alongside Gram negatives such as *E. coli* and *P. aeruginosa* (Matsukawa *et al.*, 2005). Hence, antimicrobial preclinical device testing should include a range of bacterial species commonly reported to cause infections associated with the particular device type.

Strain selection within a pathogen species is just as important. The clinical significance or relevance of the entire preclinical investigative phase can be profoundly influenced both by the choice of bacterial species and strain within that species. Considering the significant variations commonly found within bacterial populations, selecting a test strain representative of a significant fraction of the pathogenic strains causing clinical infections is a crucial aspect in preclinical studies (Campoccia *et al.*, 2008). For example, *S. aureus* is commonly isolated from infections related with several different medical devices. From a microbiological perspective, *S. aureus* virulence is often classified by toxic, adhesive or evasive parameters, in addition to antibiotic resistance (Rooijakkers *et al.*, 2005; Clarke and Foster, 2006; Otto, 2010). Since whole genome sequencing of bacteria has become much more affordable in recent years, it has been realised that the make-up of different *S. aureus* strains may vary by up to 20 % (Lindsay and Holden, 2006). Furthermore, gene expression, particularly expression of numerous virulence factors, is under the control of global regulators that are themselves highly variable between strains of *S. aureus* (Rogasch *et al.*, 2006). It is, therefore, highly likely that within a potential population of strains available for preclinical testing, significant differences between virulence potential and regulation are observed. Clinical strains of *S. aureus* with differing repertoires of virulence genes have already been associated with differing clinical progression. For example, the USA 300 MRSA strains are known to be highly toxic and capable of causing significant disease even in healthy people (Que *et al.*, 2005; Deleo *et al.*, 2010;

Otto, 2010). In contrast, less virulent but more antibiotic-resistant hospital-acquired MRSA clones are common in the U.K. and Ireland and prevalent in chronic infections in susceptible hosts (Collins *et al.*, 2010; Rudkin *et al.*, 2012). Therefore, the selection and use of antimicrobial strains in preclinical testing must consider the source of the strain, its repertoire of virulence factors, and how this correlates with the clinical application and disease in question. *In vitro* antimicrobial efficacy in a given strategy is profoundly impacted by strain selection: biased success can result from selecting weak microbial strains not relevant to clinical pathology.

Another important issue is the fact that many clinical infections, particularly chronic open wounds (Fazli *et al.*, 2009) and many musculoskeletal infections (*e.g.*, diabetic foot infections) are polymicrobial infections (James *et al.*, 2008; Peters *et al.*, 2012). Numerous *in vitro* and *in vivo* studies have shown that interspecies bacterial interactions directly impact bacterial virulence factor production and disease severity *in vivo* (Mastropaolo *et al.*, 2005; Sun *et al.*, 2008; Dalton *et al.*, 2011). Fundamental mechanisms underpinning these effects remain to be elucidated. However, the known mechanisms that underpin bacteria:bacteria communication include quorum sensing, synergistic immunomodulation, augmented virulence factor production and direct contact-dependent mechanisms. One study investigating *S. aureus* and *P. aeruginosa* polymicrobial infection in an implant-related spinal model in the rat found that these two species displayed a complex pathogenic synergy (Hendricks *et al.*, 2001). Low numbers of *P. aeruginosa* and *S. aureus* caused more infections than expected for either bacterium alone at an equivalent bacterial inoculum. Confirmatory studies have shown that Gram-negative infections do in fact potentiate Gram-positive infections (and *vice versa*) (Duan *et al.*, 2003; Dalton *et al.*, 2011). For example, it has been shown in a murine model that *P. aeruginosa* detects the presence of Gram-positive peptidoglycan and responds by increased production of virulence factors *in vivo* (Korgaonkar *et al.*, 2013). Peptidoglycan production by *S. aureus* was found to increase *P. aeruginosa* infection, and reduce numbers of Gram-positive bacteria in both a Drosophila and a murine chronic wound model (Korgaonkar *et al.*, 2013). The reduction in Gram-positive bacterial numbers was attributed to an increase in toxin production affecting the Gram-positive bacterial population.

Bio-optical imaging and bioluminescent strains in device-associated infection models

Bio-optical imaging of either bioluminescence or fluorescence signals from engineered pathogens, antibiotics and biomarkers, is emerging as an invaluable tool in device associated infection models. Bioluminescent, light-producing *S. aureus* strains are commercially available and increasingly used for *in vivo* and *in vitro* infection imaging research since they can be directly imaged in tissue or implants. The *S. aureus* strain Xen29 is derived from the pleural fluid clinical isolate ATCC 1260017, strain Xen36 is derived from the bacteraemia isolate ATCC 49525 (Pribaz *et al.*, 2012) and Xen40 is derived from the highly reported, virulent osteomyelitis clinical isolate, UAMS-1

(Elasri *et al.*, 2002). Xen29 and Xen40 showed similar concentration-dependent increases in bioluminescent signals that peaked on day 3 in a mouse model of infection and then decreased to a steady-state level that was 2- to 4-fold above background levels from 14 to 42 days (Pribaz *et al.*, 2012). In contrast, Xen36 had higher concentration-dependent increases in bioluminescent signals that peaked on day 3 then decreased to a steady-state level that was 8- to 10-fold above background levels from 14 to 42 days (Pribaz *et al.*, 2012). Higher optical emission signals observed for Xen36 compared with Xen29 or Xen40 in these models was likely due to the placement of the bioluminescent construct in a stable bacterial plasmid in Xen36 (Pribaz *et al.*, 2012) whereas Xen29 and Xen40 contain one copy of the bioluminescent construct placed in the bacterial chromosome (Kadurugamuwa *et al.*, 2003; Pribaz *et al.*, 2012). These models suggest that these *S. aureus* bioluminescent pathogens have certain phenotypic and pathogenic traits useful for *in vitro* and *in vivo* infection assays. Further validation using side-by-side comparisons with clinical isolates will be required to assert more equivalence.

In addition, fluorescent marker molecules have been validated for use in *in vivo* imaging of experimental models of infection (Sjollema *et al.*, 2010; Daghighi *et al.*, 2014) and cadaveric trials in humans have shown promise for fluorescently labelled vancomycin (van Oosten *et al.*, 2013). A recent study that combined bioluminescent bacterial strains with fluorescent observation of inflammatory process (Daghighi *et al.*, 2014) highlights the possibilities of these techniques, which are certain to become important tools in the research arena, though potentially also in the clinical diagnosis of infection.

Assessing antimicrobial efficacy and elution

The development of any antimicrobial-loaded device will also require evaluation and optimisation of antimicrobial release kinetics and local dosing efficacy and pharmacology. The optimal antimicrobial release profile has not been clearly validated for any clinical situation. Reliable pathogen killing is the desired performance yet how this outcome is best and reliably linked to antibiotic release *in vivo* is not proven. Typically, most biomaterials release antimicrobial fractionally with an initial substantial burst release, lasting from minutes to one or more days and 40-70 % of total drug load. Such a release profile may be suitable for short-term devices (*e.g.*, endotracheal tubes), or those at elevated risk of infection at time of implantation (*e.g.*, intramedullary nailing after open fracture). Antibiotic-loaded bone cement clinically used in the treatment of osteomyelitis is characterised by a substantial burst (Neut *et al.*, 2010). An alternative release profile that is regularly pursued is an extended or controlled release pattern that attempts to minimise drug burst. Typically, this may involve controlled erosion or degradation of matrix surface layers, gradually releasing antimicrobial beneath, or chemical modification of the drug to change the release from the surrounding material.

The preclinical assessment of antimicrobial-loaded devices is unfortunately fraught with challenges in determining clinical efficacy. Clearly, release kinetics of

the antimicrobial from the carrier are a function of matrix size, shape, and porosity. *In vitro* testing of convenient coupons or discs may therefore not reflect release from a large coating spread over a complex device, such as a prosthetic joint for example. Furthermore, the antimicrobial release may be significantly influenced by the composition, temperature and volume of fluid surrounding the test material. Higher release concentrations may be achieved by immersing large release matrix in a small volume of release fluid, which is an artificial situation not representative of any realistic *in vivo* situation. In a time course experiment, results may be affected if each sample point completely replaces all the surrounding release fluid *versus* small samples taken and replaced with an equal volume. Generally, infinite sink conditions are recommended for assays to provide clear understanding of the release kinetics *in vitro*. However, how this relates to the *in vivo* situation is unclear. Above all other aspects discussed presently, it would be valuable and achievable to produce guidelines on the conditions required for *in vitro* testing of biomaterials releasing antimicrobial drugs. Such guidelines are currently not available.

Challenges in Studying Device-Associated Infection *in vivo*

Requirements for *in vivo* studies
Preclinical *in vivo* testing for safety and efficacy testing of potential anti-infective interventions is required to petition regulatory bodies for possible human use. Since *in vitro* tests are incapable of replicating the complex host response to bacteria, tissue trauma and placement of a medical device, such preclinical determinations *in vivo* are required. Nonetheless, precise experimental paths needed to address regulatory concerns for antimicrobial devices in humans are frequently unclear and continuously evolving worldwide. This uncertainty and the costs associated with pursuit of any statistically validated *in vivo* tests have proven to be barriers for innovation (Busscher *et al.*, 2012, Grainger *et al.*, 2013). Despite specific regulatory considerations for combination devices, primary mode of action as a medical device must be shown to be unaffected by secondary delivery of antimicrobial agent or the antimicrobial property. Co-predicate claims are possible to assert in regulatory filings (*i.e.*, for a precedent approved similar implanted device and a precedent approved drug in the same therapeutic context) as the basis for a combination device 510k Federal Drug Administration (F.D.A.) application in the USA, but the success of this strategy as the basis for regulatory approval are unknown. Additionally, ostensible clinical trial designs are generally the same as for a new medical device, regardless of this designation, meaning that trials will be costly and extensive, limiting commercial enthusiasm for their pursuit in the face of shifting, uncertain regulatory demands. In some cases, the regulatory pathway for antimicrobial strategies (even for devices) has been designated to be as an investigational new drug, requiring more extensive testing and clinical assessments, and trial costs, frequently a "no-

go" for further testing and development on a commercial basis.

In moving antimicrobial strategies to *in vivo* testing, the critical factor contributing to infection risk is the underlying trauma or pathology that requires surgical intervention. In the case of soft and hard tissue damage caused by trauma, the local tissue effects may also include cellular necrosis, devascularisation, hypoxia, haematoma, oedema and increased intra-compartmental pressure (Gristina *et al.*, 1991; Guillou, 1993; Wichmann *et al.*, 1996). The associated compromised vascular perfusion can lead to reduced humeral and cellular immune competences at the site of injury (Hoch *et al.*, 1993). These immunological deficits can then lead to infection susceptibility independent of whether or not the wound is open or closed (Gustilo and Anderson, 1976; Krettek, 1998) and whether soft (Kalicke *et al.*, 2003) and/or hard tissues are traumatised (Wichmann *et al.*, 1996). Many of these risk factors for device-associated infection are clearly not amenable to *in vitro* investigation. Thus, *in vivo* models have proven indispensable in the preclinical testing of anti-infective interventional strategies. A comprehensive review of *in vivo* implant infection model pros and cons is not within the scope of the present review; however, numerous reviews are available on this topic (An and Friedman, 1998; Calabro *et al.*, 2013).

Basic limitations of *in vivo* models
Many infection risk factors are patient-specific, such as immune status, tissue compromise and co-morbidities. Unfortunately, many of these human factors are also challenging to introduce into *in vivo* studies (Table 2). In contrast to diverse co-morbidities and varying immune competences of human patients, the overwhelming majority of *in vivo* studies use young, healthy and often syngeneic animals, despite the fact that young healthy patients are the minority in device-associated infection. For example, syngeneic in-bred rodents are intrinsically resistant to infection, resulting in difficulty in reliably creating even short-lasting acute infections. To produce infections, these models often require either deliberate addition of a sclerosing agent or extremely high doses of bacterial inoculum (Norden, 1970). Sclerosing agents are a particularly questionable addition in bone infection studies as they may influence bone loss independent of infection status. Reports have shown that infected animals displayed similar radiographic changes to uninfected animals in receipt of sclerosing agents (Scheman *et al.*, 1941).

Similarly, super-dosing of high bacterial inocula into these animals is an equally unrealistic situation for modelling all device-associated infections, with the possible exception of open traumatic wounds. The numbers of bacteria found in operating room exposures (*e.g.*, 10^2 CFUs) are many orders of magnitude lower than the mega-doses (*i.e.*, 10^5-10^9 CFUs) of bacteria inoculated into healthy test animals. Alone, the addition of very large numbers of bacteria will elicit a large immune response, regardless of whether an infection develops or not. The requirement for such high doses to produce infection is a clear indication that the animal model used is highly

resistant to infection. This is a significant limitation in terms of replicating the clinical situation where infections are considered to arise from much lower inocula.

One interesting study investigated the incidence of infection in experimental animals included in tissue engineering research studies. It appeared that these constructs, even though they have maintained a seeded, viable eukaryotic cell population within the construct, appear to have infection rates equivalent to human patients receiving an implant (Kuijer *et al.*, 2007). The "opportunism" present in human surgical infections is thus replicated in these examples and represents an accurate, though non-practical (ethical and economical) model for opportunistic and subclinical infections.

Testing against immunologically naive animals

For the predominant pathogens causing device-associated infections, such as *S. epidermidis* and *S. aureus*, preclinical studies are regularly performed in specific pathogen-free animals. As such, the hosts have been bred with particular attention to ensure that the animals do not retain any infectious pathogens that may contribute to anomalous results. Furthermore, these species do not carry endogenous strains of these microorganisms under normal circumstances, and are unlikely to have significant transcutaneous inoculation of these particular species. The use of disease-free animals is clearly an important factor to ensure that healthy animals are used in research studies: however, it should be contrasted with the clinical reality whereby the normal patient populations are certain to have experienced numerous minor temporary septicaemia or tissue abrasions, exposing these patients to staphylococcal antigens for many years. The patient therefore will have a complex bank of immune "memory" to staphylococci. The contrast with the test animal is therefore significant, where the inoculation of bacteria directly into the wound is the first immunological exposure to this species. Such stark differences may be particularly important for investigations of passive and active immunisation strategies against pathogens, the development of sub-acute infection models, and the impressive ability of some species to seemingly repel enormous "super-dose" inocula in infection models.

Microbial virulence *in vitro*, *ex vivo* and *in vivo*

Numerous bacterial virulence factors are differentially expressed depending upon environmental conditions. These include adhesins, toxins, immune evasive molecules and even global regulatory loci (Chan and Foster, 1998; Lammers *et al.*, 2000; Vriesema *et al.*, 2000; Goerke *et al.*, 2001). MSCRAMMs are a family of staphylococcal adhesins known to play crucial roles in early stages of infection by facilitating adhesion to host tissues or the surfaces of implants after preconditioning with host proteins. *S. aureus* MSCRAMM expression was demonstrated to fluctuate with growth phase (McAleese *et al.*, 2001), growth culture environment (Massey *et al.*, 2002) or intracellular survival within neutrophils (Garzoni *et al.*, 2007). Data have also shown that MSCRAMM gene expression may be altered during *in vivo* growth that cannot be replicated by *in vitro* or *ex vivo* conditions (Sellman *et al.*, 2008). For example, it has been shown
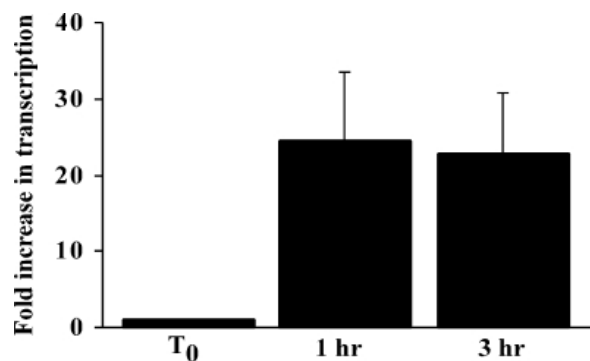
Fig. 2. Illustration of the difference between *in vitro* and *in vivo* conditions of virulence factor expression in *S. epidermidis*. sdrG is a surface-associated fibrinogen binding protein present in most strains of *Staphylococcus epidermidis*. *In vitro* (T_0) expression is low, but results show an increased transcript level 1 h following a shift from growth in nutrient broth to growth in the bloodstream of a mouse (Sellman *et al.*, 2008). (Figure reproduced by permission).

that bacterial surface adhesins of *S. epidermidis* were expressed after 30 min in a murine model (Figure 2). However, neither growth in TSB nor growth in a serum-supplemented growth medium could induce expression of the particular MSCARAMM in question (SdrG, a surface-associated fibrinogen binding protein). A similar study investigated *S. aureus* transcriptional responses to either log phase growth, stationary phase growth, or growth *in vivo* over a period 0.5 to 6 h in the murine lung. As many as 1000 gene transcripts were shown to either increase or decrease after even 30 min growth *in vivo*, in comparison with either laboratory condition (Chaffin *et al.*, 2012). Affected transcripts included nutrient acquisition and virulence factor expression and regulation, including phenol soluble modulins and alpha toxin (Chaffin *et al.*, 2012). Similar type studies have also shown transcriptional changes between *in vitro* cultured *S. aureus* and *in vivo* models of endocarditis (Xiong *et al.*, 2006) and cystic fibrosis lungs (Goerke and Wolz, 2004), highlighting that global transcriptional changes that occur in *S. aureus* upon exposure to an *in vivo* environment are not replicated by *in vitro* conditions. In a model specifically focused on medical device-associated infection, Goerke *et al.* showed that regulation of alpha toxin production was altered after growth in a guinea pig host compared with *in vitro* growth (Goerke *et al.*, 2001). Furthermore, it appeared that alpha toxin and coagulase were closely linked to *sae* (global regulator) expression *in vivo* (Goerke *et al.*, 2005), which contrasts with the *in vitro* situation where the *agr* regulator appears to play a more prominent role.

Certain "*in vivo*" niches have also been reported with regards to biofilm formation. In one example, two strains of *S. aureus* that differentially express the biofilm-forming polysaccharide intercellular adhesin (PIA) *in vitro* and *in vivo* were studied. *S. aureus* strain RN6390 produced PIA *in vitro* (only after 48 h anaerobic growth), whereas *S. aureus* Newman did not produce PIA under any *in vitro*

conditions. However, after growth in a subcutaneous tissue cage model in murine and guinea pig hosts, both *S. aureus* strains produced PIA late in the infection course, indicating an *in vivo* biomaterial associated infection specific response not detected by conventional *in vitro* growth (Fluckiger *et al.*, 2005). Similar data also show that biofilm isolates taken from human patients with cystic fibrosis display significant differential gene expression *in vivo* in comparison with *in vitro* cultured biofilms (Goerke *et al.*, 2000).

Host species-specific virulence

The relative importance of virulence factors and immune evasion factors may be significantly variable between different host species (Holtfreter *et al.*, 2010). Species-specific activity of bacterial toxins has recently emerged as a potential confounding factor in preclinical *in vivo* trials of at least some virulence factors (Loffler *et al.*, 2010). The *S. aureus* exotoxin Panton-Valentine leukocidin (PVL) is found in a majority of MRSA strains that cause CA-MRSA infections, such as necrotising pneumonia and skin and soft tissue infections (Lina *et al.*, 1999; Gillet *et al.*, 2002). In numerous mouse studies and *in vitro* studies using murine cells, PVL was not found to significantly activate or kill murine neutrophils (Voyich *et al.*, 2006; Bubeck *et al.*, 2007). However, later discoveries *in vitro* showed that PVL did activate and kill human and rabbit neutrophils, but was inactive against mouse or monkey neutrophils (Fig. 3) (Loffler *et al.*, 2010). This specificity for rabbit neutrophils is somewhat corroborated by preclinical studies *in vivo* whereby rabbits were in fact found to display differential response to infection with PVL positive and corresponding PVL negative mutants (Diep *et al.*, 2008; Cremieux *et al.*, 2009; Lipinska *et al.*, 2011), which was undetected in the murine models. The reason for the species-specific sensitivity to this toxin is unknown, but varying receptors or signal transduction pathways between species are likely to be responsible. This highlights the importance of species selection, since some animals do not necessarily correctly replicate *S. aureus* diseases in humans.

Preclinical success does not preclude clinical failure

The most prominent examples of anti-infective strategies that passed preclinical test phases yet failed in early clinical trials are numerous staphylococcal vaccines developed over the past decades (Botelho-Nevers *et al.*, 2013). A wide range of active staphylococcal vaccine targets have been selected on the basis of preclinical animals studies, and efficacy has been variable, though often promising. Unfortunately, all have failed in clinical trials to date. For example, a vaccine targeting the iron-sequestering protein, IsdB, showed promising results in preclinical *in vitro* and *in vivo* tests (Kuklin *et al.*, 2006; Brown *et al.*, 2009). Unfortunately, clinical studies could not support the use of this vaccine in cardiothoracic patients (Fowler *et al.*, 2013). Possible reasons for the failure of this and similar vaccines are discussed elsewhere (Scully *et al.*, 2014), though precisely why the discrepancy exists remains largely unexplained. Similarly, preclinical murine studies indicated that a tumour necrosis factor (TNF)-α receptor therapy showed efficacy in a mouse endotoxemia model (Mohler *et al.*, 1993), yet human clinical trials proved

less successful (Fisher *et al.*, 1996). On-going uncertainty in how antimicrobial strategies fail to translate from preclinical to clinical efficacy and the considerable costs of failure in translation have led to risk-aversion among many biomedical device commercialization efforts.

Challenges Posed by Device-Associated Infections to the Clinical Laboratory

Suitability of PK/PD principles in device-associated biofilm infections

Clinical therapies for all bacterial infectious diseases are based upon administration of antibiotics. The selection of the particular antimicrobial regimen and dosage used are based on a combination of laboratory test results, empirical selection and basic pharmacokinetic and pharmacodynamic principles. The minimum inhibitory concentration (MIC) is a familiar anti-microbiological parameter related to the minimum concentration of a particular antimicrobial agent in solution required to inhibit growth of a particular microorganism under defined, planktonic conditions. In theory, the MIC is used to estimate the likelihood of *in vivo* efficacy as it is correlated with dosing, potency and pharmacokinetics in humans and usual dosage regimens. Careful attention to pharmacodynamic principles has been shown to correlate with the treatment of some infectious diseases such as hospital-acquired pneumonia (Kim *et al.*, 2009), however, this is not necessarily true for all infections (Smith *et al.*, 2003). For example, biofilm growing bacteria do not retain the MIC values of their planktonic counterparts and, similarly, the growth phase of the bacterium may affect the MIC, or MBC (minimum bactericidal concentration) of some antibiotics (Kim and Anthony, 1981). The discrepancy between such laboratory results (MIC) and biofilm susceptibility has been conclusively shown for cystic fibrosis (Moriarty *et al.*, 2007) and orthopaedic device isolates when grown as biofilm (Molina-Manso *et al.*, 2013). This phenomenon is believed to hold true for all bacterial biofilms, at least those of medical concern. The lack of *in vitro*:*in vivo* dosing correlation for treating biofilm-related diseases has spurred development of "biofilm inhibitory concentrations" (BIC) or minimum biofilm eliminating concentration (MBEC) assays (Sepandj *et al.*, 2004). Typically, the BIC/MBEC is usually significantly higher than the MIC, as would be expected. Nevertheless, attempts have been made to tailor antibiotic dosage regimens to the BIC/MBEC rather than the MIC. In cystic fibrosis, where biofilm infections are present on and near the lining of the alveoli in the lungs of affected patients, a trend for reduced bacterial load upon completion of the MBEC-led antibiotic regimen (Keays *et al.*, 2009) was found in early studies, however, later studies could not identify a significant effect (Moskowitz *et al.*, 2011).

Lack of efficacy of single antibiotic agents has led to the use of antibiotic combination therapies for many infectious diseases. Antibiotic combinations are potentially synergistic and investigations have sought to answer whether antibiotic therapy tailored to synergistic combinations display improved treatment outcomes.
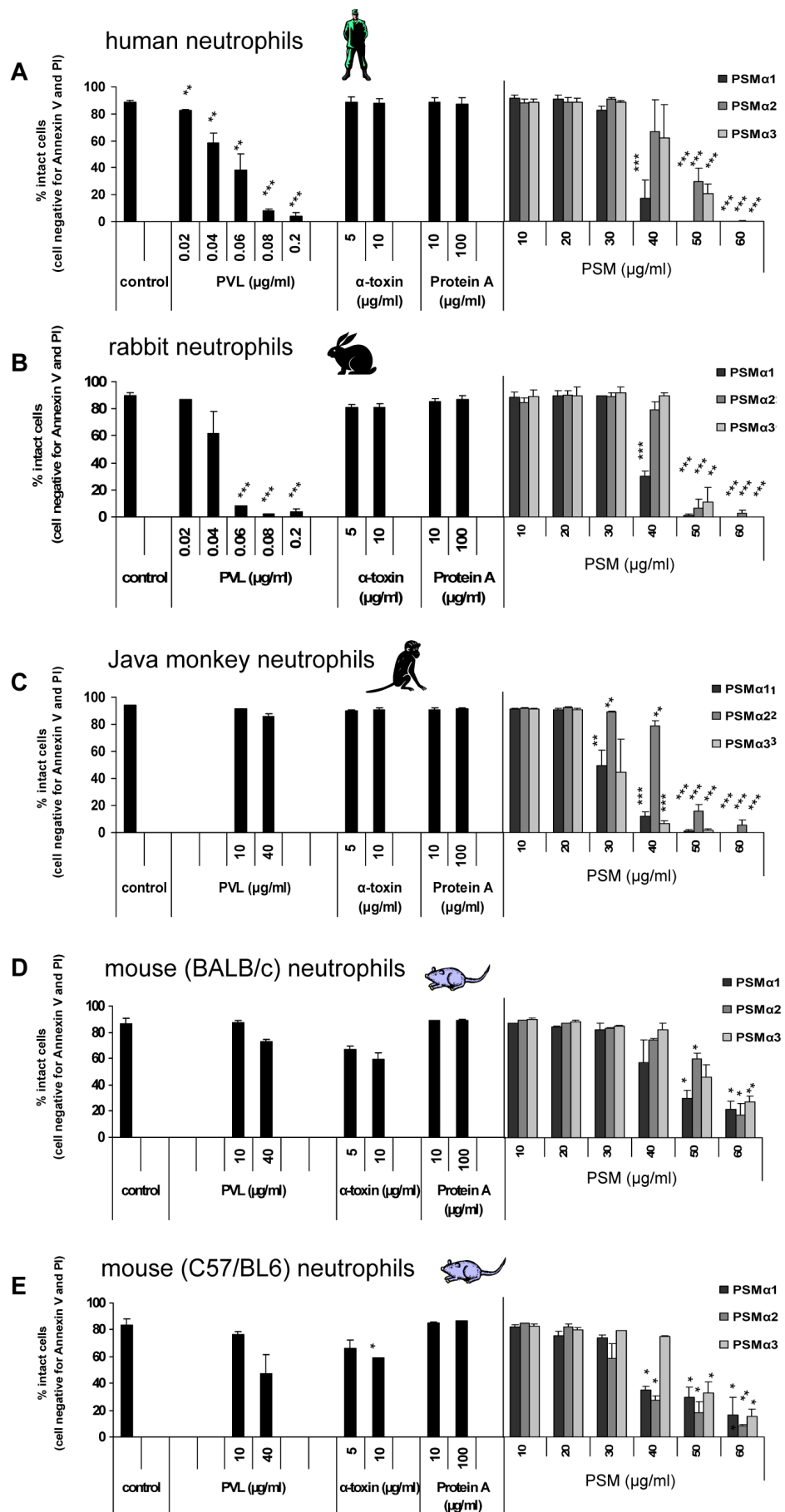
Fig. 3. The cytolytic effect of purified *S. aureus* virulence factors on neutrophils from different species (Loffler *et al.*, 2010). Species differences in cytolytic activity highlight the importance of species selection in *in vivo* studies. (Figure reproduced by permission).

Unfortunately again, despite many claims to efficacious antimicrobial synergy for combination drugs, clinical antimicrobial efficacy of antibiotic combinations did not improve treatment outcomes over and above empiric therapy for cystic fibrosis patients (Aaron *et al.*, 2005), although some prophylactic effect against septicaemia in transplant patients was observed (Haja *et al.*, 2012). In contrast, antibiotic combinations have been shown to offer protection against infection when present on catheters without any observed increase in antibiotic resistance (Ramos *et al.*, 2011; Brooks *et al.*, 2013).

One interesting approach to developing an *in vitro* assessment of antibiotic efficacy against biofilm and mimicking the normal *in vivo* fluctuations in local antibiotic concentrations was reported by Widmer *et al*. (Widmer *et al.*, 1990) Their strategy involved growing bacterial biofilms on small glass beads and exposing them to fluctuating antibiotic concentrations predicted based on normal human drug pharmacokinetics. The authors were able to identify antibiotic combinations that could be shown to result in eradication of biofilm *in vitro* (Widmer *et al.*, 1990). Replicating these treatment scenarios in the guinea pig exhibited a correlation between the regimens found to work *in vitro* with clinical outcomes *in vivo*. This approach has not been extensively repeated, despite demonstrated utility in clinical situations, and warrants further investigation and utilisation.

The detection of biofilm infections in the clinical microbiology laboratory
The clinical microbiology laboratory is tasked with assessing the presence, identity and antibiotic resistance pattern of bacteria in clinical specimens. In the realm of medical device-associated infections, a slowly emerging realisation is that these infections are frequently biofilm infections and require specific biofilm-detection techniques. Not all infections produce biofilms, but biofilms are increasingly implicated in difficult-to-treat implant-associated infections (Busscher *et al.*, 2012). Numerous studies have shown that sampling from the explanted device, by targeting the biofilm on the surface, increases the detection rate of bacteria in comparison with directly sampling tissues (Tunney *et al.*, 1999; Trampuz *et al.*, 2007). The method of choice for detecting implant infections is surgical explant sonication, with subclinical or quiescent infections sometimes detected by this method when infection was not suspected by the treating physician (Tunney *et al.*, 1999; Trampuz *et al.*, 2007). Similarly, it has been shown that by extending bacterial culture times, the detection rate of numerous pathogens may be increased (Schafer *et al.*, 2008), particularly slow-growing, fastidious pathogens such as *Propionibacterium acnes*. In addition, to enable detection of pathogens in the clinical laboratory, each microorganism causing the infection must obviously be capable of growth in the culture media regularly in use in clinical microbiology laboratories. Broadly non-selective and enrichment broths and agars, such as blood agar and thioglycolate broth, are commonly used. The most commonly isolated microbes from implant-related osteomyelitis, *S. aureus and S. epidermidis,* are eminently culturable in standard growth media. Unfortunately, an estimated 90 % of bacterial species are not culturable by conventional nutrient media or may be in a viable but non-culturable state (Oliver, 2010). Thus, a significant proportion of bacterial species, at least in theory, may be missed due to inappropriate detection techniques. This problem is further compounded by the realisation that bacteria growing within a biofilm may also be non-culturable, even less fastidious microbes such as *S. aureus* (Palmer *et al.*, 2011), through mechanisms that to date remain unexplained.

Through the use of more sophisticated detection techniques, the true prevalence of bacterial infection in medical device-associated infection may be emerging (Palmer *et al.*, 2011). For example, by combining biofilm sonication from explanted orthopaedic implants and non-culture polymerase chain reaction (PCR)-based techniques, the detection of bacterial contamination around failed (septic and "aseptic") implants has been shown to be increased (Tunney *et al.*, 1999). Similar data has been replicated numerous times since, even in the absence of PCR data, further highlighting the clinical relevance of sonication (Trampuz *et al.*, 2007). More recently, the IBIS system (Ecker *et al.*, 2008), a mass spectrometry-based technology developed for rapid detection of potential bioterrorism-related microbes, was also found to be capable of identifying the bacteria present in wound tissue with greater sensitivity than culture or PCR (Palmer *et al.*, 2011; Howe *et al.*, 2013). Using this technique, it has been shown that large numbers of a diverse array of microbes may colonise tissues adjacent to orthopaedic implants, and which due either to biofilm formation or fastidious growth requirements, do not grow in laboratory conditions. The clinical implications of such work are potentially very significant, though it remains to be seen how practices in the clinical microbiology laboratory may be affected, since such equipment is out of the range of most hospital laboratories from a cost and operational perspective. It should also be recognised that many of the molecular techniques described provide confirmation only of the presence of bacterial DNA, and not proof of a viable bacterium. There is significant risk that an infection that has been cleared may be DNA-positive for dead bacteria, which is a significant uncertainty. Potentially, centralised reference laboratories could serve to provide definitive diagnosis of bacterial contamination of tissues in all cases of hardware failure or removal, whether an infection is clinically suspected or not. Nevertheless, it is clear that current conventional bacteriological practices are likely to be significantly under-reporting clinical infections. This subsequently leads to under-reporting in the literature, further exacerbating the lack of evidence to support research into the methods and assessing their outcomes.

Summary and Conclusions

With increasing prevalence of multi-drug resistant bacteria in the hospital setting, clinicians are already faced with treating device-associated infections using a diminishing arsenal of anti-infective tools. To survive regulatory scrutiny to enter the market as a product, let

alone achieve a clinically measureable impact, new anti-infective interventions must be subjected to preclinical test regimens that robustly and consistently provide clinically relevant evaluation. Neither *in vitro* nor *in vivo* testing currently provides a satisfactory level of proof to reliably predict efficacy for each subsequent step in the product development process. Presently, it seems that current testing protocols are not fit as a reliable screen prior to clinical implementation. In order to achieve the clinical goals of both reducing incidence and improving treatment of device-associated infections, improved validation, testing, and interpretation standards must be set. Consensus is unavailable and currently no formal scientific or medical bodies are vested in achieving this ideal. Cross-disciplinary research networks aimed at providing fundamental targets for preclinical testing and based on representative clinical demands shall be required to advance towards this goal. Currently, the research output on the topic suffers from notable lack of consensus, vast disparity in testing protocols and limited correlations with clinical realities.

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Discussion with Reviewers

J. Wenke: The authors suggest changes in *in vitro* and pre-clinical testing to survive regulatory scrutiny. Authors state that consensus is needed on how to do the required tests. How do the authors envisage that such consensus will be reached, and more importantly, convince the scientific (and regulatory) community of this?

Authors: Guideline documents are available for numerous clinical and basic laboratory assays. The bodies publishing such guidelines, such as the Clinical and Laboratory Standards Institute (CLSI), and the International Organization for Standardization (ISO) draw upon the expertise available within the practicing professionals in the field. Recently, a consensus document has been published on the diagnosis of prosthetic joint infection, again published with the input of professionals active in the field. A similar process could clearly be suitable for generating and publicising preferred protocols in the area of anti-infective device preclinical testing.

H.C. van der Mei: Hardware infections are both fairly common and very challenging to treat, and it is clear that the current *in vitro* and *in vivo* assessments may not be the most predictive of clinical efficacy. There are many different variations for anti-infection hardware for different clinical issues, which would likely require different preclinical assessment approaches. What approach would you suggest for preclinical evaluation of an intramedullary nail that has a durable coating that slowly elutes an antimicrobial? Please assume that the application is for open fractures of the tibial diaphysis.

Authors: The development of such a device has precedence in the European market with the development of the ETN ProTect antimicrobial tibial nail by DePuy Synthes. The preclinical testing of this device has been published in the literature, with several studies establishing the efficacy of the coating in preventing infection in preclinical *in vivo* models. The ETN ProTect nail provides a good example of a product tested in a rat model, which revealed a 100 % reduction in bacterial numbers. As such, this model represents a positive example of a preclinical test phase accurately predicting clinical success, as seen by published data since its release for human medicine.