ANTIMICROBIAL DRUGS IN FIGHTING AGAINST ANTIMICROBIAL RESISTANCE: PREPARATION AND IN VITRO ANTIMICROBIAL PROPERTIES OF PRP

Susanta kumar panda[1], Garima Pandey[2]
Department of Microbiology, Maharishi University of Information Technology (U.P)

Abstract

The outbreak of antimicrobial resistance, together with the lack of newly developed antimicrobial drugs, represents an alarming signal for both human and animal healthcare worldwide. Selection of rational dosage regimens for traditional antimicrobial drugs based on pharmacokinetic/pharmacodynamics principles as well as development of novel antimicrobials targeting new bacterial targets or resistance mechanisms are key approaches in tackling AMR. In addition to the cellular level resistance (i.e., mutation and horizontal gene transfer of resistance determinants), the community level resistance (i.e., bilofilms and persisters) is also an issue causing antimicrobial therapy difficulties. Therefore, anti-resistance and antibiofilm strategies have currently become research hotspot to combat antimicrobial resistance. Although metallic nanoparticles can both kill bacteria and inhibit biofilm formation, the toxicity is still a big challenge for their clinical applications. In conclusion, rational use of the existing antimicrobials and combinational use of new strategies fighting against antimicrobial resistance are powerful warranties to preserve potent antimicrobial drugs for both humans and animals. Implant-associated infection is becoming more and more challenging to the healthcare industry worldwide due to increasing antibiotic resistance, transmission of antibiotic resistant bacteria between animals and humans, and the high cost of treating infections. In this study, we disclose a new strategy that may be effective in preventing implant-associated infection based on the potential antimicrobial properties of platelet-rich plasma (PRP). Due to its well-studied properties for promoting healing, PRP (a biological product) has been increasingly used for clinical applications including orthopaedic surgeries, periodontal and oral surgeries, maxillofacial surgeries, plastic surgeries, sports medicine, etc. PRP could be an advanced alternative to conventional antibiotic treatments in preventing implant-associated infections. The use of PRP may be advantageous compared to conventional antibiotic treatments since PRP is less likely to induce antibiotic resistance and PRP's antimicrobial and healing-promoting properties may have a synergistic effect on infection prevention. It is well known that pathogens and human cells are racing for implant surfaces, and PRP's properties of promoting healing could improve human cell attachment thereby reducing the odds for infection. In addition, PRP is inherently biocompatible, and safe and free from the risk of transmissible diseases. For our study, we have selected several clinical bacterial strains that are commonly found in orthopaedic infections and examined whether PRP has *in vitro* antimicrobial properties against these bacteria. We have prepared PRP using a twice centrifugation approach which allows the same platelet concentration to be obtained for all samples. We have achieved consistent antimicrobial findings and found that PRP has strong *in vitro* antimicrobial properties against bacteria like methicillin-sensitive and methicillin-resistant *Staphylococcus aureus,* Group A *Streptococcus*, and *Neisseria gonorrhoeae*. Therefore, the use of PRP may have the

potential to prevent infection and to reduce the need for costly post-operative treatment of implant-associated infections.

Keywords: antimicrobial resistance, biofilm, persisters, antimicrobial drug, nanoparticles.

1. INTRODUCTION

Antimicrobial resistance (AMR) is one of the ultimate fears to the health of humans and animals worldwide. Use of antimicrobial drugs in humans or animals results in the emergence and dissemination of resistant bacteria, and overuse or abuse of them makes this situation worse. Thus, it is important to simultaneously preserve effective antimicrobials as long as possible as well as continue to employ them for the service of human and animal health (Chang et al., 2015).

The dissemination of AMR has not been paralleled by the development of novel antimicrobials. This is due to that the process of drug discovery and clinical trials of new antimicrobials takes a long time, and only a few new agents have recently been approved for use. These situations prompt the efforts to develop alternatives to traditional antimicrobials, as described in our previous review (Cheng et al., 2014). However, some of the alternatives are only used for the prevention of bacterial infections (e.g., vaccines); some show indirect effect against pathogens (e.g., immunomodulators, feed enzymes); some are of complex composition (e.g., probiotics, plant extracts), thus the effects vary greatly; and the antimicrobial peptides, such as one of the bacteriocins, lantibiotics, have been reported causing bacterial resistance (Draper et al., 2015).

In this review, we briefly focus on old and novel antimicrobial agents in tackling AMR. The AMR occurs on two levels, the cellular level resistance (mutation and horizontal gene transfer (HGT) of resistance determinants) and the community level resistance (biofilm and persister cells) (Penesyan et al., 2015). The studies reviewed suggest that only rational use of existing old antimicrobial drugs and combinational use of anti-resistance or antibiofilm strategies with antimicrobials as well as continuing development of new antimicrobial agents could fight against AMR. Several characteristics of PRP indicate that PRP may also have antimicrobial properties [6-9]. PRP contains a large number of platelets, a high concentration of leukocytes (which may possess host-defense actions against bacteria and fungi), and multiple antimicrobial peptides [7,8,10]. In a recent study of a large cohort of cardiac surgical patients, it was revealed that the intraoperative use of PRP-gel during wound closure significantly decreased the incidence of superficial and deep sternum infection [11]. For these reasons and observations, we **hypothesized** that PRP, besides its well-studied healing-promoting properties, has antimicrobial properties. The potential advantages of using PRP to prevent infection may include: (**i**) PRP is less likely to induce resistance compared to conventional antibiotic treatments. (**ii**) PRP also has properties that promote healing which may have a synergistic effect on infection prevention; PRP's healing-promoting properties could provide a seal to prevent bacterial attachment thereby reducing the odds for infection as pathogens and human cells are racing for implant surfaces [12,13]. (**iii**) PRP is inherently biocompatible, and safe and free from the risk of transmissible diseases. Our long-term goal is to use PRP as a new approach to prevent implant-associated infections. The **aim** of this study was to prepare PRP using a twice centrifugation approach, to examine PRP's *in vitro* antimicrobial properties, and to describe the protocols for evaluating such antimicrobial properties.

2. DEVELOPMENT OF NEW ANTIMICROBIALS

The current antimicrobials, mainly derived from natural sources, inhibit cellular processes such as cell wall biosynthesis, DNA replication, and protein synthesis. With the worldwide emergence of AMR, there is renewed interest in the investigation of alternative essential cellular processes, including bacterial central metabolic pathways, as the drug targets for the next generation of antimicrobials (Murima et al., 2014). For examples, bedaquiline is an antitubercular drug targeting the F_0F_1 ATP synthase (Andries et al., 2005). Like bedaquiline, Q203, an optimized imidazopyridine amide compound, selectively inhibits the respiratory cytochrome *bc1* complex in mycobacteria regardless of architectural conservation of the *bc1* complex in many species (Pethe et al., 2013). The inhibition of the bacterial divisome, mainly by targeting the central cell division mediator FtsZ, has been accepted as a promising strategy for antimicrobial attack by either interfering with

the natural dynamics and functions of FtsZ during the cell cycle or activating a bacterial protease to degrade FtsZ, thus causing bacterial death in a suicidal manner (Sass and Brötz-Oesterhelt, 2013). The mode of action of alkyl gallate is a combination of direct targeting of FtsZ and permeabilization of bacterial membranes, which is a promising hit for the further development of antibacterials (Król et al., 2015). Recent efforts have also been devoted to developing drugs that interrupt the assimilation of iron by bacteria, a process that is vital to cellular homeostasis (Foley and Simeonov, 2012). The unique asymmetric outer membrane in Gram-negative bacteria, which acts as a permeability barrier that protects the cell from external stresses such as the presence of antimicrobials, has become an attractive drug target. A novel β-hairpin macrocyclic peptide, JB-95, exhibits an ability to selectively disrupt the outer membrane through interactions with selected β-barrel outer membrane proteins including BamA and LptD, but not the inner membrane of *E. coli* (Urfer et al., 2016). Furthermore, the bacterial protein secretion pathway is a target for eliminating or disarming pathogens. Targeting the Sec-pathway for novel antimicrobial agents focuses on two key components: SecA, the ATP-driven motor protein responsible for driving preproteins across the cytoplasmic membrane and the Type I signal peptidase which is responsible for the removal of the signal peptide to allow the release of the mature protein from the membrane (Rao et al., 2014). Except for the above inhibitors targeting resistance, drugs in already-known classes such as new β-lactams, quinolones, aminoglycosides, and tetracyclines have been designed to escape from many of the known resistance mechanisms. BAL30072, a siderophore monosulfactam similar to aztreonam, exhibits antibacterial activity against most species of aerobic Gram-negative bacteria (Page et al., 2010). It is stable toward metallo-β-lactamases and is a poor substrate for many serine carbapenemases. Several new quinolones, such as avarofloxacin, delafloxacin, finafloxacin and the desfluoroquinolone nemonoxacin, which show enhanced activity against fluoroquinolone-resistant Gram-positive bacteria including MRSA are in clinical development (Page and Bush, 2014). A modified aminoglycoside, plazomicin, has been demonstrated activity against both Gram-negative and Gram-positive bacterial pathogens (Zhanel et al., 2012). Modified tetracyclines, such as tigecycline, omadacycline and eravacycline are of interest for their activity against many MDR *Enterobacteriaceae* and *Acinetobacter* spp., including isolates expressing tetracycline-specific efflux and ribosomal protection proteins (Sutcliffe, 2011).

2.1. APPROACHES TO COMBAT BIOFILMS

The approaches to combat biofilms are extensively reviewed by Beloin et al. (2014). During the biofilm development, the bacteria initially adhere to a surface that ultimately leads to colonization and infection by pathogenic bacteria. Therefore, reducing adhesion is a strategy to prevent biofilm formation and related infections (Veerachamy et al., 2014). Recently, non-specific inhibition of adhesion vs. targeting specific adhesions has been developed to reduce bacterial adhesion. Quorum sensing (QS), which controls many important physiological processes such as biofilm development, is a widespread intercellular form The deep research on the mechanism of biofilm formation leads to the emergence of numerous promising antibiofilm approaches. However, the conversion of experimental data into clinical settings is time-consuming and to some extent unsatisfactory. Non-biocidal anti-adhesive or anti-virulence strategies face the diversity of bacterial phenotypes and may only be active against a subpopulation of bacteria encountered in clinical practice, therefore limiting their overall efficacy. *In vitro* biofilms are probably structurally different from *in vivo* biofilms (Lebeaux et al., 2013). Currently, due to the diversity of the *in vivo* conditions leading to the complexity of clinical biofilm situations, the diversity of persister phenotypes is unknown. Most of the studies use rodent models, but these *in vivo* models may not properly replicate real clinical state. Furthermore, as for clinical trials, rigorous statistical analysis is compulsory in order to avoid any false positive results. Most importantly, molecules identified *in vitro* should be validated using *in vivo* models not only for the antibiofilm activity but also non-toxicity.

2.2. METALLIC NANOPARTICLES (NPS)

Physiochemical properties of nanomolecules as antimicrobial agents are widely used in human and veterinary medicine. Metallic NPs are of great interest for use as

potential antimicrobial agents because of their unique optical, electronic, and magnetic properties (Kandi and Kandi, 2015). The electrostatic interaction of NPs with negatively charged bacterial surfaces draws the particles to the bacteria and promotes their penetration into the membrane, causing membrane disruption, bacterial flocculation, and a reduction in viability. The generation of reactive oxygen species (ROS) is also a mechanism of antibacterial activity of NPs (Thekkae Padil and Cerník, 2013).

3. *In vitro* Antimicrobial Test of PRP Using Kill Curve Assay

1. Using a sterile inoculating loop, add several colonies of *S. aureus* from its overnight plate culture into 5 ml of Mueller Hinton broth (MHB) in a plastic tube. Vortex briefly and then incubate the sample for 2 hr at 37 °C. Next, the optical density of the bacterial media was determined using a spectrophotometer and adjusted to an optical density equal to ~1 x 10^8 CFU/ml based on the pre-determined standard curve.
2. Make a 100x dilution using PBS to obtain 1 x 10^6 CFU/ml and place the inoculums on ice.
3. Set up and label sterile, disposable 5 ml round-bottom polystyrene tubes, and prepare the following sample groups as indicated in **Table 1** for a final volume of 2 ml in each tube.
4. Add PRP, PPP, or PBS first to the polystyrene tubes, followed by the thrombin solution for activation (gel formation). Next, add MHB and then the *S. aureus* inoculums (1 x 10^6 CFU/ml) to obtain the final concentration of 1 x 10^5 CFU/ml.
5. Incubate the tubes at 37 °C with orbital agitation at 150 rpm.
6. At pre-determined time points (*e.g.* 0, 1, and 2 hr), mix the solutions in each tube via repeat pipetting (this step is important since bacteria may be trapped inside the PRP gel). Take 10 µl of sample, dilute serially with sterile 0.9% saline, and pipette a 100 µl aliquot of each dilution onto a Tryptic soy agar (TSA, with 5% sheep blood) plate for CFU counting.
7. Culture the agar plates overnight at 37 °C, then count and record the plate colonies. Plot data on a logarithimic scale with time (hr) on the x-axis and CFU/ml on the y-axis.

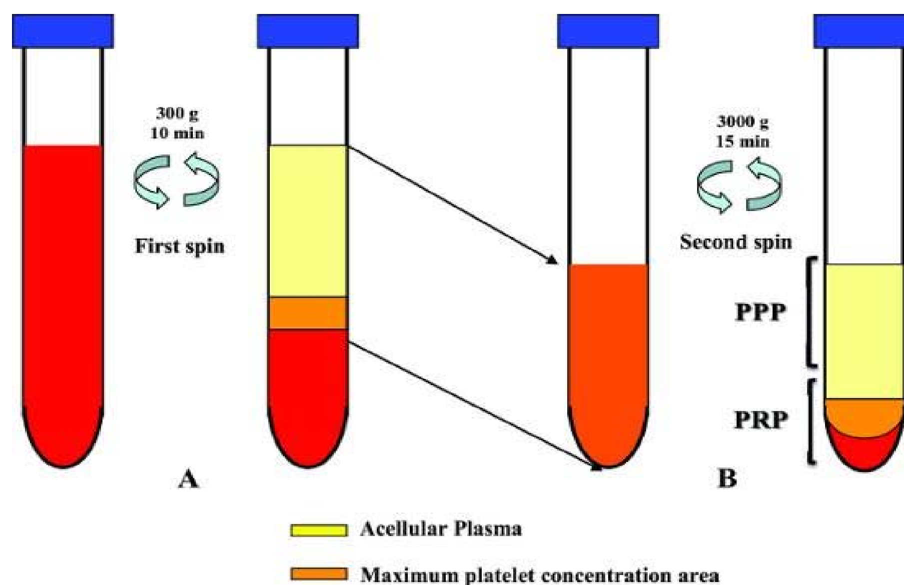

Figure 1. PRP preparation using a twice centrifugation procedure. (**A**) First centrifugation. After the first centrifugation, three layers are formed, and the top two layers (*i.e.* plasma and buffy coat layers) and 2-3 mm of the bottom layer (*i.e.* red blood cell layer) are transferred to a second sterile centrifuge tube. (**B**) Second centrifugation. After the second centrifugation, the top layer is transferred to a new sterile tube and designated as PPP. The remaining is adjusted with PPP to a platelet concentration of 2.0 x 10^6 platelets/µl and designated as PRP.

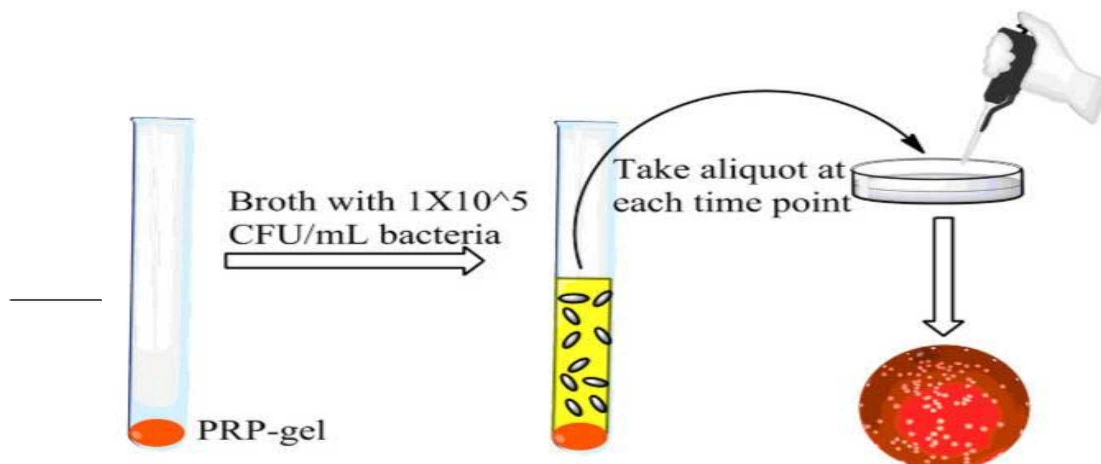

Figure 2. Experimental set-up for assessing the antimicrobial properties of PRP using the kill curve assay. First, PRP or PPP is added to the test tubes, and immediately activated with thrombin solution. Next, MHB is added followed by bacterial inoculums. Aliquots of samples are taken at different time points and plated for CFU counts.

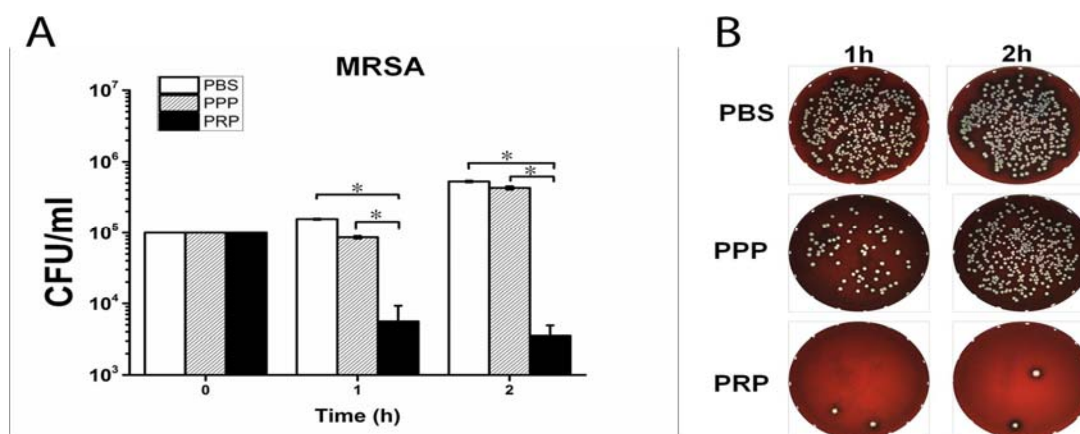

Figure 3. PRP, PPP, or PBS are placed in a sterile 5 ml polystyrene tube along with thrombin, MHB broth, and MRSA inoculum and then incubated at 37 °C with orbital agitation at 150 rpm. At pre-determined time points (*i.e.* 1 and 2 hr), aliquots of samples are taken and plated for CFU counts. (**A**) CFU data and (**B**) representative plate images at 10^{-2} dilution. Significant reduction (~100-fold at 2 hr) of MRSA growth is obtained using PRP compared to PPP and PBS controls. This is true for bacteria like MSSA, Group A *Streptococcus*, and *Neisseria gonorrhoeae* as well.

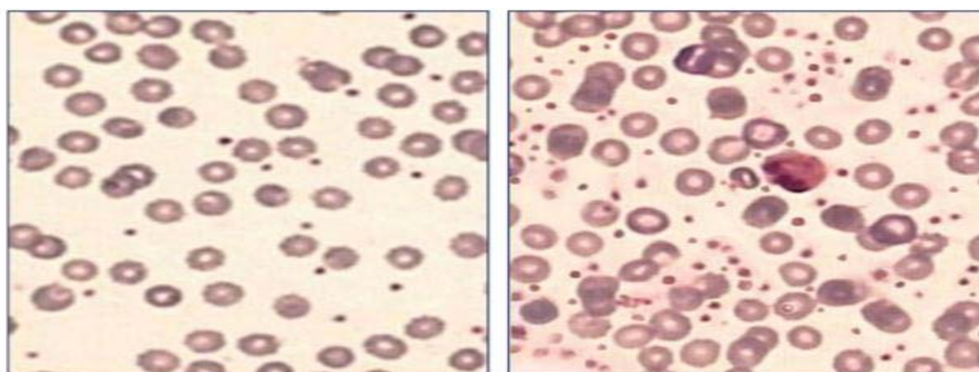

Figure 4. Blood smears of whole blood (left) and PRP (right). PRP prepared from the twice centrifugation approach has ~10 times the number of platelets compared to whole blood.

PRP is reproducibly prepared using a twice centrifugation approach (**Figure 1**). PRP is found to present strong (up to 100-fold reduction in CFUs) *in vitro* antimicrobial properties against methicillin resistant *S. aureus* (MRSA) (**Figure 3**), which is commonly found in hospitals worldwide[14]. Similarly, PRP has strong antimicrobial properties against methicillin sensitive *S. aureus* (MSSA), Group A *Streptococcus*, and *Neisseria gonorrhoeae*.

The twice centrifugation approach allows acquisition of PRP with the same platelet concentration (*i.e.* 2.0×10^6 platelets/μl) but concentrated (~10 times above the baseline in blood; **Figure 4**) and allows us to obtain consistent antimicrobial findings; no significant differences in CFU findings among PRPs from different individual animals (*i.e.* rabbits) have been observed.

4. CONCLUDING REMARKS

The paradox of antimicrobial drugs is that through their use, they not only inhibit an infection but also select for the emergence and spread of AMR, directly counteracting their long-term efficacy. Considerable inappropriate use of both prophylactic and therapeutic antimicrobials in human and veterinary medicine highlights an urgent need for antibiotic stewardship initiatives. At the present time, rational use of existing antibiotics based on PK/PD dosage-regimen is a key strategy in tackling AMR, thus to preserve potent antimicrobials for both humans and animals. At the same time, we should never stop discovering novel inhibitors with new bacterial targets and digging the treasure box of "old" and "forgotten" antimicrobials. Compounds showing profound anti-resistance and antibiofilm effects are in research hotspot, but they still have limitations. Combining existing antimicrobials with compounds that inhibit their specific resistance mechanisms would be a good choice. Although metallic NPs can both kill bacteria and inhibit biofilm formation, the toxicity is still a big challenge for their clinical applications (dos Santos et al., 2013). With single-drug therapy, there is always a selective advantage to resistance; specific combinations of drugs can inhibit bacterial growth while disfavoring resistance to the individual components. These approaches can be used to invert the selective advantage of resistant bacteria competing with their sensitive cousins, or even drive a resistant bacterial population back toward drug sensitivity (Baym et al., 2016). Besides, screening and developing multiple-target inhibitors as "resistance-resistant" antimicrobials can reduce the effects of target mutation (Oldfield and Feng, 2014). The natural products have also been a prolific and unsurpassed source for new lead antimicrobial compounds, but target identification and validation has remained a major bottleneck (Farha and Brown, 2016). Functional genomics techniques are proved to be indispensible for *in vitro* target authentication and elucidating mechanism of action of novel antimicrobials (Khan and Khan, 2016). Since most of the new strategies described in this review are only at the early basic experimental stage, their potential for clinical applications requires more extensive investigations. In this study, 50 ml whole blood was used to obtain approximately 5 ml PRP. If blood volume is a concern, pooled blood from multiple animals may be used to prepare PRP. If clots form during blood draw and/or centrifugation, most likely some platelets are activated which will result in low platelet yield. Therefore, sufficient anticoagulants and gentle but thorough mixing are important steps for successful PRP isolation.

References:

[1] Gristina, A.G. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science*. **237**, 1588-1595 (1987).

[2] Gristina, A.G. & Costerton, J.W. Bacterial adherence to biomaterials and tissue. The significance of its role in clinical sepsis. *J. Bone Joint Surg. Am.* **67**, 264-273 (1985).

[3] Everts, P.A., *et al.* Reviewing the structural features of autologous platelet-leukocyte gel and suggestions for use in surgery. *Eur. Surg. Res.* **39**, 199-207, [pii] 000101743 doi:10.1159/000101743 (2007).

[4] Marx, R.E. Platelet-rich plasma (PRP): what is PRP and what is not PRP? *Implant. Dent.* **10**, 225-228 (2001).

[5] Toscano, N. & Holtzclaw, D. Surgical considerations in the use of platelet-rich plasma. *Compend. Contin. Educ. Dent.* 29, 182-185 (2008).

[6] Cieslik-Bielecka, A., Gazdzik, T.S., Bielecki, T.M., & Cieslik, T. Why the platelet-rich gel has antimicrobial activity? *Oral Surg.*

Oral Med. Oral Pathol. Oral Radiol. Endod. **103**, 303-305, author reply 305-306, [pii] S1079-2104(06)00809-2 doi:10.1016 /j.tri ple . 2006.08.034 (2007).

[7] Yeaman, M.R. The role of platelets in antimicrobial host defense. *Clin. Infect. Dis.* **25**, 951-968, quiz 969-970 (1997).

[8] Tang, Y.Q., Yeaman, M.R., & Selsted, M.E. Antimicrobial peptides from human platelets. *Infect Immun.* **70**, 6524-6533 (2002).

[9] El-Sharkawy, H., *et al.* Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. *J. Periodontol.* **78**, 661-669, doi:10.1902/jop.2007.060302 (2007).

[10] Krijgsveld, J., *et al.* Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. *J. Biol. Chem.* **275**, 20374-20381, doi:275/27/20374 [pii] (2000).

[11] Trowbridge, C.C., *et al.* Use of platelet gel and its effects on infection in cardiac surgery. *J. Extra Corpor. Technol.* **37**, 381-386 (2005).

[12] Gristina, A.G., Naylor, P., & Myrvik, Q. Infections from biomaterials and implants: a race for the surface. *Med. Prog. Technol.* **14**, 205-224 (1988).

[13] Vardanyan, Z., Gevorkyan, V., Ananyan, M., Vardapetyan, H., and Trchounian, A. (2015). Effects of various heavy metal nanoparticles on *Enterococcus hirae* and *Escherichia coli* growth and proton-coupled membrane transport. *J. Nanobiotechnol.* 13, 69. doi: 10.1186/s12951-015-0131-3

[14] Klevens, R.M., *et al.* Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA*. **298**, 1763-1771, [pii] 298/15/1763 doi:10.1001/jama.298.15.1763 (2007).

[15] Foster, T.E., Puskas, B.L., Mandelbaum, B.R., Gerhardt, M.B., & Rodeo, S.A. Platelet-rich plasma: from basic science to clinical applications. *Am. J. Sports Med.* **37**, 2259-2272, [pii] 37/11/2259 doi:10.1177/0363546509349921 (2009).

[16] Carlson, N.E. & Roach, R.B., Jr. Platelet-rich plasma: clinical applications in dentistry. *J. Am. Dent. Assoc.* **133**, 1383-1386 (2002).

[17] Man, D., Plosker, H., & Winland-Brown, J.E. The use of autologous platelet-rich plasma (platelet gel) and autologous platelet-poor plasma (fibrin glue) in cosmetic surgery. *Plast. Reconstr. Surg.* **107**, 229-237, discussion 238-229 (2001).

[18] Fridkin, S.K., *et al.* Epidemiological and microbiological characterization of infections caused by *Staphylococcus aureus* with reduced susceptibility to vancomycin, United States, 1997-2001. *Clin. Infect. Dis.* **36**, 429-439, [pii] CID21222 doi:10.1086/346207 (2003).

[19] Jackson, C.R., Fedorka-Cray, P.J., Davis, J.A., Barrett, J.B., & Frye, J.G. Prevalence, species distribution and antimicrobial resistance of enterococci isolated from dogs and cats in the United States. *J. Appl. Microbiol.* **107**, 1269-1278, doi:JAM4310 [pii] 10.1111/ j.1365-2672.2009.04310.x (2009).

[20] Murray, C.K., *et al.* Recovery of multidrug-resistant bacteria from combat personnel evacuated from Iraq and Afghanistan at a single military treatment facility. *Mil. Med.* **174**, 598-604 (2009).

[21] Durr, M. & Peschel, A. Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. *Infect Immun.* **70**, 6515-6517 (2002).