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Minireview

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Abstract: The gold standard for the diagnosis of bacterial infections in clinical samples is based on culture tests that are time-consuming and labor-intense. For these reasons, an extraordinary effort has been made to identify biomarkers as the tools for sensitive, rapid and accurate identification of pathogenic microorganisms. Moreover, biomarkers have been tested to distinguish colonization from infection, monitor disease progression, determine the clinical status of patients or predict clinical outcomes. This mini-review describes *Pseudomonas aeruginosa* and *Staphylococcus aureus* biomarkers, which contribute to pathogenesis and have been used in culture-independent bacterial identification directly from patient samples.

Keywords: bacteremia; biomarkers; cystic fibrosis; endocarditis; osteomyelitis; virulence factors.

Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are among the major causes of severe nosocomial infections. These bacteria are included in the group of so-called ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species), capable of "escaping" antibiotic treatment due to increasing multi-drug resistance (Pendleton et al. 2013). World Health Organization (WHO) has placed ESKAPE pathogens on the list of antibiotic-resistant bacteria for which the research and the development of new antibiotics should be prioritized (Tacconelli et al. 2018). The Gram-negative rod *P. aeruginosa*, which is ubiquitous in nature, can cause infections among immunocompromised and critically ill patients, resulting in high rates of mortality and morbidity. It is associated with various infections such as (burn-) wound infections, bloodstream infections, respiratory tract infections in cystic fibrosis, and urinary tract infections. Gram-positive *S. aureus* expresses an astounding arsenal of virulence factors to establish itself within its infectious niche, unlike many other bacterial pathogens, which often rely only on one or a few toxins to cause disease. It is a common pathogen causing in the community mostly skin and soft tissue infections, and in medical facilities also severe infections, such as respiratory tract, sepsis, pneumonia, surgical site, prosthetic joint and cardiovascular infections (Uhlemann et al. 2014).

The rapid detection and correct identification of *P. aeruginosa* and *S. aureus* are crucial for the implementation of timely, appropriate treatment of infection. The gold standard for the diagnosis of infections in clinical samples is based on bacterial culture incubation, isolation, identification and biochemical testing that are time-consuming (generally taking from over 24 h up to several days) to obtain a positive result. For these reasons, the rapid and accurate identification of pathogenic microorganisms using bacterial biomarkers has become the subject of intense research. Moreover, biomarkers not only allow for pathogen identification but also distinguish colonization from infection to reduce antibiotic exposure, monitor disease progression, determine the clinical status

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of patients, predict clinical outcomes and are useful as point care diagnostic tests. In this mini-review, we focus on these *P. aeruginosa* and *S. aureus* biomarkers, which contribute to pathogenesis and have been used in culture-independent bacterial identification directly from patient samples.

Quorum sensing biomarkers of *P. aeruginosa* in cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene, encoding an anion channel that primarily affects the lungs and digestive system (Riordan et al. 1989). *P. aeruginosa* is the principal pathogen in the airways of CF patients. Once the CF lung has been colonized, eradication of *P. aeruginosa* is challenging, often unsuccessful and infection can last years or even decades. There are significant clinical efforts aimed at rapid detection of new colonizations and monitoring of lung changes to delay the onset of chronic infection (Davidson et al. 2012).

Quorum sensing (QS) is a bacterial cell-to-cell communication mechanism that regulates the expression of virulence factors in a population density-dependent manner (Papenfort and Bassler 2016). QS relies on the release of autoinducers, small signaling molecules (Figure 1). The detection of a minimum threshold concentration of an autoinducer results in an alteration in gene expression. *P. aeruginosa* possesses three major QS systems, namely *las, rhl* and *pqs*, which are hierarchically organized (Williams and Cámara 2009). The *las* system positively regulates the other two systems, so it is regarded as being at the top of the signaling hierarchy. The *las* system is composed of the transcriptional regulator LasR and the LasI synthase, which produces the signal molecule *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL). The *rhl* system consists of the transcriptional regulator RhlR and the RhlI synthase responsible for the production of the signal molecule *N*-butanoyl-L-homoserine lactone (C_4-HSL). The *pqs* system is composed of the transcriptional regulator PqsR and the signal molecules, 2-alkyl-4 (1h)-quinolones (4-hydroxy-2-heptylquinoline [HHQ] and 2-heptyl-3,4-dihydroxyquinoline [PQS]), which are synthesized by the proteins encoded by the *pqsABCDE* and *phnAB* operons and the *pqsH* gene. The signal molecules activate their respective cognate receptors, LasR, RhR and PqsR, which subsequently induce transcriptional expression of numerous genes responsible for virulence and biofilm formation (Figure 2).

Elevated expression of *P. aeruginosa* QS dependent virulence factors is correlated with exacerbations of lung disease in patients with CF (Jaffar-Bandjee et al. 1995). Moreover, QS signaling molecules (QSSMs) not only regulate the expression of virulence factors but also play a direct role in the pathogenesis by interaction with host cell signal pathways and modulation of immune cell biology (Liu et al. 2015). Thus, the identification and quantification of QSSMs could provide valuable information concerning changes in clinical status. Several studies showed that QSSMs were detectable in the sputum, urine and plasma of patients with CF (Erickson et al. 2002; Struss et al. 2013). The first study aimed to test the hypothesis that QSSMs may be correlated with the clinical status of CF patients with chronic *P. aeruginosa* infection was performed by Barr and colleagues (Barr et al. 2015). Analysis of the samples obtained from 60 CF patients with chronic *P. aeruginosa* infection revealed that the concentrations of alkyl quinolone molecules in the sputum, plasma and urine were positively correlated with the *P. aeruginosa* bacterial cell density. Among the eight tested alkyl quinolones in this study, plasma concentrations of 2-nonyl-4-hydroxy-quinoline (NHQ) correlated with clinical status and NHQ was suggested to be the biomarker for *P. aeruginosa* infection in CF lungs (Barr et al. 2015). The same group investigated 2-alkyl-4-quinolones as early biomarkers of pulmonary infection caused by *P. aeruginosa*. Among six 2-alkyl-4-quinolones tested on 176 adults and 68 children with CF, the most promising biomarker with the greatest diagnostic accuracy was HHQ (Barr et al. 2017). HHQ detected in the plasma can be used as an early biomarker for pulmonary infection particularly in young children. The diagnosis of these infections pose challenges that are not encountered in adults. Early detection of pulmonary *P. aeruginosa* infection will allow to timely initiate the eradication therapy.

Redox-active metabolites of *P. aeruginosa*

Pseudomonas aeruginosa secretes several redox-active phenazine metabolites, including pyocyanin, which has toxic effects on human cells and other microbes (Pierson and Pierson 2010). Pyocyanin is a major virulence factor of *P. aeruginosa* and plays important role in establishing chronic and acute infections. *P. aeruginosa* is the only microorganism that synthesizes pyocyanin (Reyes et al. 1981), for which reason this molecule is a good biomarker for identifying affected patients for treatment in the early stages of infection. Wilson and coworkers proved for the first time *in vivo* production of phenazines by *P. aeruginosa*

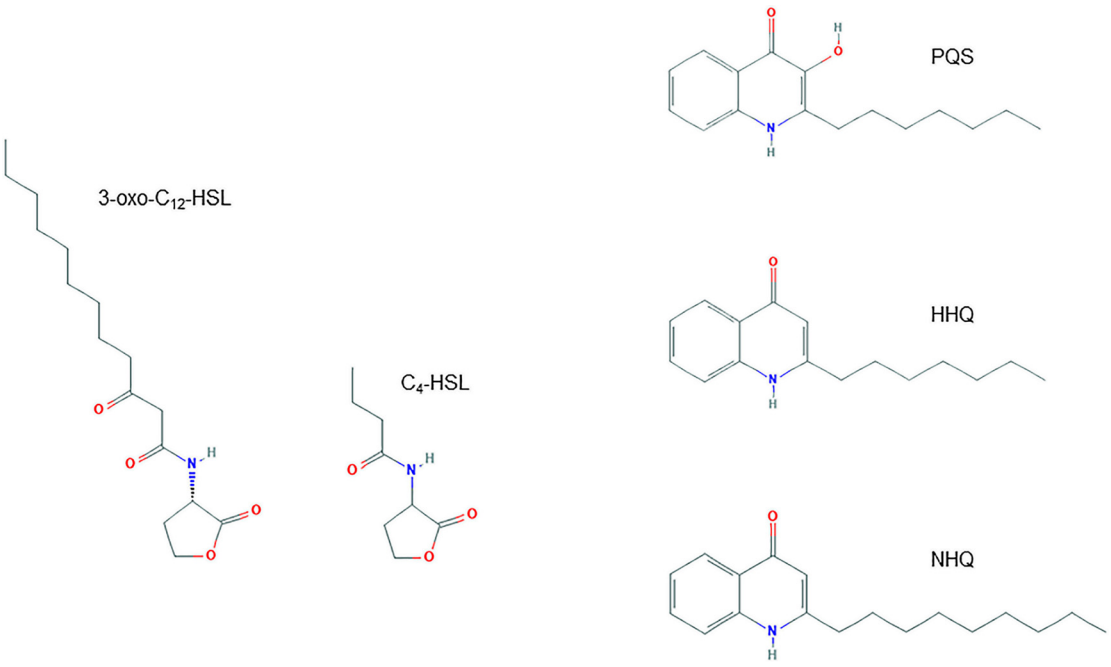

Figure 1: Chemical structures of *P. aeruginosa* quorum sensing (QS) signals. *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL); *N*-butanoyl-L-homoserine lactone (C_4-HSL); 2-heptyl-3,4-dihydroxyquinoline (PQS); 4-hydroxy-2-heptylquinoline (HHQ); 2-nonyl-4-hydroxy-quinoline (NHQ).

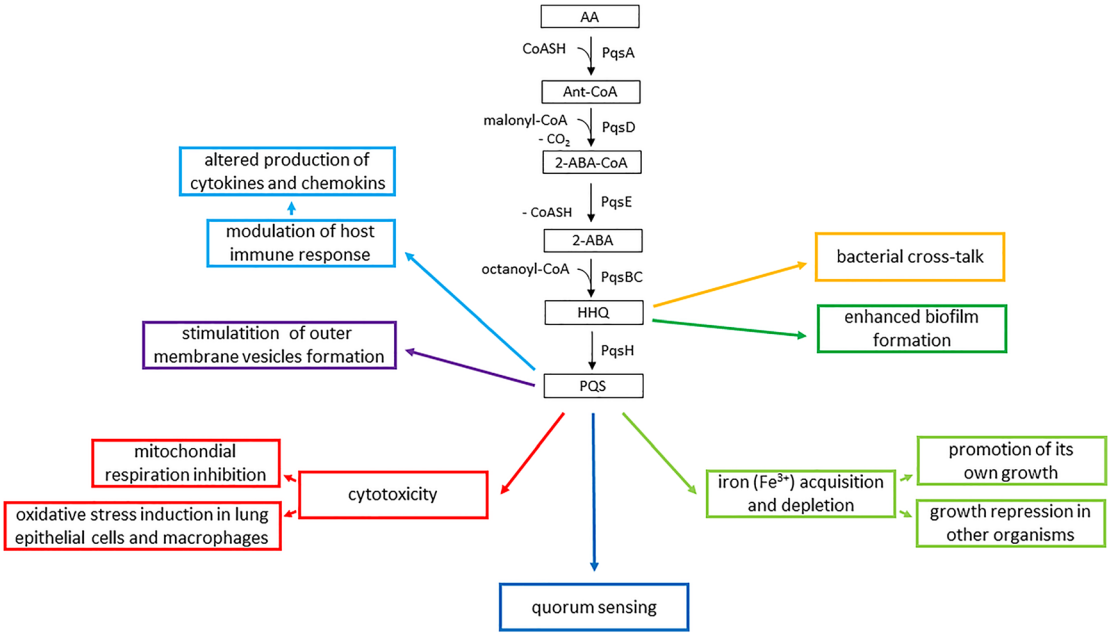

Figure 2: Biosynthesis and multifunctionality of 4-quinolones of *P. aeruginosa*, HHQ and PQS.
The PqsABCDE proteins are responsible for biosynthesis of HHQ. The monooxygenase PqsH catalyses the conversion of HHQ to PQS. Intermediates and products of the biosynthetic pathway: AA, anthranilic acid; CoASH, coenzyme A; Ant-CoA, anthraniloyl-coenzyme; malonyl-CoA; CO_2, carbon dioxide; 2-ABA-CoA, 2-aminobenzoylacetyl-coenzyme A; 2-ABA, 2-aminobenzoylacetate; HHQ, 4-hydroxy-2-heptylquinoline; PQS, 2-heptyl-3,4-dihydroxyquinoline.

in the respiratory tract (Wilson et al. 1988). They measured the levels of pyocyanin and 1-hydroxyphenazine directly from sputum collected from 13 colonized patients (8 bronchiectasis and 5 CF) and five patients with bronchiectasis

not colonized by *P. aeruginosa*. After sample extraction and purification, pyocyanin and 1-hydroxyphenazine were identified by their HPLC-UV profiles in 9 of 13 patients with cystic fibrosis or bronchiectasis colonized by *P. aeruginosa*, while no phenazines (n = 3) or marginal UV responses (n = 2) were detected in the samples from the control group. Although the data obtained by Wilson et al. (1988) are relevant, the number of patients is too small to conclude on the importance of pyocyanin as a biomarker. Despite the interest in the development of analytical techniques for phenazines detection, other studies to date aimed at measuring human fluids spiked with pyocyanin or in the detection of pyocyanin in *P. aeruginosa* cultures (Webster et al. 2014). Thus, there is a need to carry out well designed clinical studies using larger patient cohorts.

Hydrogen cyanide as biomarker of *P. aeruginosa* in cystic fibrosis

Pseudomonas aeruginosa releases hydrogen cyanide (HCN)/cyanide (CN^-), a potent inhibitor of cytochrome *c* oxidase in human cell mitochondria and also inhibits multiple metalloenzymes (Cooper and Brown 2008). CN^- is produced by *P. aeruginosa* to presumably compete for space with CN^- sensitive microorganisms. HCN/CN^- has been used as a biomarker of the presence of *P. aeruginosa* in the airways of patients with CF disease. Therefore, monitoring its concentration profile can provide useful, timely information for disease diagnosis and treatment decisions. However, there were discordant reports regarding the value of CN^- as a biomarker for *P. aeruginosa* infection status. Ryall and colleagues showed that CN^- was detected only in the sputum of *P. aeruginosa* positive patients with CF and non-CF bronchiectasis (Ryall et al. 2008). Gilchrist et al. (2015) confirmed that exhaled breath HCN could be used as a specific biomarker of *P. aeruginosa* infection in children with CF. Opposite conclusions were drawn from a study by Stutz et al. (2011). They revealed that CN^- in bronchoalveolar lavage (BAL) had a poor diagnostic value for *P. aeruginosa* in children with CF. The CN^- levels in BAL were related to the level of neutrophilic inflammation. Eiserich et al. (2018) indicated that CN^- is a transient species in the inflamed CF airway and challenges exist in the determination of overall CN^- production. Thus, stable metabolic products of CN^-, such as cyanate (ONC^-), or carbamoylated proteins, may be proper biomarkers of total CN^- levels in CF airways.

Rapid detection of *S. aureus* bloodstream infections via nuclease activity

Staphylococcus aureus is a leading cause of nosocomial bloodstream infections worldwide. *S. aureus* bacteremia (SAB) is associated with high rates of morbidity and mortality. Blood culture methods currently remain the best approach to identify microorganisms when bacteremia is suspected. However, SAB diagnosis takes a day or longer due to the low bacterial loads, ranging from below 1 colony-forming unit (CFU) per mL in 27% of episodes of bacteremia, up to over 100 CFU/mL in severe infection cases (Yagupsky and Nolte 1990).

Micrococcal nuclease (MNase) or thermostable nuclease (TNase) is specifically secreted by *S. aureus*. The *nuc* gene encoding thermostable nuclease precursor has been used for decades as PCR target to identify and distinguish *S. aureus* (Brakstad et al. 1992). Thus, the detection of enzymatic activity of MNase can serve as a diagnostic biomarker for *S. aureus* identification. MNase is a nonspecific nuclease that can produce short oligonucleotide fragments through hydrolyzing single and double-stranded DNA (ssDNA and dsDNA) as well as RNA (Hörz and Altenburger 1981). The digestion of DNA or RNA by MNase occurs preferentially at the AT-rich or AU-rich regions (Hörz and Altenburger 1981).

Burghardt and coworkers reported a rapid, inexpensive, culture-independent assay based on detection of MNase activity directly from patient plasma samples in less than 3 h (Burghardt et al. 2016). The assay consisted of three main steps. As MNase is a thermostable nuclease, in the first step of the assay, a heat treatment that inactivates MNase-inhibitory antibodies present in plasma was carried out. This step increased the detection sensitivity of MNase by several orders of magnitude. Subsequently, to maximize the sensitivity of the assay, MNase was concentrated from heat-treated plasma supernatants by immunoprecipitation with a monoclonal antibody that did not inhibit MNase activity. Finally, detection of MNase enzymatic activity was achieved using a nuclease-activatable fluorescent oligonucleotide probe. The MNase assay was evaluated on 17 human plasma samples from individuals with SAB confirmed in a culture test. In 16 of them, higher MNase assay signals were detected compared to plasma samples obtained from individuals exhibiting no signs of active infection, or from individuals with non-*S. aureus* bacteremia. This result showed a high degree of sensitivity of MNase assay.

Very recently, Lopez-Alvarez and colleagues examined in our hospital (University Medical Center Groningen) the potential of the nuclease-activatable P2&3TT probe for the detection of *S. aureus* directly from clinical blood culture samples (López-Álvarez et al. 2020). The probe consists of an oligonucleotide comprising 2′-o-methyl modified uridines flanking a pair of unmodified deoxythymidines, which is coupled to fluorescein amidite on the 5′-end and the ZEN and Iowa Black RQ quenchers on the 3′-end (López-Álvarez et al. 2020). Activation of this probe by MNase cleavage leads to the emission of a readily detectable, dose-dependent fluorescence signal. The turnaround time of the assay is less than 2 h. This MNase activity assay was tested in 17 blood cultures identified as *S. aureus*-positive by conventional culture-based methods. Probe activation was observed in all 17 blood cultures of SAB patients, while incubation of the probe with non-bacteremic blood or non-*S. aureus*-positive blood cultures did not result in probe activation. Thus, the MNase assay with the P2&3TT probe provides rapid and accurate detection of *S. aureus* in clinical blood culture samples.

With the advent of nanotechnology, some ultrasensitive biosensors have been developed to determine the MNase activity, including quantum dots, gold–silver and magnetic nanoparticles or graphene oxide nanosheets (Samani et al. 2021). Due to the excellent features of biosensors showing accurate identification and detection performance combined with speed, simplicity and inexpensiveness, they are becoming one of the most important methods for the detection of biomolecules. We expect that in a short time, these ultrasensitive biosensors for the detection of the MNase activity will have a high clinical impact.

Although the previous studies showed that the culture-independent assays based on detection of MNase activity could provide needed specificity for the detection of *S. aureus*, homologs of the *nuc* gene were also found in other species of the *S. aureus* complex (Grossmann et al. 2021). Therefore, we cannot exclude a possibility that infections caused by *Staphylococcus schweitzeri* (possessing nuc_M) or uncommon coagulase-negative staphylococci may yield false-positive identifications since they were not examined previously (Burghardt et al. 2016; López-Álvarez et al. 2020).

Cell-free DNA as a biomarker of metastatic reservoirs of infection

In SAB patients, *S. aureus* can spread haematogenously into various tissues such as heart and bone tissue, resulting in metastatic reservoirs of infection. *S. aureus* bacteremia-associated endocarditis or osteomyelitis are difficult to treat and require a much longer treatment duration with antibiotics compared to uncomplicated *S. aureus* bacteremia. It is difficult to verify the clearance of infection since the serial collection of tissue from metastatic reservoirs during antibiotic treatment is often not possible. Insufficient treatment of metastatic infection is associated with the relapse of *S. aureus* bacteremia and cause serious complications. Two weeks of antimicrobial treatment is recommended in case of uncomplicated SAB, while infective endocarditis and osteomyelitis typically require six weeks or longer (Liu et al. 2011). Therefore, rapid biomarker tests with higher sensitivity to detect persistent bacteremia or relapsing infection would be valuable for optimal antibiotic treatment.

The biomarker value of *S. aureus* cell-free DNA (cfDNA) for clinical use was evaluated recently in two studies by the same research group (Guimaraes et al. 2019; Gutierrez et al. 2019). Bacterial DNA can be released from the cells as a result of antibiotics activity or as the immune response of the host. Extracelullar DNA contributes to pathogenesis being a major structural component in the biofilm matrix of *S. aureus*. Moreover, bacterial DNA is also a potential driver of inflammation (Liaw et al. 2016). *S. aureus* cfDNA was measured by quantitative polymerase chain reaction (PCR) in baseline serum samples from a cohort of patients with complicated *S. aureus* bacteremia and correlated with clinical outcomes. *S. aureus* cfDNA was detectable considerably longer than the duration of detection of *S. aureus* by blood cultures (Gutierrez et al. 2019). There was a relationship between foci of infection and time to clearance of *S. aureus* circulating DNA. Also, the study showed an association between higher bacterial DNA load and a more severe clinical course like organ dysfunction or hypotension. *S. aureus* cfDNA was prognostic for mortality as cfDNA was significantly higher in patients who died within 90 days of positive diagnostic blood culture (Guimaraes et al. 2019). High levels of *S. aureus* cfDNA were prognostic for persistent bacteremia as cfDNA at enrollment was significantly higher in patients who subsequently developed persistent SAB compared to those patients who resolved SAB. Examination of relationships between cfDNA and infection foci revealed that in patients with infective endocarditis cfDNA was significantly elevated when compared with other sources of SAB.

Monitoring levels of bacterial cfDNA by quantitative PCR is a clinically feasible candidate biomarker for the identification of patients at higher risk for complicated SAB and poor outcomes. Such a risk stratification biomarker can be used as an adjunctive tool for blood cultures assuring best management options. However, future studies

in larger prospective cohorts of patients with invasive *S. aureus* infections are needed to address the usefulness of cfDNA quantification for guiding antibiotic treatment length and monitoring clearance of tissue infection foci.

Proteolytic signatures as biomarkers of healing wound status

An acute wound is a wound that occurs suddenly but then heals in a timely manner. Chronic ulcers or non-healing ulcers are wounds characterized by prolonged (a range of four weeks to three months) inflammation, defective re-epithelialization and impaired connective tissue remodeling (Krishnaswamy et al. 2017). Non-healing wounds are a significant burden on the affected patient and healthcare system and the incidence of non-healing ulcers is expected to increase as the population in developed countries ages. Wound infection with bacterial pathogens negatively impacts the wound-healing process and increases the risk of loss of limb and life (Edwards and Harding 2004).

Swab culture and antibiotic susceptibility testing are the most frequently applied methods of confirming wound infection before antibiotics are given to the patient. However, wound culture may not distinguish between colonization and wound infection (Copeland-Halperin et al. 2016). Both acute and chronic wounds can be colonized without clinical signs of infection. For colonized wounds, it is important to monitor the wound for any signs of change that may indicate the transition stage before it reaches infection.

Thrombin plays a key role in wound healing. Hemostasis is the initial phase of wound healing and facilitates series of enzymatic activations that lead to active thrombin, which in turn acts as a serine protease that converts soluble fibrinogen into insoluble strands of fibrin as well as catalyzing many other coagulation-related reactions. Thrombin also regulates the expression of several genes, which play a role in signal transduction, inflammation and wound healing (López et al. 2014). Moreover, C-terminal peptides of thrombin, released upon proteolysis of the enzyme, exert antimicrobial effects against Gram-positive and Gram-negative bacteria (Papareddy et al. 2010). Non-healing ulcers are associated with an elevated level of proteases and excessive proteolytic activity has been correlated with disturbances in the wound healing processes (Trengove et al. 1999). Therefore, proteolytic peptide patterns could serve as biomarkers of healing activity and infection.

Saravanan and colleagues established thrombin-derived C-terminal peptides using human and bacterial proteinases followed by mass spectroscopy analysis and correlated the peptide patterns presence in human wound fluids from acute and non-healing ulcers (Saravanan et al. 2017). First, they determined the peptide patterns by *in vitro* proteolytic digestion of thrombin with endogenous human proteinases, neutrophil elastase and cathepsin G. Both enzymes generated a large number of fragments. In parallel, they analyzed *in vitro* degradation of thrombin by bacterial proteinases such as elastase of *P. aeruginosa*, and aureolysin and V8 protease of *S. aureus*. These bacteria are commonly present in non-healing ulcers and infected burn wounds. Saravanan and colleagues revealed that distinct peptide patterns were generated *in vitro* by the human and bacterial proteinases. Notably, the peptide sequences HVFRLKKWIQKVIDQFGE and FYTHVFRLKKWIQKVIDQFGE were found to be unique products of human and bacterial protease activity, respectively. Next, thrombin-derived peptide patterns were established in human wound fluids from acute surgical wounds and chronic ulcers co-infected with *P. aeruginosa* and *S. aureus*. Comparison of *in vitro* and *in vivo* generated thrombin C-terminal fragments allowed to identify the corresponding peptide sequences in wound fluids from acute and non-healing ulcers. Importantly, the peptide sequence FYTHVFRLKKWIQKVIDQFGE (unique product of bacterial protease activity) was only identified in wound fluids from non-healing ulcers co-infected with *P. aeruginosa* and *S. aureus*.

As proteinase activity is so fundamentally linked to the healing status of wounds, it creates a possibility of using these peptides as biomarkers of colonization or infection stage in future diagnostics based on mass spectrometry analysis, immunoassays, aptasensors or other sensor-based technologies.

Future challenges for the development of biomarkers

The studies described in this mini-review represent the early steps to determine if the identified biomarkers can be used for rapid detection and correct identification of *P. aeruginosa* and *S. aureus* in clinical practice. A biomarker should answer a final question related to a particular state, or the prognosis, of infection in individuals. Thus future studies should focus on measuring identified biomarkers in a larger sample size with a wide range of disease severities. One area for further investigation is testing biomarkers in control populations. Critical barriers that can hamper validation progress can also

include the lack of standardization in specimen collection methods, especially when the results between laboratories will be compared, in addition to improper handling and storage of samples, which can drastically alter detectable levels of biomarkers. Another challenge is elucidating the clinical relevance of low levels of biomarkers; therefore, appropriate specimens, test assays, and cutoff levels for specific biomarkers are also warranted. Finally, to adapt a biomarker measurement to routine clinical practice would also require development of a widely available, easy-to-use and inexpensive laboratory or point of care tests.

Conclusion and outlook

In this mini-review, we provided an overview of pathogen virulence factor-related biomarkers to detect *P. aeruginosa* and *S. aureus* infections with high clinical importance. Application of such tests potentially has additional diagnostic value compared to the conventional microbial culture alone as they provide direct information on the infectious processes. Besides, biomarker assays are culture-free, allowing for rapid detection of infections. Several studies showed excellent candidate biomarkers for detecting infections using both clinical characteristics and microbial culture results as a comparison. Future prospective randomized-controlled trials may evaluate the effects of biomarker-guided treatment on clinical outcomes and antibiotic stewardship endpoints.

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