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Short Communication

SHORT COMMUNICATION

Donor platelet plasma components inactivate sensitive and multidrug resistant *Acinetobacter baumannii* isolates

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One sentence summary: Protein fractionation of donor platelet plasma reveals that the primary inhibitors of *Acinetobacter baumannii* growth are complement and a heat-stable fast form of alpha-2 macroglobulin.

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ABSTRACT

Acinetobacter baumannii is an environmentally resilient healthcare-associated opportunistic pathogen responsible for infections at many body sites. In the last 10 years, clinical strains resistant to many or all commonly used antibiotics have emerged globally. With few antimicrobial agents in the pharmaceutical pipeline, new and alternative agents are essential. Platelets secrete a large number of proteins, including proteins with antimicrobial activity. In a previous study, we demonstrated that donor platelet supernatants and plasma significantly inhibited the growth of a reference strain of *A. baumannii* in broth and on skin. This inhibition appeared to be unrelated to the platelet activation state. In this study, we demonstrate that this growth inhibition extends to clinical multidrug resistant isolates. We also demonstrate that there is no relationship between this activity and selected platelet-derived antimicrobial proteins. Instead, the donor plasma components complement and alpha-2 macroglobulin are implicated.

Keywords: platelets; plasma; growth inhibition; antimicrobial peptides; multidrug resistance; *Acinetobacter baumannii*

Acinetobacter baumannii is primarily found in healthcare settings worldwide (Dijkshoorn, Nemec and Seifert 2007) and causes pneumonia and bacteremia as well as central nervous system, skin, bone and soft tissue infections (Peleg, Seifert and Paterson 2008). Isolates can be resistant to all commonly used gram-negative antimicrobial agents. With very few treatment options, the development of new antimicrobial agents is imperative (Boucher *et al.* 2013; Roca *et al.* 2015).

Platelet gel or platelet-rich plasma is composed of concentrated activated platelets in donor plasma. When activated, platelets secrete a variety of proteins and other molecules, including antimicrobial peptides (AMPs). These AMPs include platelet factor 4 (PF-4)/CXCL-4 (Yeaman *et al.* 1997), the proteolytic derivatives of platelet basic protein (PBP/CXCL-7/β-thromboglobulin), thrombocidins 1 and 2 (TC-1and TC-2) (Krijgsveld *et al.* 2000), RANTES/CCL-5 (Tang, Yeaman and Selsted 2002), thymosin β4 (Tβ4) (Tang, Yeaman and Selsted 2002) and

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IL-8/CXCL-8 (Yount *et al.* 2007). A previous study established that human donor platelet gel supernatants exhibited antimicrobial properties against *A. baumannii* (Edelblute *et al.* 2015). Here, we hypothesize that platelet-produced AMPs are responsible for this inhibition and investigate individual platelet gel components.

One-day expired single-donor platelets were purchased from the American Red Cross (Norfolk, VA). Washed platelets were activated with 100 US units mL^{-1} bovine thrombin or by the application of nanosecond pulsed electric fields (5 pulses, 30 kV/cm 300 ns, 2 Hz, PEFs) as previously described (Edelblute *et al.* 2015). After clotting, the samples were centrifuged and the supernatants were assayed to avoid direct platelet activation by bacteria (Cox, Kerrigan and Watson 2011). In this study, the separated plasma was referred to as 'donor plasma' to distinguish it from normal human plasma.

Regardless of activation state, platelet supernatants significantly inhibited the growth of several clinical *A. baumannii* isolates. This inhibition of individual isolates was consistent across the donor samples (Fig. 1). Quiescent and PEF-activated, but not thrombin-activated, supernatants significantly inhibited the growth of isolate 2 with respect to the control. Isolates 3, 4 and 8 were sensitive to quiescent and thrombin-activated, but not PEF-activated, supernatants. The growth of isolates 5 and 7 was significantly inactivated by all supernatants. Isolates 1, 6 and 9 were not significantly inhibited by any platelet supernatant. This activity was stable after supernatant exposure to 56°C for 30 minutes.

Interestingly, donor plasma significantly inhibited the growth of eight of the nine clinical isolates, including the six MDR isolates. Therefore, the concentration of the inactivating agent or agents must be highest in the donor plasma. Heat inactivation reduced growth inhibition from 97 to 62% ($P < 0.001$) (data not shown), implicating the activity of complement, an innate, heat labile plasma component with non-specific antibacterial activity. Several groups have demonstrated that serum complement kills *A. baumannii* reference strains, including ATCC 19606, and clinical isolates (Cervi and Monetto 1996; Garcia *et al.* 2000; Kim *et al.* 2009; King *et al.* 2009). The loss of killing after heat exposure implicates activation of the alternative pathway (Cervi and Monetto 1996; Kim *et al.* 2009; King *et al.* 2009). However, the residual activity in donor plasma must be attributed to a heat-stable component.

Inhibition of *A. baumannii* by AMPs was first described in 2000 (Giacometti *et al.* 2000), and platelet-derived AMPs could account for the heat-stable antimicrobial activity. Four of these proteins were quantified in platelet gel supernatants and in donor plasma (Fig. 2). Levels of RANTES, NAP-2/TC-1 and IL-8 were significantly increased by thrombin activation with respect to quiescent platelet gel supernatants. Although PEF application induced platelet aggregation as observed previously (Zhang *et al.* 2008), no significant increase in AMP levels was detected. Donor plasma contained a significantly reduced concentration of RANTES but a significantly greater concentration of PF-4 than the supernatants, initially implicating PF-4 as an inactivating agent. Platelet-produced PF-4 and its derived peptides are well-studied AMPs (Yeaman *et al.* 1997; Yeaman 2010) particularly against *Staphylococcus aureus*.

Growth inhibition of *A. baumannii* (ATCC 19606) and control strains *B. subtilis* (ATCC 6633) and *E. coli* (ATCC 25922) by recombinant proteins was assayed at the highest mean concentration detected in any sample type (Fig. 3a). Since increases in concentration for the quantified analytes varied between 2-fold (RANTES), 3-fold (NAP-2/TC-1), 7-fold (IL-8) and 2000-fold (PF-4) over normal serum or plasma levels and Tβ4 was not quantified, this protein was tested at concentration of approximately 10-fold its normal serum concentration (Weller *et al.* 1988) In initial studies, PF-4 significantly inhibited the growth of *A. baumannii* and *B. subtilis*. However, when additional formulations of PF-4 were tested at the same concentration, including an alternative human PF-4, mouse PF-4 and PF-4 (58-70), a peptide with known antimicrobial activity (Darveau *et al.* 1992), no growth inhibition was detected (Fig. 3b).

To better identify the heat-stable source of growth inhibition, a supernatant sample was fractionated with size exclusion chromatography. Activity was found in the fractions eluted between 7 and 10 ml of elution volume. These fractions inhibited growth by 30% (Fig. 4) and corresponded to an apparent molecular weight ranging between 400 and 800 kDa. SDS PAGE analysis of the active fractions revealed the presence of multiple proteins, so ion exchange chromatography was applied to the pooled active fractions to reduce this number for identification analysis.

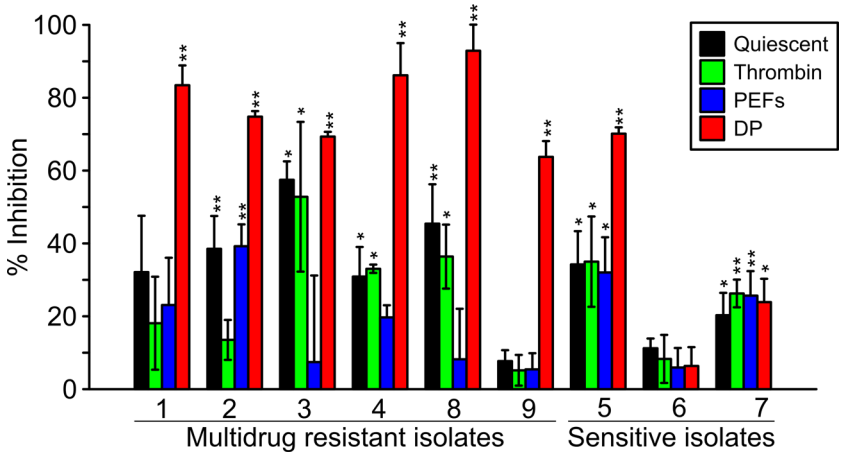

Figure 1. Growth inhibition of clinical *A. baumannii* isolates by platelet supernatants and donor plasma. Multidrug sensitive and resistant clinical *A. baumannii* isolates were cultured from urine, wounds, sputum or the nose of different patients at a local hospital. Identification and antibiotic susceptibilities were determined using a Vitek 2 system (bioMerieux, Durham, NC). Growth of 1000 colony forming units incubated 18 hours with peptides in Mueller Hinton broth (Hancock 2001) was monitored by changes in OD_{620} and compared to control growth. Quiescent, quiescent platelet supernatant; thrombin, thrombin-activated platelet supernatant; PEFs, PEF-activated platelet supernatant; DP, donor plasma. Mean ± SEM, $n = 5$–6 donors per group. $^{*}P < 0.05$; $^{**}P < 0.01$ with respect to quiescent supernatants.

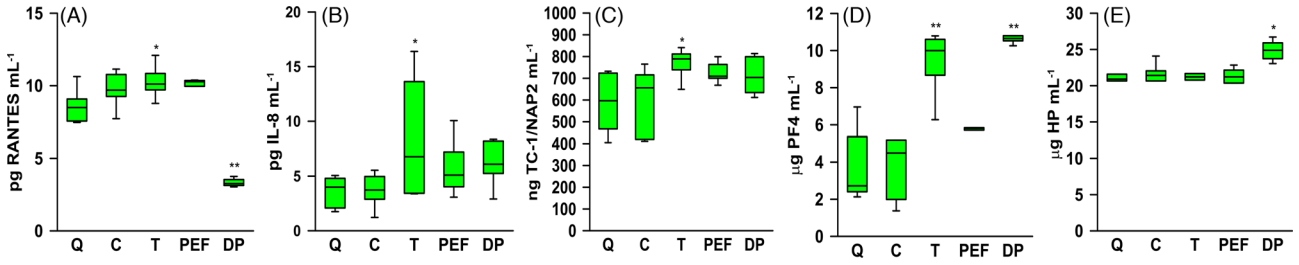

Figure 2. Quantification of AMPs in platelet gel supernatants and platelet plasma. Proteins were quantified using a bead-based multiplex assay (EMD Millipore, Billerica, MA, MAGPIX platform, Luminex Corp., Austin, TX). A, RANTES; B, IL-8; C,CXCL7/NAP2; D, PF-4; E, HP. Q, quiescent platelet supernatant; C, supernatant from platelets enriched with $CaCl_2$; T, thrombin-activated platelet supernatant; PEFs, PEF-activated platelet supernatant; DP, donor plasma. Mean ± SEM, $n = 5$–6 donors per group. $^{*}P < 0.05$; $^{**}P < 0.01$ with respect to quiescent supernatants.

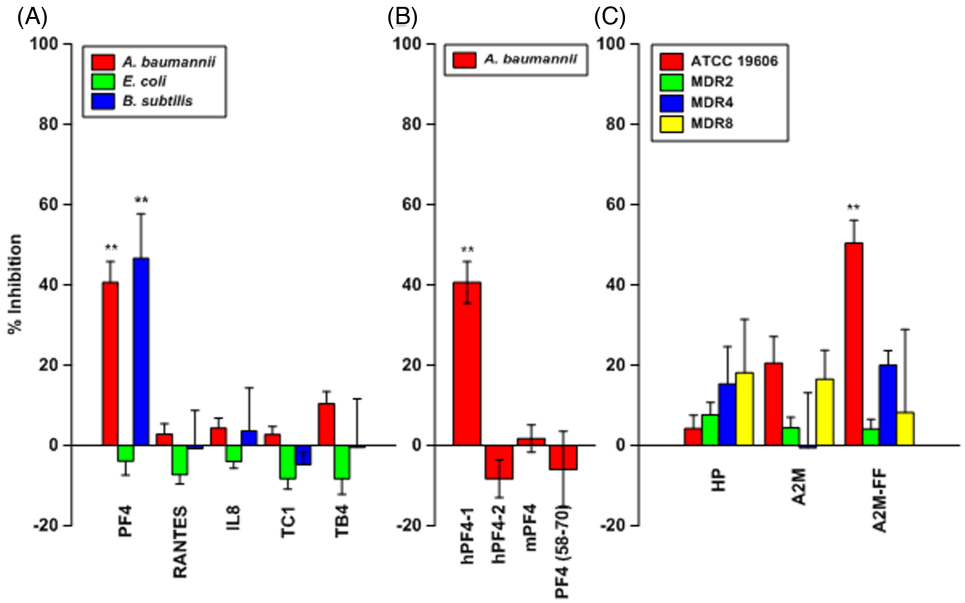

Figure 3. Growth inhibition by proteins or peptides. A, recombinant AMPs with *A. baumannii* (ATCC 19606), *E. coli* (ATCC 25922) or *B. subtilis* (ATCC 6633); B, recombinant PF-4 formulations with *A. baumannii* (ATCC 19606); C, proteins identified by HPLC ECI-MS/MS with *A. baumannii* (ATCC 19606) or selected clinical isolates. Recombinant IL-8, RANTES and Tβ4 (PeproTech, Rocky Hill, NJ), human or mouse PF-4 (PeproTech, Rocky Hill, NJ or R&D Systems, Minneapolis, MN), human PF-4 (58-70) (Bachem, Torrance, CA), purified HP (Sigma Aldrich, St. Louis, NJ) and custom-synthesized TC-1 (NeoBioLab, Woburn, MA) were reconstituted in PBS containing 0.1% BSA. Purified A2M and A2M-FF were buffered by the manufacturer (Athens Research, Athens, GA). $^{**}P < 0.01$ with respect to controls containing the appropriate buffer.

Activity that inhibited growth by 40% was detected in two peaks eluted in a NaCl gradient. High-performance liquid chromatography/electrospray tandem mass spectrometry (University of Texas Health Science Center, San Antonio, TX) identified two proteins, alpha-2-macroglobulin and haptoglobin (HP).

HP is a natural bacteriostat affecting iron metabolism (Eaton *et al.* 1982). Since HP levels in donor plasma were more than 10-fold over those of normal plasma (Fig. 2), this concentration was assayed for growth inhibition of the reference strain and three clinical isolates. HP did not inhibit *A. baumannii* growth (Fig. 3c).

A2M is a non-specific protease inhibitor that occurs in a native form and in a proteolytically cleaved, electrophoretically fast form (A2M-FF) (Rehman, Ahsan and Khan 2013). This protein was not quantified; however, it is not found in the platelet proteome (Senzel, Gnatenko and Bahou 2009; Burkhart *et al.* 2012; van Holten *et al.* 2014; Zufferey *et al.* 2014), so the normal plasma concentration of native A2M was assayed for each form. While native A2M did not inhibit growth, two different lots of A2M-FF inhibited the growth of the reference strain by 50%. This activity was heat stable, supporting the concept that this protein comprised the heat-stable growth-inhibiting component of donor plasma. However, neither form of A2M significantly inhibited the growth of the clinical isolates. While native A2M has a half-life in the circulation of several hours, A2M-FF is immediately endocytosed by hepatocytes and macrophages, resulting in a half-life of 2–4 minutes (Rehman, Ahsan and Khan 2013). Since hepatocytes are not present in donor plasma and macrophages are present in only small numbers, A2M-FF may accumulate in expired platelet plasma and potentially other stored serum or plasma sample types. These proteins may stick to platelets and therefore be present in platelet supernatants.

The potential antimicrobial mechanism of A2M is unknown. The possibility of the direct generation of AMPs was investigated computationally using by HHpred homology modeling (Soding 2005) and structure comparison in 3D-BLAST (Yang and Tung 2006). No apparent AMP motifs (Fjell *et al.* 2012) were found. Alpha-2 macroglobulin shares about 35–40% homology to complement molecules C3, C4 and C5, which, during activation, generate AMPs known as anaphylatoxins (Pasupuleti *et al.* 2007). Several conserved domains are shared by A2M and C3, C4 and

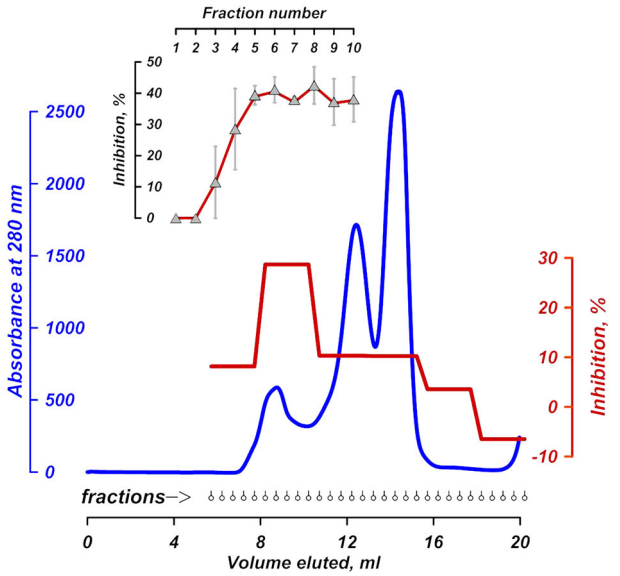

Figure 4. Purification of the antimicrobial components of human platelet supernatants. Samples were separated by size exclusion chromatography (Superdex 200, GE Healthcare, Pittsburgh, PA, USA). Antimicrobial activity was assayed through entire elution volume, initially in the pools of five fractions and then in individual fractions. A characteristic elution profile is depicted in blue, antimicrobial activity of corresponding pool fractions is overlayed in red. The inset demonstrates the antimicrobial activity in individual fractions ($n = 3$).

C5. However, the canonical anaphylatoxin (ANATO) domain is not found in the A2M sequence (DELTA-BLAST, National Center for Biotechnology Information, USA). It is worth noting that the region of the putative ANATO domain is not available for structural analysis. This region is disordered in the only available A2M crystal structure (Protein Data Bank ID 4ACQ). Consequently, the possibility of anaphylatoxin-like AMP production is uncertain and requires further investigation.

In this study, we describe growth inhibition of a reference strain and several clinical *A. baumannii* isolates by donor platelet plasma components. As previously published, complement, a heat-labile plasma component, was responsible for a percentage of this inhibition. Unexpectedly, platelet-derived AMPs did not significantly inhibit growth. Sample fractionation, protein identification and activity analysis implicated the fast form of A2M in the growth inhibition of the reference strain only. These results highlight the necessity of screening for potential antimicrobial effects with clinical rather than reference strains. *A. baumannii* in particular is a genetically fluid organism able to acquire virulence and resistance determinants (Gordon and Wareham 2010; Karah *et al.* 2012); some of these determinants may contribute to the resistance to growth inhibition observed in these isolates. The overall level of inhibition observed in donor plasma was not recapitulated by the individual components, therefore, undiscovered components, acting additively or synergistically, probably contribute to growth inhibition.

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Conflict of interest. None declared.

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