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Thesis Title: Investigations into biofilm formation by colistin resistant *Acinetobacter baumannii* and identification of compounds with antibiofilm properties

Keywords: Colistin, biofilm, biofilm associated protein (Bap), alexidine dihydrochloride, *A. baumannii*

FINDINGS

This study explores the impact of colistin resistance-associated mutations on various aspects of *A. baumannii*. Specifically, the research focuses on four pairs of *A. baumannii* isolates, ATCC 19606, BC-5, 3-137, and HK-45, and developed their laboratory adapted colistin resistant counterparts. The investigation explores several key parameters, including the growth rate, antimicrobial resistance profiles, genetic mutations contributing to biofilm formation, *in vitro* biofilm formation capacity, and the expression of genes linked with biofilm formation. The potential role of virulence determinants, specifically genes linked with biofilm formation, in contributing to the higher rates of treatment failure during infections by highly resistant *A baumannii* isolates is an area that needs further exploration. By examining these aspects, the study aims to comprehensively understand how colistin resistance influences various phenotypic traits in *A. baumannii*. The findings of this study revealed that the biofilm formation ability of colistin resistant *A. baumannii* strains BC-5R and HK45R remained unaffected exhibited point mutations in the *pmrB* gene. However, strain 3-137R, which harbored nonsense mutations in *lpxC* and *lpxA*, showed a significant reduction in biofilm formation ability owing to the loss of synthesis of lipid A and, hence, the LPS of the outer membrane. Additionally, 3-137R demonstrated a colistin-dependent phenotype, exhibiting impaired growth in the absence of the antibiotic. Further analysis of the expression of biofilm-associated genes (*ompA, bfmR, abaI, pgaC, bap*), virulence genes (*recA, blsA*), and the lipid A-modifying gene (*pmrC*) provided insights into the molecular mechanisms underlying these phenotypic changes. OmpA, an outer membrane porin that is involved in the efflux of antibiotics

was overexpressed in all the resistant isolates. *bfmR* encodes for the regulatory protein of the BfmRS two-component system that was overexpressed in 3-137R. *pmrC* was upregulated in 19606R and BC-5R, isolates harbouring mutations in the *pmrAB* operon. *pgaC*, *bap*, and *abaI* genes responsible for synthesizing poly-N-acetyl glutaraldehyde, biofilm-associated protein, and quorum sensing molecule, acyl homoserine lactone (AHL), respectively, were upregulated in BC-5R while downregulated in 3-137R and 19606R, that exhibited decreased biofilm formation ability.

In this study, we attempt to identify differences among types of Bap to understand the evolutionary changes among *A. baumannii* that may favor biofilm formation and, subsequently, the pathogenesis. The role of different Bap types among the *A. baumannii* population in deciding the biofilm formation ability was determined using *in vitro* studies. The potential of Bap types to interact with host cell receptors, CEACAM-1 present on the epithelial cells and PIgR expressed on the mucosal surface of epithelial cells of pharynx and gut region was determined using *in silico* approaches. On analyzing 7338 genome sequences of *A. baumannii* isolates from the NCBI database, it was observed that Bap or Bap-like proteins (BLP) were present in 6422 (87.52%) isolates. Further categorization revealed that 12.12% carried Type-1 Bap, 68.44% had Type-2, 6.91% had Type-3, 0.054% had Type-6 or SDF-Type, and 12.51% lacked Bap or BLP. Most isolates with Type-1, Type-2, and Type-3 Bap corresponded to ST1, ST2, and ST25, respectively. Phylogenetic analysis suggested that Type-1 Bap is the most ancient, while Type-3 and SDF-Type have evolved recently. Investigation into the interaction of predicted Bap structures with human CEACAM-1 and PIgR indicated that Bap, particularly with its BIg13 and BIg6 domains, interacts with the N-terminal domain of CEACAM-1, involving Arg43 and Glu40, which are implicated in CEACAM-1 dimerization. Furthermore, the recently evolved Type-3 and SDF-Type Bap exhibited enhanced interaction with CEACAM-1 and PIgR. This suggests that the evolution of Bap has conferred increased virulence characteristics to *A. baumannii* by enhancing its interaction with CEACAM-1 and PIgR. Through *in silico* approaches, our study explores the evolutionary, physicochemical, and structural features of *A. baumannii* Bap, elucidating its crucial role in mediating interactions with human CEACAM-1 and PIgR through detailed structure modeling. These findings significantly contribute to our understanding of evolutionary and pathogenic role *A. baumannii* Bap and emphasize its importance in pathogenesis. Using *in silico* approaches, this study successfully characterizes Bap and unravels its crucial role through detailed structure

modelling. The findings of this study have significant implications for the development of more effective treatments against *A. baumannii* infections.

Antimicrobial resistance to all the commercially available antibiotics have been reported among *A. baumannii* strains of clinical and environmental origin from various parts of the globe. Conventional antibiotics such as colistin, ciprofloxacin, and imipenem, even at a thousand times higher than bactericidal concentrations, were insufficient to eradicate strong *A. baumannii* biofilms. For identifying antibiofilm compounds against *A. baumannii*, we utilized the open drug discovery approach and screened 400 compounds from the Pandemic Response Box provided by MMV and DNDi to identify compounds exhibiting antibacterial and antibiofilm activity against reference *A. baumannii* strains ATCC 19606 and BC-5. This screening employed a highly robust resazurin assay. *In vitro* screening unveiled thirty compounds with MIC $\leq$ 50μM, demonstrating growth inhibitory effects against the planktonic state. Among these, five compounds, MMV396785, MMV1578568, MMV1578574, MMV1578564, and MMV1579850, exhibited the capacity to diminish metabolically active cells within the biofilm state. Notably, MMV396785 showcased promising antibacterial and antibiofilm activity, with MIC, MBIC, and MBEC values of 3.125 μM, 12.5, and 25-100 μM against the tested *A. baumannii* strains, respectively. This compound inhibited biofilm formation by 93% and eradicated pre-formed biofilms by 60-77.4%. Additionally, MMV396785 significantly reduced the surface area and thickness of biofilms, as observed using confocal laser scanning microscopy. Molecular investigations through qRT-PCR unveiled the downregulation of biofilm-associated genes when exposed to 50 μM MMV396785 across all tested strains. Thus, this study identifies the novel compound MMV396785 as a potential candidate exhibiting *in vitro* antibacterial and antibiofilm efficacy against *A. baumannii*.