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Darwinian selection analysis of the two-component system PmrAB indicates there could be lingering delay in emergence of colistin resistance in *Acinetobacter baumannii*

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Running title: Mutational analyses show delay in emergence of colistin resistance in *Acinetobacter baumannii*

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Abstract

Investigations on the selection pressure acting on point mutations in PmrAB two-component system may provide insights into the fate of colistin resistance in *Acinetobacter baumannii*. In this study, we performed Darwinian selection analysis of occurrence of non-synonymous mutations and synonymous mutations in *pmrAB* at each amino acid site. We analyzed PmrAB sequences in 3113 draft genomes of *A. baumannii* obtained from RefSeq database. Nearly all the amino acid substitutions previously reported in PmrAB occur at sites that are fairly conserved and under either neutral or purifying selection. Further, the sites with high levels of polymorphisms in PmrAB were found to be under neutral selection. Strong negative evolutionary selection pressure is also observed at sites throughout both PmrA and PmrB. Notably, there was no sign of positive selection. Mutations that cause colistin resistance are neither adaptive nor polymorphic. Some of them are rather deleterious. These conditions

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might be maintaining the incidence of colistin resistance in *A. baumannii* under check. In addition, because of the purifying selection on PmrAB, it is highly unlikely that these mutations will get fixed by random genetic drift. Therefore, in the context of colistin resistance, natural selection plays only a minor role and we argue that in future, *A. baumannii* may not be able to emerge as a colistin-resistant organism, since the documented mutations are not sustainable and hence, not able to successfully disseminate. Therefore, the concerns raised about continuing the usage of colistin for the treatment against *A. baumannii* infections are unnecessary

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Introduction

Single nucleotide polymorphisms (SNPs) contribute greatly towards the microevolution of a bacterium (Chouard, 2010; Reznick & Ricklefs, 2009). They are likely to occur more frequently in fast-growing bacteria, consequently, these bacteria are able to adapt to their environment comparatively faster without any significant associated costs. These mutations also have a profound effect on drug resistance and pathogenesis. In recent years, colistin resistance has been found to be due to SNPs in the genes encoding the two-component system (TCS) PmrAB, as well as in the *lpx* operon, that are responsible for the production and regulation of lipopolysaccharide (LPS) in bacterial cell walls (Moffatt et al., 2010; Snitkin et al., 2013; Thi Khanh Nhu et al., 2016).

PmrAB is thought to regulate lipid-A biosynthesis of gram negative outer membranes (Boll et al., 2016). Lipid-A is a constituent of the outer membrane LPS, which is the target of colistin. Colistin resistant strains of *A. baumannii* have modifications and/or loss of LPS from their outer membrane, with concomitant mutations in the *pmrCAB* and/or *lpx* operon (Boll et al., 2016; Carretero-Ledesma et al., 2018). Emergence of such resistance is worrisome, since colistin is the last-choice drug against multidrug resistant *A. baumannii*. The objective of this study was therefore to identify the hyper-variable regions of the TCS PmrAB in *A. baumannii* to gain insights into their contribution towards the structure and function of this TCS. Since point mutations in PmrAB have been implicated in resistance to colistin, sequence analysis of PmrAB in the context of the evolutionary selection pressure acting on these mutations may provide insights into the fate of colistin resistance in *A. baumannii*. We report that, both PmrA and PmrB are under strong purifying evolutionary selection pressure. In addition, there was no sign of adaptive evolution. Mutations that cause colistin resistance are neither adaptive nor polymorphic. Some of them are rather deleterious. Therefore, it is highly unlikely that these mutations will get fixed and *A. baumannii* may not be able to emerge as a colistin-resistant organism

Methods

Antibiotic susceptibility testing

Minimum inhibitory concentration (MIC) for colistin were performed according to European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2017). E-test (Hi-media, Mumbai) was used for colistin MIC determination. The reference strain *A. baumannii* ATCC 19606 and quality control strain *Escherichia coli* ATCC 25922 were included in the assays as reference controls.

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Mutational analysis of pmrA

Mutations in *pmrAB* have been known to be associated with colistin resistance. We amplified *pmrA* genes from two isolates namely PKAB15 and PKAB19 from our collection (Supporting Information), which showed higher MIC values using the *in house* designed primers 5'-AAG CCA ACA AAC TAA ACA AAA-3' and 5'-GCT TGC TCA ACA GGT GGA AC-3' and the amplicons generated were custom sequenced (Macrogen Inc., Seoul, South Korea). The origin, molecular characters and other clinical details of the isolates were given in **Table 1**

As *pmrAB* loci is known to be associated with colistin resistance, the present study attempted to catalogue the mutations in PKAB15 and PKAB19. Mutations at protein level indicate occurrence of non-synonymous mutations. Therefore, the translated sequences of PmrAB of PKAB15 and PKAB19 were aligned to 3113 translated PmrA sequences extracted from whole genome sequences of *A. baumannii* available in the RefSeq database.

Literature search for mutations reported in colistin resistant isolates

This study reviewed previous literatures that have identified non-redundant and non-synonymous mutations in *pmrAB* in colistin resistant *A. baumannii* isolates (up to December 2018) (**Table S1, Supplementary file S1**). Reports of *pmrAB* point mutations without any mention of corresponding colistin resistance phenotypes were excluded from this study. Amino acid substitutions reported in colistin resistant mutation phenotype that appear in colistin sensitive strains were also excluded. The mutations found in the literature were mapped into the amino acid sequence and analysed in the context of natural selection at the respective sites of the mutations.

Selection analysis of pmrAB

To study the strength of the selection pressure at each site of PmrA and PmrB, we performed a selection analysis. Given a codon-aligned multiple nucleotide sequence alignment corresponding to a protein sequence, the selection analysis counts the number of non-synonymous mutations (*dN*) at each amino acid position and compares it with the number of synonymous mutations (*dS*) at that site, tested on a phylogenetic tree. The extent of selection pressure at a codon can be estimated by comparing the rate of occurrence of synonymous mutations to that of non-synonymous mutations.

In total, 3113 draft genome sequences of *A. baumannii* (as available up to December 2018) were retrieved from National Centre for Biotechnology Information database. Genomes of strains of *A. baumannii* isolated during time-span of more than 30 years (1984 - 2018) were included in the analysis and such a long-term of isolation period should be sufficient enough to answer a query of evolution in *A. baumannii.*

Coding sequences for *pmrA* and *pmrB* were extracted from a standalone BLAST+ database of the downloaded genome sequences by 'tBLASTn-Fast' search algorithm of BLAST+ v2.8.1. An E-value cut-off of 1e-10 and 'max_target_seqs' value (maximum target sequences to keep) of 3113 were kept for the tBLASTn search. Hits with <90% identity were filtered out from tabular output before extracting the coding sequences. Sequences with indels and truncated / partial sequences were removed manually prior to alignment. Only complete coding sequences free from indels were included in the study. The resulting coding sequences were utilized to perform codon based multiple sequence alignment by MEGA7 software with the MUSCLE algorithm (Edgar, 2004; Kumar et al., 2016). For this purpose, the coding

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sequences were translated into protein sequences, followed by multiple protein sequence alignment and reverse-translation of the alignments into nucleotide sequences. As a result, the alignment was based on triplet codons instead of individual nucleotides, which would allow identification of synonymous and non-synonymous mutations. The codon-aligned multiple sequence alignment files of all *pmrA* and *pmrB* coding sequences are available in **Supplementary file S2.**

The codon-based multiple sequence alignment files were introduced into the Datamonkey server for selection analysis. The Datamonkey server is the web interface for the HyPhy package for the analysis of molecular evolution (Delport et al., 2010; Sergei L. Kosakovsky Pond & Frost, 2005; Pond & Muse, 2005). Single-Likelihood Ancestor Counting (SLAC) method was chosen for the estimation of synonymous and non-synonymous mutation rates at each site. SLAC was preferred to other options based on its simplicity and speed, as well as its performance on closely related sequences (Pond & Frost, 2005). Duplicate sequences were removed at this stage of the sequence submission portal of the web server. The neighbor joining tree based on HKY85 nucleotide substitution model, which was the best fit model, have been chosen for the estimation of branch lengths and substitution rates for SLAC (Hasegawa et al., 1985). Model comparison is an implicit automated procedure of the web-server, which as described in Pond et al. (Pond & Frost, 2005). A p-value of <0.05 from a two-tailed extended binomial distribution was used as a cut-off for the significance of positive selection against a neutral null model. Positive selection was detected in terms of the difference between non-synonymous and synonymous mutations, which was expressed as $dN - dS$ value, normalized with the total length of the phylogenetic tree as measured in terms of the number of expected substations per nucleotide per site. A site with amino acid mutations in >1% (≈30) genomes was considered as polymorphic. Results of codon-wise mutation and selection analysis and the corresponding p-values are provided in **Supplementary file S3.**

The SLAC method is equivalent to the site model of the CodeML program of the PAML package (Yang, 2007). Therefore, we performed an adaptive selection analysis under the site model (Model M2a), against a nearly-neutral evolution null model (Model M1a) in CodeML (PAML v4.9) (Jeffares et al., 2015; Yang, 2007). Briefly, the file so generated after the removal of duplicates was parsed into RaxML for the construction of phylogenetic tree with the option 'ML + Rapid bootstrap', under 'GTRGAMMAI' (general time reversible-gamma, invariant) model with 1000 bootstrap repeats. The bootstrap and branch length values were kept enabled ('BS brl' enabled) (Stamatakis, 2014). *pmrA* of *A. junii* strain 65 and *pmrB* of *A. nosocomialis* strain M2 were used as outgroups to for the phylogenetic analysis of *pmrA* and *pmrB* of *A. baumannii*, respectively. The tree files and the sequence files were used as inputs to CodeML. The likelihood ratio test statistics were obtained from the log-likelihood values of individual models, as presented in the result files of the program, and were compared with the critical χ^2 values corresponding to p-value 0.05 in order to reject or accept the null hypothesis. The phylogenetic trees, sequence files, CodeML control files and the result files have been provided in **Supplementary file S2**.

To demonstrate that our approach of selection analysis is consistent and reliable, we intended to apply the same methodology to a gene of other bacteria that is thought to be under adaptive evolution in the context of antibiotic resistance. We chose the gene coding for DNA gyrase subunit A (*gyrA*) of *Escherichia coli* and *Salmonella enterica* , the product of which is the target for quinolone class of antibiotics, for this purpose (Hershberg, 2017; Katz & Hershberg, 2013). We applied within species codon-based selection analysis as described

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above on *gyrA* sequences of 533 genomes of *E. coli* and 553 genomes of *S. enterica* randomly downloaded from the Refseq genome database. Raw alignments can be found in **Supplementary file S4**.

Results

Mutation hotspots in PmrAB are not responsible for colistin resistance

In our observations, two of our clinical isolates, PKAB15 and PKAB19 were identified to be colistin resistant. We amplified and performed sequencing of the *pmrA* genes from these two isolates. One non-synonymous mutation in each of these isolates was identified in *pmrA*, when compared to the reference strain *A. baumannii* ATCC17978. Both of these mutations (D10N in PKAB15, and R212L in PKAB19) change charged amino acids with uncharged ones which, we believe might have significant alterations on the structure of the protein.

Across all the 3113 genomes investigated in the study, 43 sites with non-synonymous mutations were identified in *pmrA* (Supplementary file S3). Most of the mutations occur in the signal receiver domain and the region connecting the receiver domain to the DNA binding domain (Figure 1). Of these, five were in highly polymorphic sites. The DNA binding domain recorded as few as seven sites with non-synonymous mutations, four of which had rare mutations while one was among the five most polymorphic sites. A well-conserved DNA binding domain emphasizes the importance of genes that are regulated by PmrAB, and therefore, it cannot afford many mutations in this region.

One hundred fifty sites with amino acid substitutions in the PmrB protein sequence were found in our study, of which, 64 sites had rare mutations, each occurring in ≤0.1% of the genomes analysed (Supplementary file S3). Eighteen of the sites were highly polymorphic. Most of these belong to the two trans-membrane regions and the intracellular domain connecting them. The HATPase_c domain, which binds the ATP moiety required for auto-phosphorylation of the sensor kinase, is the most conserved domain. In particular, with the exception of a few rare mutations, the ATP binding domain, Mg^{2+} binding domain and the G-X-G motif were well conserved (Figure 2). The Mg^{2+} metal cofactor and the G-X-G motif line the ATP binding pocket and are therefore crucial for the function of PmrB. Clearly, mutations in these sites can severely compromise its function. Most of the amino acid substitutions previously reported in PmrAB in isolates of *A. baumannii* that are resistant to colistin were found to be non-polymorphic in our analysis. To date, four sites in *pmrA* and 26 sites in *pmrB* have been described as having non- synonymous mutations in the isolates resistant to colistin (**Table S1, Supplementary file S1**). Only one of the four resulting amino acid substitutions in PmrA and only three of the 26 in PmrB belong to polymorphic sites. In PmrA, the only substitution (S119T) at a polymorphic site, which has been shown to correlate with colistin resistance, occurs in the region that connects the domain of the signal receptor to the DNA binding domain. In the case of PmrB, two of the three polymorphic sites in PmrB of colistin-resistant isolates have frequent substitutions A142V and P170Q; both sites fall into the intracellular domain of the protein. P360Q mutation occurs in the third polymorphic site, which lies in the ATPase domain of PmrB. Since the amino acid substitutions previously described in colistin resistant isolates of *A. baumannii* are sporadic observations, we can argue that both PmrA and PmrB are constrained when accommodating non-synonymous SNPs. This also explains why the emergence of colistin resistance has not been a common phenomenon to be observed in the case of *A. baumannii*. We therefore examined the selection pressure at each amino acid site of the TCS, to see if there is negative selection acting on them, which could be the cause of the underlying mutation constraints.

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Both PmrA and PmrB are subject to negative selection pressure

The extent of selection pressure at a codon can be estimated by comparing the rate of occurrence of synonymous mutations (*d*S) to that of non-synonymous mutations (*dN*). For a site under neutral selection, synonymous and non-synonymous mutations will occur at similar rate and therefore, $dN \approx dS$. However, if a site is subjected to high selective pressure, the site is under purifying selection, which allows only synonymous mutations, in which case $dN < dS$. On the other hand, a site with adaptive selection will gain more non-synonymous substitutions than synonymous mutations, resulting in $dN > dS$.

To estimate the degree of the selection pressure acting on each amino acid site in PmrA and PmrB, we performed a selection analysis and expressed it in terms of the difference between the rates of non-synonymous and synonymous mutations ($dN - dS$). We found significant negative selection (also known as 'purifying selection') all through both the protein sequences. In PmrA, 43 sites out of 224 are subjected to a strong negative selection pressure. Therefore, these sites and the regions around them remain conserved (Figure 1, bottom panel). Functionally important sites, both in the signal receiver domain and in the DNA binding domain, exhibit a much more negative selection than the rest of the sequence. While no signs of positive selection were found in PmrA, sites with high levels of polymorphism were found to be under neutral selection instead. Three of the four mutated sites (M12K/M12I, P102H, S119T and L206P) that are reported in colistin-resistant isolates of *A. baumannii* belong to these high polymorphic regions, which are under neutral selection.

Similarly, 52 sites out of 444 sites are under significant negative selection in PmrB. Sites under negative selection were well distributed throughout the protein sequence. As in the case of PmrA, these sites and regions around them are fairly conserved in PmrB. In particular, the extracellular region, which spans the functional sites of the HATPase_c, HisKA and HAMP domains, has far fewer mutations than the rest of the molecule, due to the presence of the majority of the sites under negative selection therein. Notably, though most of these sites are under negative selection, the functional domains therein observed to congregate most of the amino acid substitutions (Figure 2). We observed such negative selection was associated with colistin resistance in all the previous reports. As many as 23 out of the 26 amino acid substitutions in PmrB that occur in colistin-resistant isolates fall in the functional domains. However, these mutations occur mainly on sites that are under neutral selection, each of them being delimited by the sites under negative selection in the functional domains (Figure 2). On the contrary, there exists a site at 153 of PmrB that is under significant positive selection. This site has $dN - dS$ value of 9.48851 ($p = 0.013$). Surprisingly, this site has never been reported to be mutated in colistin resistant strains of *A. baumannii*. This site belongs to one of the transmembrane domains of PmrB. The fact that it has so far never caused colistin resistance indicates that the positive selection gain may not overcome the cost of colistin resistance. Since the site belongs to a transmembrane domain, it is also possible that the mutations that occur at this site might serve an advantage in terms of structural stability, rather than having a functional role. Further, future studies employing site directed mutagenesis experiments could throw additional information about this site.

The SLAC method is equivalent to the site model of the equally well-accepted program CodeML of the PAML package, but both employ different sets of procedures and algorithms (Yang, 2007). To examine the consistency of the obtained results, we performed the same selection analysis with CodeML. CodeML produced similar results to that we obtained with HyPhy; it did not find any sign of significant positive selection in any of the sites of PmrAB. This increases the reliability of the study and strengthens our results.

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Taken together, most non-synonymous mutations in PmrAB TCS that are held responsible for colistin resistance in *A. baumannii* occur at non-polymorphic sites and at sites under neutral selection (Figure 3). Therefore, it is plausible to believe that resistance to colistin, when occurred due to mutations in PmrAB, is not disseminated owing to the lack of positive selection. Fixation of these mutations due to random genetic drifts seems unlikely, since the organism spends a short period of time under colistin exposure and severe selection pressure in the body of the patient.

Selection analysis of gyrA of E. coli and S. enterica was able to detect signatures of adaptive evolution that validates our approach

Resistance to quinolones in *E. coli* requires multiple mutations in different genes including gyrase A (*gyrA*) to achieve a clinically relevant level. It is more likely that elevated rate of mutations and their accumulation could be an important factor in resistance development. The requirement for multiple mutations suggests that there is strong selection for resistance mutations invariably associated with unusually high mutation rates occurring due to antibiotic exposure. Mutations at sites Ser-83 and Asp-87 in gyrase A are the most frequently reported to be responsible for quinolone resistance in Enteric bacteria (Domínguez et al., 2002; Everett et al., 1996; Lindgren et al., 2003; Vila et al., 2001, 1994). Quinolones are known to interact with the complex of DNA and DNA gyrase rather than with the enzyme alone. One study correlated that mutation at site Ser-83 to Trp leads to reduction of drug binding to the protein-DNA complex to 60-fold less than that of wild-type gyrase A (Willmott & Maxwell, 1993). Interestingly, selection analysis of *gyrA* of *E. coli* and *S. enterica* performed by us detected only these two sites (S83 and D87) to be under adaptive evolution, which is a very significant observation. This validates our approach of selection analysis with the required sensitivity, which detected all signatures of purifying mutations and the absence of adaptive mutations in PmrAB, unlike the observations on *gyrA* mutations.

Discussion

The emergence and spread of multidrug-resistant ESKAPE group of pathogens including *A. baumannii* have prompted many health professionals to assume that we are on the verge of a post-antibiotic era. Colistin and to some extent, tigecycline appear to be the only treatment options for treating infections caused by multidrug-resistant *A. baumannii*. In addition, the notable efficacy of colistin against tigecycline-resistant *A. baumannii* advocates colistin as the last option against the organism. However, reports of colistin resistance in *A. baumannii* have raised unprecedented concerns, as it is thought that resistance to colistin may become widespread in the future. Contrary to this belief, we have shown in this work that resistance to colistin may not become a general phenomenon in near future and these concerns are therefore unwarranted. Based on reduced fitness and virulence of colistin-resistant *A. baumannii* isolates obtained by experimentally induced mutations, Lo´pez-Rojas *et al.* also hypothesized that dependence on colistin is still safe from the evolutionary point of view (Lo´pez-Rojas et al., 2011). However, they also cautioned that, though the synonymous mutations are initially not adaptive, compensatory mutations may attenuate the reduced fitness. In contrast, using selection analysis, our investigation provided more direct evidence that mutations that cause colistin resistance are neither adaptive nor polymorphic and therefore, may not take over the global population.

In *A. baumannii*, resistance to colistin occurs in two mechanisms: enzymatic modification of lipid A moieties of the bacterial cell wall, either by the addition of phosphoethanolamine or

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galactosamine, or hyperacylation, and complete loss of cell wall lipo-polysaccharide (LPS) due to inactivation of lipid A biosynthesis (Beceiro et al., 2011; Boll et al., 2015; Henry et al., 2011; Moffatt et al., 2011, 2010; Pelletier et al., 2013). Colistin resistance by both mechanisms is the result of deletions and/or substitutions in the PmrAB two-component system (Adams et al., 2009; Arroyo et al., 2011; Beceiro et al., 2011). Resistance to colistin due to mutations in PmrAB is the most common mode of colistin resistance.

In this study, we collected 3113 draft genomes from the RefSeq database to evaluate the degree of natural selection acting on each site of PmrAB two-component system. Of the 3113 draft genomes, only a small percentage (≈0.61%, n = 19) of the genomes belonged to Colistin resistant strains. The dosage used for those resistant strains during *in vivo* treatment was 80–160 mg every 8 h for >60 kg bodyweights that may reach serum concentration of up to a maximum of 5mg/L, while in *in-vitro* experimental studies it ranged from 1 to >256 µg/µl. Thus, it includes resistances that have arisen both during patients' treatment as well as by laboratory induction.

The availability of only few genomes corresponding to colistin resistance strains gives an inherent strength to the study. Relying on a large number of genomes from Colistin resistant strains would ignore sites that are under adaptive evolution, as such mutations at these sites tend to reach fixation due to the convergent and parallel evolution resulting from colistin exposure. Consequently, adaptive evolution at such sites are no longer detected by computational methods (Crandall et al., 1999). A similar scenario is when the samples belong to the same population and have been exposed to similar environmental conditions (Kryazhimskiy & Plotkin, 2008). The presence of only a small percentage of the genomes representing Colistin resistant isolates in our investigation avoids this possibility, while at the same time allowing the inclusion of genomes reported worldwide over a wide range of time scale and ensuring a heterogeneous set of samples/populations.

Most of the amino acid substitutions in PmrAB reported to date are either from longitudinal isolates of patients or induced in laboratory conditions. Reports of clinical isolates bearing colistin resistance are scant. In addition, it has also been observed that the colistin resistant phenotype reverts back to susceptible phenotype on withdrawal of colistin in laboratory conditions (Adams et al., 2009; Cheah et al., 2016). The central theme of the study is how likely a long term emergence of colistin resistance in *A. baumannii* is. The colistin exposure to *A. baumannii* lasts as long as the treatment of the patient, and the resistance (if arises) reverts back to sensitive phenotype on withdrawal of colistin (Adams et al., 2009; Snitkin et al., 2013). To evaluate long term emergence of colistin resistance, we need to examine what happens post-exposure to colistin, or in the absence of colistin treatment (we could then qualify these mutations are stable enough to sustain long term resistance to colistin). The majority of genomes comprising of colistin susceptible strains in the data take account of the above condition. Further, with no evidence of compensatory mutations linked to colistin resistance identified till date, this strengthens our supposition that colistin resistant mutations may never get fixed in the global population of *A. baumannii* (Lesho et al., 2013; Lo´pez-Rojas et al., 2011; Rolain et al., 2011). This observation may not apply to the populations in micro-environments, such as a laboratory cultures.

Apparently, resistance to colistin is achieved by a cost of fitness. Studies in the past have shown the reduced fitness in colistin resistant isolates, both in terms of growth rate under laboratory conditions and reduced virulence (Beceiro et al., 2014; Lo´pez-Rojas et al., 2011; López-Rojas et al., 2013). In addition, to compensate for the loss/modification of the LPS, the transcriptome and the corresponding proteome are largely shifted towards expression of

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genes responsible for membrane integrity and biogenesis (Boll et al., 2016; Cheah et al., 2016; Henry et al., 2011). A recent work confirms that surface lipoproteins are overexpressed and localized profusely on the cell surface, which could compensate for the loss of membrane integrity. These studies highlight the excessive cost of colistin resistance, and therefore, mutations in PmrAB may be deleterious and subject to strong purifying selection.

To access the degree of natural selection acting on each of the amino acid site of both PmrA and PmrB, we used a well-established method that compares the rate of occurrence of non-synonymous mutations (*dN*) and synonymous mutations (*dS*) in each site of a codon-aligned coding sequence, tested on a phylogenetic tree (Goldman & Yang, 1994; Kimura, 1977). In this study, this value has been expressed as $dN - dS$. A value of $dN - dS$ close to zero implies that a site is under neutral selection and a negative value indicates a purifying mutation. In some cases, this gives a positive value for $dN - dS$, suggesting that a site is under adaptive evolution.

PmrAB mutation analysis in conjunction with domain analysis suggests that the signal receptor domain and the DNA binding domain, as well as the functional sites therein are ideally conserved in PmrA (Figure 1). This indicates that PmrA transduces a very specific signal, input from PmrB and in turn regulates a limited and non-degenerate set of genes. On the other hand, the transmembrane domains as well as the intracellular domain of PmrB are highly variable (Figure 2). Other domains such as the kinase and ATP binding domain are comparatively conserved and contain more sites with purifying selection. Since there is only one intracellular domain delimited by transmembrane α-helices, this domain must be recruiting the response regulator(s). Since this domain is variable, we can state that it recruits more than one response regulators and/or transcription factors, resulting in cross-talks between parallel signal transduction pathways, one of which is the cognate PmrA-mediated signalling. More detailed investigations at the molecular level are needed to corroborate this hypothesis.

A significant number of sites in both PmrA and PmrB are under purifying selection. In addition, we have also identified mutation hotspots, sites that are exceptionally polymorphic. Interestingly, sites with mutations that cause colistin resistance are not polymorphic sites. Rather, colistin resistance mutations are largely under neutral selection. In addition, a few of colistin resistance mutations are also under purifying selection (Figure 3). We suggest that these conditions maintain the incidence of colistin resistance under check. Further, loss of fitness due to colistin resistance resulted from the combined effect of neutral colistin resistance mutations and the purifying selection acting on the overall protein sequences ensure that the colistin resistance remains uncommon. The reasons for this loss of fitness include physiological costs in terms of slow growth rate, loss of membrane integrity and its compensation, and reduced virulence. In addition to this, because of the high selection pressure exerted by the host's immune system and the presence of drugs, the short exposure of the bacterium to colistin in the host, it is highly unlikely that random genetic drift can fix these mutations. These observations explain us why reports of colistin-resistant isolates of *A. baumannii* have been sporadic and they might remain so. In the near future, *A. baumannii* may not be able to emerge as a colistin-resistant organism, unless other mechanisms such as plasmid-mediated (*e. g.*, *mcr*-1) resistance emerge.

The other mechanism of development of Colistin resistance is due to mutations in *lpxACD* of *A. baumannii*. However, unlike mutations in *pmrAB*, where virtually all mutations that cause Colistin resistance are due to substitutions, mutations reported in *lpxACD* are due to large indels, non-sense mutations and insertion sequence-mediated gene disruptions that cause

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complete loss of cell wall lipopolysaccharide (Carretero-Ledesma et al., 2018; Girardello et al., 2017; Moffatt et al., 2010). There is no direct method to analyse signatures of selection pressure from such mutations. However, it has already been reported through growth curve and virulence assays that fitness costs associated with *lpx* mutations are even higher than those associated with of *pmr* mutations (Beceiro et al., 2014) and therefore, *lpxACD* mutants are unsustainable in the long run.

The computational method of selection analysis has precisely detected the sites under adaptive evolution in *gyrA*, an observation which is supported by a wealth of previous literature about quinolone resistance in bacteria. However, this approach has a limitation when studied from samples of a homogenous population as it under-represents the underlying positive selection (Kryazhimskiy & Plotkin, 2008). A large number of mutations in a single homogenous population are likely to be transient mutations that may not have fixed in that population. On the other hand, the positively selected adaptive mutations may tend to get fixed and take over the entire population. In such context, signs of positive selection may be missed during the analysis of a homogenous population. In this study, inclusion of draft genomes that have been reported all over the world over a wide range of time scale alleviates this limitation and ensures that we have taken a heterogeneous set of samples/populations that include different lineages.

Conclusion

The anticipation and management of natural selection is an important aspect of the fight against multidrug resistance (Lesho et al., 2013). This was the central theme of this work in the context of the degree of natural selection acting on PmrAB TCS, which has been implicated in resistance to colistin. We showed that in case of resistance to colistin, natural selection plays only a minor role, if at all, and therefore, continuing use of colistin could still be a safe option. Besides as we see, *lpxACD* mutations leading to colistin resistance is also unsustainable in the long run. Therefore, the concerns raised by healthcare professionals regarding rapid emergence of colistin resistance and about continuing the usage of colistin for the treatment against *A. baumannii* infections are unwarranted.

Nucleotide sequence accession numbers

Nucleotide sequences of *pmrAB* from *Acinetobacter baumannii* strains PKAB19 and PKAB15 have been deposited in GenBank under accession numbers MH925082 and MH925083, respectively.

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Conception and design, analysis and interpretation, writing of the article, provision of materials, patients, and resources; **PK**: Supervision of the entire work, Conceptual design of the experiment, analysis and interpretation, writing of the article, provision of materials, patients, resources and funding.

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Figure Legends

Figure 1. Mutation analysis of PmrA of *A. baumannii*.

Top panel – The domain analysis (predicted by HMMER)(Finn et al., 2011). The secondary structure was predicted by Phyre2 web server (Kelly et al., 2015). The black triangles show functional sites of the protein (predicted by the Conserved Domain Database)(Marchler-Bauer et al., 2011). A-Active site, P-Phosphorylation site, D-Dimerization interface, R-Intermolecular recognition site. Barrels and arrows represent α-helices and β-sheets, respectively. Numbers in brackets denote the start-end positions of the corresponding domains.

Middle panel – Positions and numbers of non-synonymous mutations across all the genomes under the analysis.

Bottom panel – Selection along the protein sequence expressed in terms of normalized $dN - dS$ (calculated by Datamonkey web server)(Sergei L. Kosakovsky Pond & Frost, 2005). Asterisks indicate sites under negative selection with $p \leq 0{\cdot}05$. Triangles show the sites identified in colistin resistant isolates in previous literatures.

Figure 2. Mutation analysis of PmrB of *A. baumannii*.

Top panel – The domain analysis (predicted by HMMER)(Finn et al., 2011). The secondary structure was predicted by Phyre2 server and trans-membrane (yellow barrels) by TMHMM (Kelly et al., 2015; Sonnhammer et al., 1998). The black triangles show functional sites of the protein (predicted by the Conserved Domain Database) (Marchler-Bauer et al., 2011). AB-ATP binding site, M-Mg^{2+} binding site, G-GXG motif. Barrels and arrows represent α-helices and β-sheets, respectively. Yellow coloured regions of the α-helices denote transmembrane domains. Numbers in brackets denote the start-end positions of the corresponding domains.

Middle panel – Positions and numbers of non-synonymous mutations across all the genomes under the analysis.

Bottom panel – Selection along the protein sequence in terms of normalized $dN - dS$ (calculated by Datamonkey web server) (Sergei L. Kosakovsky Pond & Frost, 2005). Asterisks indicate sites under negative selection with $p \leq 0{\cdot}05$, green asterisk representing a site with positive selection. Triangles show the sites identified in colistin resistant isolates in previous literatures.

Figure 3. Venn diagram depicting the amino acid substitutions resulting from non-synonymous mutations in A) *pmrA*; and **B)** *pmrB*, which are responsible for resistance to colistin in *A. baumannii* are under neutral selection, most of which are non-polymorphic sites. Numbers in each set indicate the number of sites falling under the corresponding category.

Table 1

Resistant Determinants

	bla$_{IMP-1}$	*bla*$_{OXA-23}$	*bla*$_{OXA-51}$	*bla*$_{OXA-58}$	*bla*$_{NDM-1}$	*bla*$_{PER-1}$	*bla*$_{ADC}$	*adeB*	*aac(3)-I*	*aac(6)-Ib*	*aph(3)-I*	*ant(3)-I*	*rmtC*	*armA*
PKAB15	+	+	+	-	-	+	-	+	-	-	-	-	-	-
PKAB19	-	+	+	-	-	-	-	+	-	-	+	-	-	-

Biofilm production and Antimicrobial susceptibility [a]

	MEM (mg/L)	CAZ (mg/L)	CRO (mg/L)	AMK (mg/L)	GEN (mg/L)	TOB (mg/L)	TGC (mg/L)	CST (mg/L)	Biofilm[b]
PKAB15	>256(R)	>256(R)	>256(R)	>256(R)	>256(R)	>256(R)	0.75(S)	3(R)	+
PKAB19	32(R)	32(R)	>256(R)	8(S)	256(R)	<2(S)	0.38(S)	3(R)	++

[a]R – Resistant, I – Intermediate, S – Sensitive, according to EUCAST Guidelines(EUCAST, 2017)

[b]+ – Weakly positive ($OD_{Control} < OD < 2xOD_{Control}$), ++ – Strongly positive ($OD > 3xOD_{Control}$)

MEM – Meropenem, CAZ – Ceftazidime, CRO – Ceftriaxone, AMK – Amikacin, GEN – Gentamicin, TOB – Tobramycin, TGC – Tigecyclin, CST – Colistin

Figure-1

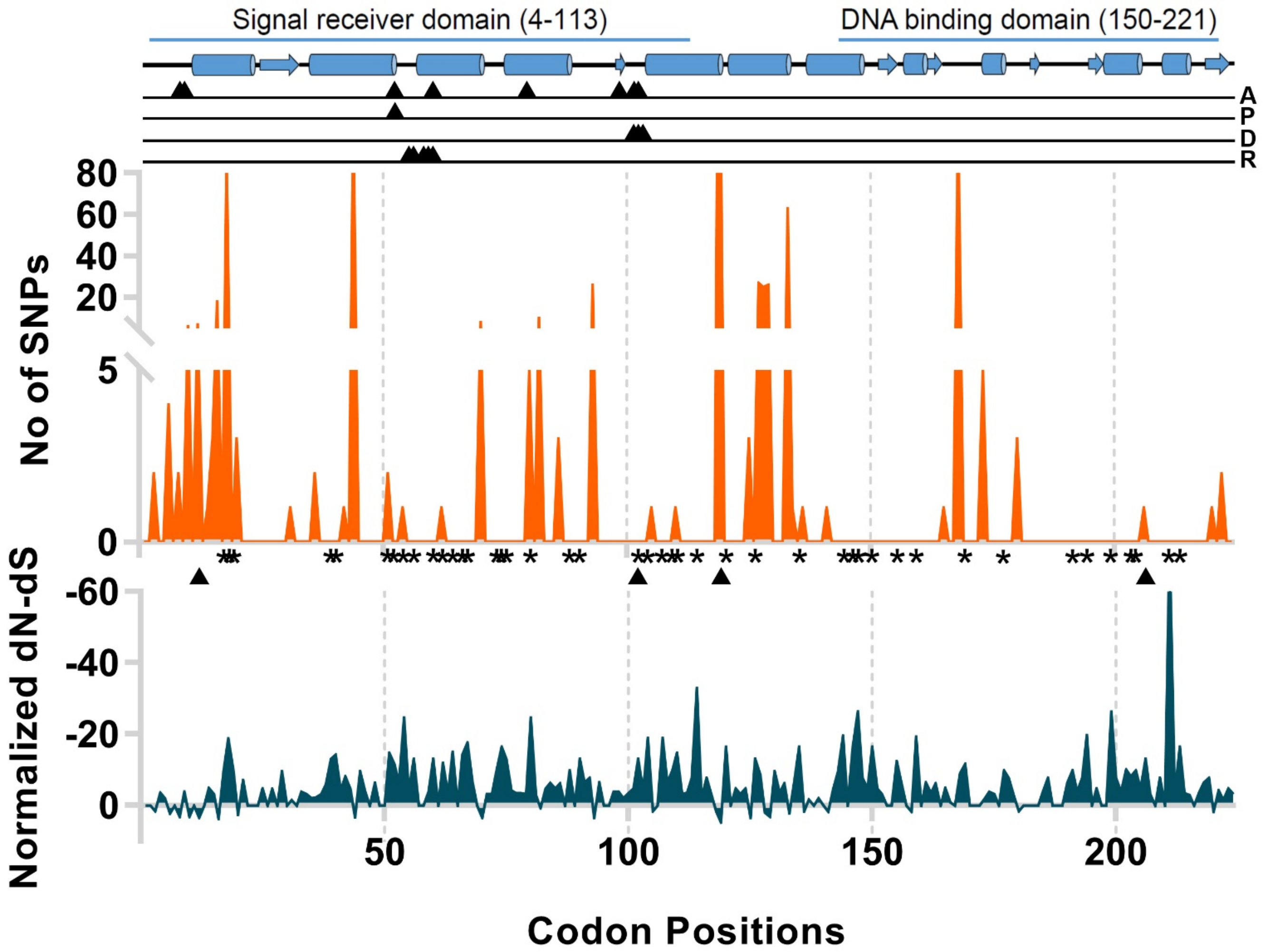

Figure-2

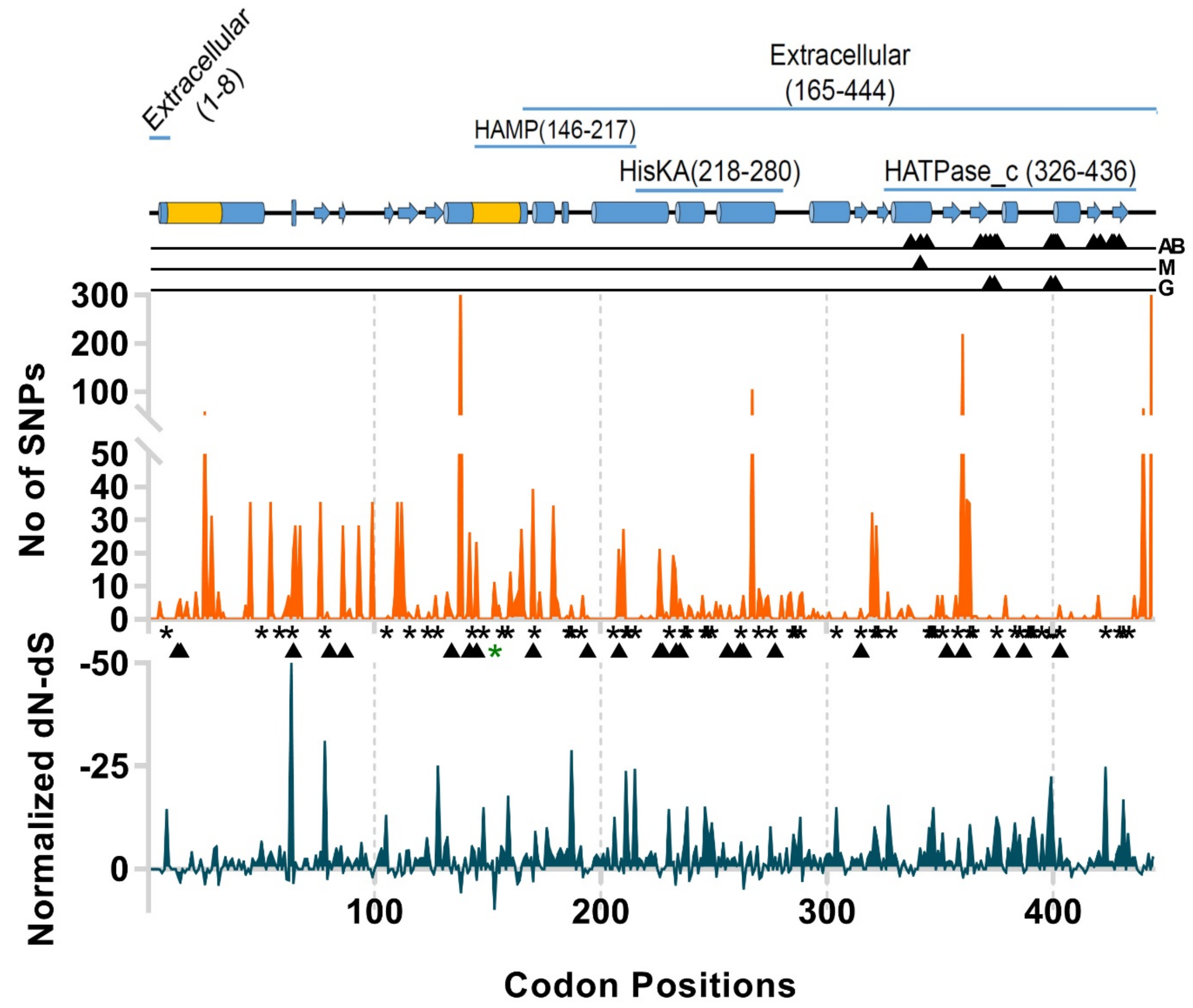

Figure-3