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Original Research

The highly conserved domain of RND multidrug efflux pumps in pathogenic Gram-negative bacteria

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Abstract: RND (Resistance-Nodulation-Division) family transporters have a vital role in both intrinsic and acquired multi-drug resistance in Gram-negative bacteria. It is important to find a conserved domain in the RND family between different pathogenic bacteria for diagnostic and therapeutic purpose. Total sequences of three-component system RND efflux pumps were retrieved from NCBI nucleotide and protein database and were subjected to conservation and variation analysis using the multiple sequence alignment feature of the CLC workbench. The phylogenetic tree for main transporters was drawn and the three-dimensional structure was also evaluated. From the sequence conservation analysis, highly conserved residues with 282 base pair (94 amino acid) long were identified. The location of the highly conserved domain is positioned in the domain 1 crystallographic structure of AcrB *Escherichia coli* and MexB *Pseudomonas aeruginosa*. The main transporter component phylogenetic tree shows the clusters of different genotypes and their evolutionary association. Each of three components of RND proteins is crucial for drug efflux, and the absence of even one component makes the entire complex totally nonfunctional. Therefore, this highly conserved region can be used to disable the RND multidrug efflux pumps. In addition, this highly conserved can also be used for diagnostic aspects.

Key words: RND multidrug efflux pumps; Conserved domain; Gram-negative bacteria.

Introduction

The resistance with efflux pump of the RND (Resistance-Nodulation-Division) superfamily are found ubiquitously throughout the Bacteria, Archaea and Eukaryotes. RND efflux pumps (such as AcrB of *Escherichia coli* and MexB of *Pseudomonas aeruginosa*) plays a significant role in the innate resistance of Gram-negative bacteria to multiple classes of structurally distinct antimicrobials, including those that are clinically relevant(1, 2). Those belonging to the RND family play a most important role in resistance of Gram-negative bacteria to a wide range of toxic compounds, including antibiotics, biocides, and heavy metals(3, 4). Gram-negative bacteria are protected by an outer membrane, therefore efflux transporters of the RND family are situated within the inner membrane and function in complex with two other proteins, an outer membrane channel and a periplasmic adaptor protein, to form a tripartite efflux pump spanning both the inner and outer membrane(5, 6). The RND family efflux pumps comprise the following: a transporter (efflux) protein (e.g., AcrB), which is located in the inner (cytoplasmic) membrane; a periplasmic adaptor protein (also known as a membrane fusion protein) (e.g., AcrA); and an outer membrane protein channel (e.g., TolC)(7-9). The development of powerful homology identification and molecular biology techniques over the past decade has aided in the establishment of protein structure-function and evolutionary relationships using sequence information alone (10-12). Sensitive search procedures that employ sequence-based and structure-based profiles have been shown to be effective in the reliable detection of remote evolutionary relationships, So it has a lot of uses in finding conserve domains and protein structures(13, 14). Focusing on efflux pumps in gram-negative bacteria can afford a new platform for antibiotic discovery and could expedite the need for new broad-spectrum antibiotics. The role of RND systems in both antimicrobial resistance and virulence makes them attractive targets for new drugs aimed at inhibiting their function, a suitable candidate for vaccine, modifications in immunogenicity by genetic manipulation and other immunological usages(15, 16). The aim of the present study was the accurate analysis the consensus sequence of RND multidrug efflux pumps components genes in the most common and important clinical bacterial species, and study the highly conserved residues in the arrangement of the nucleotide and amino acid sequences and draw a phylogenetic tree.

Materials and Methods

Drawing consensus sequence

The sequences of three-component system (periplasmic adaptor proteins, main transporter and Outer membrane protein channel) of RND family in most common and important clinical gram-negative pathogens were retrieved from NCBI nucleotide and protein database (http://www.ncbi.nlm.nih. gov). These sequences were from strains of *Acinetobacter baumannii, Escherichia*

Table 1. RND family efflux pumps caused multidrug resistance in more common and important clinical gram-negative pathogens.

Bacteria	PAP	Main transporter	OMF
Acinetobacter baumannii	*AdeA* KX154813.1[a]	AdeB KX154813.1[a]	*AdeC* KX154813.1[a]
Escherichia coli	*AcrA* CP021179.1[a]	AcrB CP021179.1[a]	*TolC* CP021179.1[a]
Klebsiella pneumonia	*AcrA ARPQ01000166.1*[a]	AcrB ARPQ01000166.1[a]	-
Proteus mirabilis	*AcrA JOVJ01000004.1*[a]	AcrB JOVJ01000004.1[a]	-
Pseudomonas aeroginosa	*MexA* NC_002516.2[a]	MexB NC_002516.2	*OprM* NC_002516.2[a]
Serratia marcescens	*SdeA* AY168756.2[a]	SdeB AY168756.2[a]	-
Stenotrophomonas maltophilia	*SmeA* AF173226.1[a]	SmeB AF173226.1[a]	-
Shigella dysenteriae	*AcrA CP000034.1*[a]	AcrB CP000034.1[a]	-
Shigella sonnei		RND transporter permease subunit CP019689.1[a]	

[a]: accession number. PAP: periplasmic adaptor proteins. OMF: outer membrane factor.

coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei. (table 1)

Multiple Sequence Alignment and analyze 3D structure

Sequences were aligned using CLC workbench software (multiple alignment nucleotide and peptide for each component of RND systems separately). Poorly conserved regions were detected and removed and short sequence (282bp or 94 amino acid) from the highly conserved region of three-component system of RND family (high conserved region observed in main transporter component) were selected from the multiple sequence analysis.

The highly conserved peptide region was found in the existing three-dimensional structures in the NCBI protein database.

Phylogenetic Analysis

To draw a phylogenetic tree of the main transporter component of RND family genes belonging to different genotypes we used a sequence of a strain as the representative of each bacterium. All 9 sequences were first aligned in the CLC workbench software and the aligned file was then subjected to the UPGMA method to draw a phylogenetic tree by UPGMA method. (The assessment was performed by Discovery Studio 2017).

Results

From the most common and important clinical gram negative pathogens that have been reported to be resistant by RND multidrug efflux pumps, the following bacteria were selected based on existing sequence of RND genes in NCBI nucleotide database: 22 *Acinetobacter baumannii*, 40 *Escherichia coli, 19 Klebsiella pneumonia, 11 Proteus mirabilis, 35 Pseudomonas aeruginosa, 10 Serratia marcescens, 15 Stenotrophomonas malto-*

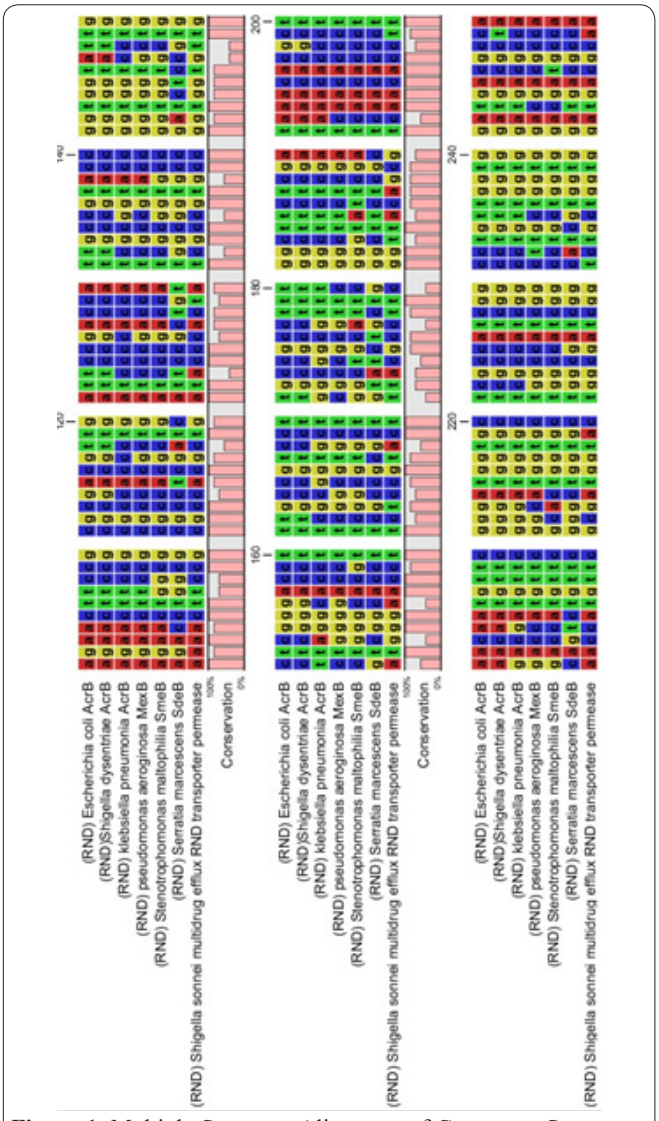

Figure 1. Multiple Sequence Alignment of Consensus Sequences of the main transporter conserve region in bacterial species: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei.*

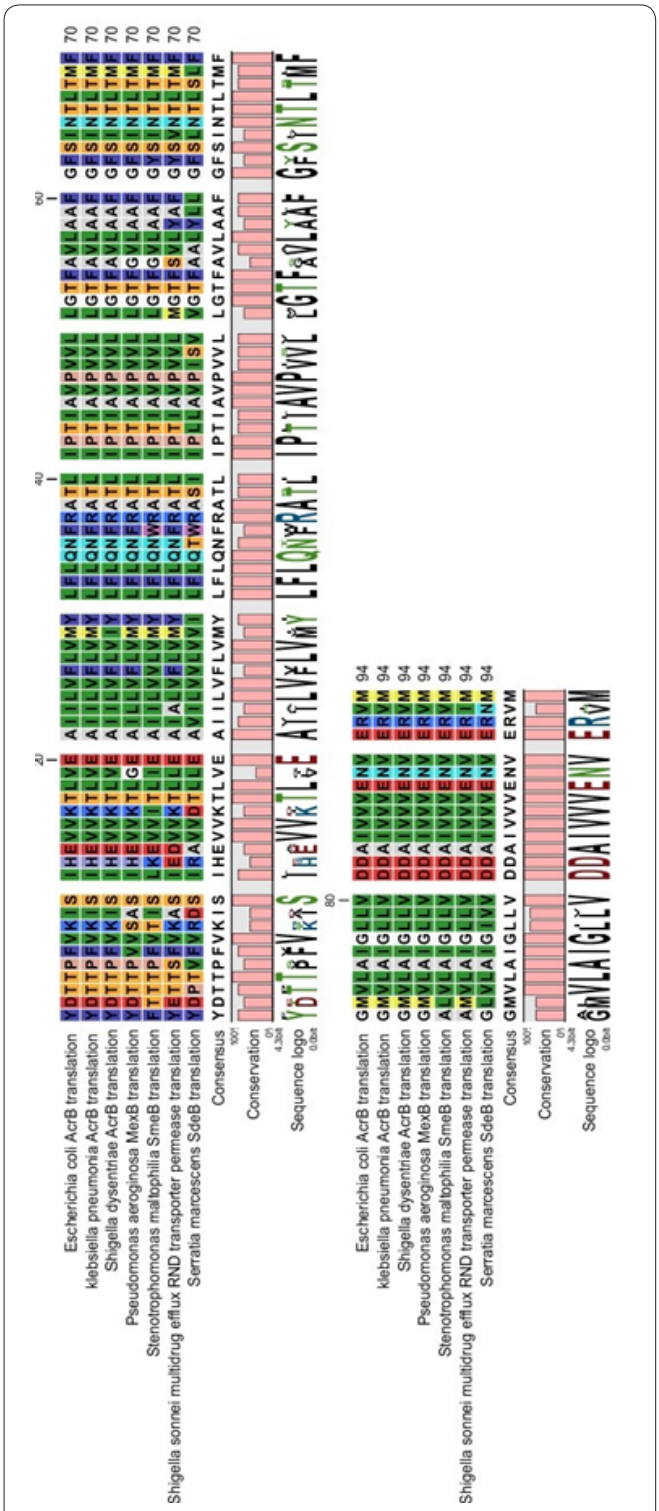

Figure 2. Multiple Sequence Alignment of translated Sequences (amino acids) of the main transporter conserve region in bacterial species: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei*).

philia, 13 Shigella dysenteriae, 8 Shigella *sonnei*. It was observed by multiple sequence alignment that different strains of each species have a homology of about 99% in the component of RND multidrug efflux pumps encoding genes. Therefore, we randomly selected a sequence of one strain from each bacterial species for further assessments. The components of the encoding RND multidrug efflux pump genes (periplasmic adaptor proteins, main transporter and Outer membrane protein channel) were separately aligned in these species. The sequences of periplasmic adaptor proteins and Outer membrane protein channel were very diverse in different bacterial species. A highly conserved sequence with 282 base pair long were perceived with alignment main transporter in bacterial species: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei* (The conserved area was not found in *Acinetobacter baumannii and Proteus mirabilis*) (Figure 1). For more precise analysis, the amino acids of the highly conserved area were aligned, and there was with high homology in mentioned bacterial species. (Figure 2) The location of the highly conserved domain is from amino acid 327 to 420, and this area is positioned in the domain 1 crystallographic structure of AcrB *Escherichia coli* and MexB *Pseudomonas aeruginosa*. Highly conserved area on the available crystal structure of main transporter (AcrB *Escherichia coli* and MexB *Pseudomonas aeruginosa)* was determined, this region is with yellow color. (Figure 3). Phylogenetic trees of the main transporter of 9 bacterial species showed clusters built on the basis of evolutionary relatedness. It can be inferred that main transporter of RND multidrug efflux pumps are evolutionarily related in bacteria species: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei*. The evolutionary tree of the main transporter of RND multidrug efflux pumps is shown in Figure 4.

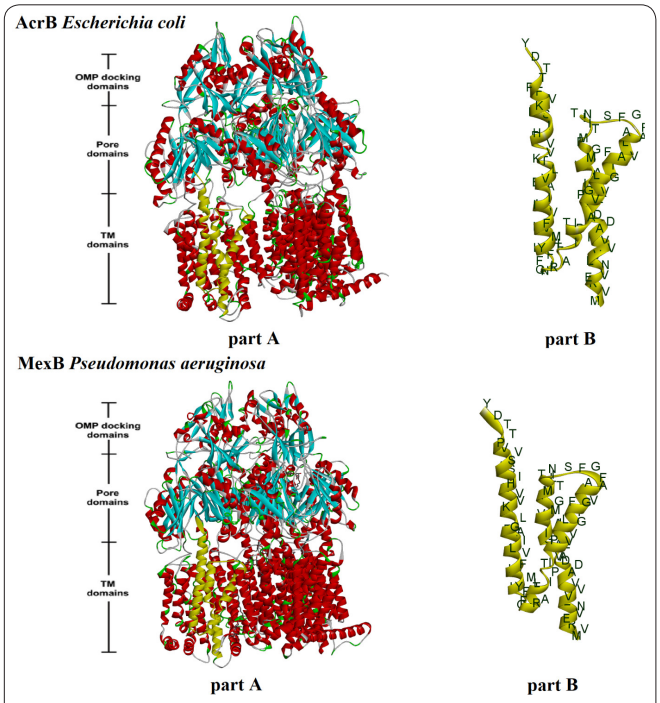

Figure 3. Detection of highly conserved region on crystal structure of AcrB *Escherichia coli* and MexB *Pseudomonas aeruginosa*; part A: conserve region on complete structure, part B: selected region of conserve area (yellow color show highly conserve region.

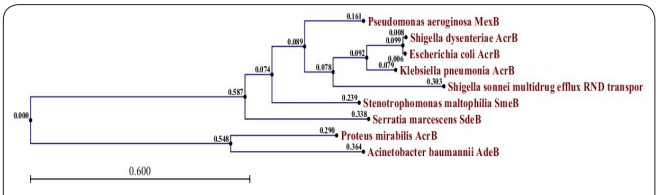

Figure 4. Phylogenetic Tree of 9 Sequences Belonging to the main transporter of different bacteria species was constructed in CLC Workbench Software by the UPGMA Method.

Discussion

RND (Resistance-Nodulation-Division) family transporters are organized as three-component system in various species, and common especially among Gram-negative bacteria and catalyze the active efflux of many antibiotics and chemotherapeutic agents (17-19). In this study, the nucleotide and amino acid sequences of the three components of the RND family of most common and important clinical gram negative bacteria were extracted from NCBI and evaluated. In the main transporter components, a highly conserved sequence with 282 base pair long were perceived in different bacterial species: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei.* Importantly, each of these three component proteins is crucial for drug efflux, and the absence of even one component makes the entire complex totally nonfunctional (19-21). Consequently, changes in the main transporters can disable RND multidrug efflux pumps. The main transporters capture its substrates also from within the phospholipid bilayer of the inner membrane or the cytoplasm. The crystal structures of the two RND main transporters, AcrB of *E. coli* and MexB of *P. aeruginosa*, have been reported earlier (22-25). These RND main transporters are organized as trimers, where each monomer exhibits a complex topology consisting of 12 transmembrane α-helix (TMs) (TM1–TM12) and periplasmic domains fold into six subdomains: PN1, PN2, PC1, and PC2, which build the pore domain, and DN and DC, which build the docking domain to the Outer Membrane Factor (OMF)(23, 24). The highly conserved domain (72 amino acid) is located in PN2, TM2, TM3 and TM4. TM4 is surrounded by other TMs and harbor well-conserved charged residues, which most likely mediate proton translocation and have been shown to be indispensable for the proper function of the protein, since the mutation of these residues leads to a complete loss of drug resistance (24), this highly conserved region can be used for making the RND multidrug efflux pump completely unfunctional. Therefore, eliminating or manipulating of this highly conserved region by different genetic-based methods, can deactivate RND multidrug efflux pumps in *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei.* In addition, the highly conserved domain of main transporter component of RND multidrug efflux pumps can be used for the diagnostic aspects. For example, we can design a primer for this domain and we can identify RND multidrug efflux pumps in mentioned Gram-negative pathogens. Phylogenetic trees of the main transporter of these nine bacterial species showed clusters built on the basis of evolutionary relatedness. The main transporters in two species (*Escherichia coli* & *Shigella dysenteriae*) are more closely related than other species, and it can be inferred that main transporters of RND multidrug efflux pumps are evolutionarily related in this bacterial species: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Serratia marcescens, Stenotrophomonas maltophilia, Shigella dysenteriae and Shigella sonnei*. It can also be inferred that main transporter of RND multidrug efflux pumps in the mentioned bacteria share a common ancestry. But the relation of main transporters in *Acinetobacter baumannii* and *Proteus mirabilis* are far from other species. RND (Resistance-Nodulation-Division) family transporters are organized as a three-component system and each of three components of RND proteins is crucial for drug efflux, and the absence of even one component makes the entire complex totally nonfunctional, so this highly conserved region can be used to disable the RND multidrug efflux pumps. In addition, the highly conserved domain of main transporter component of RND multidrug efflux pumps can be used for the diagnostic aspects.

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Conflict of interest
None declared.

Ethical approval
Not applicable.

Informed consent
Not applicable.

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