In silico characterization of putative drug targets in Staphylococcus saprophyticus, causing bovine mastitis

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Abstract:
The bovine mastitis caused by coagulase negative staphylococci (CNS) has increased in many herds of urban and rural areas of India. Emergence of multi drug resistant bacteria has further made its management more complex and serious. Therefore, innovation of novel specific drug for the treatment of disease caused by particular organism remained to be a challenge. Hence, in the present study a bacterium was isolated from milk of the cow with bovine mastitis and was identified as S. saprophyticus, 44 pathways of S. saprophyticus retrieved (KEGG) from web server were found to be non homologous to the host Bos taurus, out of which 39 pathways were found to be in cytoplasm, 2 in cell wall and 3 in the cell membrane. The knowledge of the present study could make the drug discovery easier which have high affinity to the target site of the causative organism.

Keywords: Bovine mastitis, S. saprophyticus, Phylogenetic tree, KEGG, Metabolic pathway, Drug.

Background:
Mastitis is one of the serious diseases causing huge loss to the dairy industry worldwide. Bacteria involved in bovine mastitis infections are S. aureus, Coagulase negative staphylococci (CNS), E. coli, Serretia marcescens, Bacillus subtilis, Streptococcus species etc [1]. Bovine mastitis has remained a major infectious disease which is difficult to manage and control it. Further emergence of multi drug resistant bacteria causing mastitis has made its management more complex. It is more serious in the countries like India which is the highest milk producer in the world which requires an urgent attention and novel methods of intervention to control the disease. In this context that all the drugs selected for the treatment of bovine mastitis against a particular infectious agent has to be screened through the in silico study by using the bioinformatics tools and will have to be notified through a database dedicated for this purpose which help in identifying the high prevalence of bovine mastitis. Coagulase negative staphylococcus is one of the most important gram positive bacteria, which have a potential to infect both animals and humans. Coagulase negative Staphylococci (CNS) are increasing as causes of bovine intramammary infection throughout the world in recent years the treatment of staphylococcal mastitis still remains difficult in spite of using of antibiotics with a high in vitro efficacy. The in silico based approach involves a series of screening of proteins that can be used as potential drug targets and vaccine candidates [2]. Using such approach, novel targets have been identified successfully for various pathogens [3-9]. Therefore in the present study we have selected CNS for the in silico investigation of drug target pathway in the bacterial cell and characterization of proteins involved in the metabolic pathways and identifying location of pathway in the bacterial cell.

Methodology:
Identification of bacteria
CNS has been isolated from the milk of the cow with bovine mastitis and was identified by staining, colony morphology on a specific media, catalase and coagulase tests.
Molecular identification of the bacteria
Molecular identification of CNS was done by using 16s rRNA primers forward 5'CAG GCC TAA CAC ATG CAA GTC 3' and reverse 5'GGG CCG AGT GTA CAA GGC 5' primers used in the study were designed by using Primer 3, isolation of DNA was carried out by [10] and confirmed by 16s rRNA.

Prediction of target for the drug discovery
Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used as a source of metabolic pathway information [11], all the metabolic pathways of the S. saprophyticus were retrieved from the KEGG. Pathways which do not appear in the host but present in the pathogen have been considered as pathways unique to pathogen according to KEGG database annotation [12]. The corresponding amino acid sequences of the proteins involved in the pathway were retrieved from the KEGG database. They were subjected to a Basic Local Alignment Search Tool (BLAST) search against the non-redundant database with the e-value inclusion threshold set to 0.005. The search was restricted to proteins from Bos taurus through an option available in the BLAST program. This analysis was carried out for the detection of non homologues pathways in the host, which do not have hits below the e-value inclusion threshold of 0.005 and were picked out as potential drug targets. All the non homologues pathway genes were believed to be essential genes of the pathogen. Essential genes are those indispensable for the survival of an organism, and their functions are therefore, considered a foundation of life [13]. These crucial genes were absent in the host [14]. All the essential pathways amino acid sequences were subjected to the PSORT (http://www.psort.org/psortb/index.html) analysis for the prediction the location of the pathway in the cell. This is required to find out the surface membrane proteins which could be probable drug targets. Further these surface membrane pathway protein sequences were characterized by using Peptide Statistic (PEPSTAT) program which is available online via European Molecular Biology Open Software Suite (EMBOSS) web server [15].

![Figure 1: Sequence of 16s r RNA](image)

Results:
Identification of bacteria
The bacteria used in the present study could be identified as S. saprophyticus based on the colony morphology, gram staining and biochemical test. The identification of isolates as S. saprophyticus were confirmed by PCR amplification of the gene encoding the 16s rRNA. The sequence of the 16s r RNA gene is shown (Figure 1). Identification of species was further confirmed by computational analysis through BLAST.

Identification of potential drug targets
A total of 99 pathways of S. saprophyticus were retrieved from KEGG database (http://www.kegg.jp/kegg-bin/search_pathway_text) out of which 44 pathways were non homology to the host Bos Taurus Table 1 (see supplementary material). As bovine mastitis causative agents are emerging multi drug resistant, the discovery of an alternative treatment for this disease has profound scope and significance. Drug discovery process is time consuming and multistep process which includes test on many models and clinical trials. One of the major reasons for drug failures in bovine mastitis treatment is the indiscriminate use of drug without performing the in vitro sensitivity test to the causal organism [16, 17]. It’s reported that drug resistant in Coagulase negative staphylococcus is also of growing concerned in bovine mastitis, poor drug-affinity and pharmacokinetic properties of lead compounds. Computer aided method is a rapid and significant screening approach because it selects the lead molecules with good pharmacological and druggish properties.

Prediction of targets distribution in the cellular components
ALL the 44 pathways amino acid sequence were Subjected to PSORT analysis (http://www.psort.org/psortb/index.html) for prediction of location of pathway in cell, out of 44 pathways, 2 pathways were found to be in cell wall (4%), 3 pathways were located in cell membrane (7%) and 39 pathways in cytoplasm (89%). Similar studies have been carried out by [12] for finding the location of sub cellular components by using Proteome Analyst Specialized Sub Cellular Localization (PA-SUB) Server v2.5 [18]. This is required to find out the cell wall proteins which could be probable drug targets. Folate biosynthesis pathway and Beta lactamase pathways were found to be present in the cell wall of the bacteria, these both cell wall Proteins were characterized by a PEPSTAT program [19]. The abundance of each amino acid in the pathways and their molecular percent with respect to dayhoffstat and properties of residues is given in Table 2 & 3 (see supplementary material).

Folate biosynthesis pathway
Folic acid is necessary for the biosynthesis of DNA. Bacteria that are sensitive to a drug that inhibits an enzyme necessary for the biosynthesis of folic acid in bacteria are unable to acquire folic acid from their environment. In the presence of folic acid inhibitor they are unable to synthesize the folic acid essential for cell growth and multiplication. Drugs that inhibit the folic acid biosynthesis pathway of pathogenic bacteria do not harm their hosts, however, hosts (Bos taurus, human) acquire folic acid from their diet which lack the enzyme necessary for synthesizing folic acid [20]. Folate biosynthesis pathway comprises a very important hydrolase enzyme responsible for removal of phosphate group from the environment, presence of phosphate group in the environment prevents the passage of organic molecules through the membrane [21], and however inhibition of alkaline phosphatase enzyme in the prokaryotic system makes the bacteria unable to take up the organic matter. Hence cause the death of bacteria. Therefore, folate biosynthetic pathway was believed to be the best and safe target for designing the drug against the S. saprophyticus causing bovine mastitis.

Beta-lactamase precursor pathway
Nowadays methicillin resistant staphylococcus (MRS) is of serious concern. This is due to the failure of beta – lactam ring to bind to the Penicillin Binding Proteins (PBP). PBP helps to cross link the polymer of repeated units of N-acetylgalactosamine (NAG) and N- acetylmuramic acid (NAM), which is the final step in the biosynthesis of cell wall. However, beta-lactam antibiotics are becoming ineffective against pathogenic bacteria.
The most common reason is due to the production of beta-lactamase enzyme which catalyzes the hydrolysis of the antibiotics through the formation of carboxyl group degrading beta-lactam ring. Hydrolyzed antibiotics lose its activity or binding affinity towards the PBP hence no effect against bacteria. Resistant bacteria possessing beta lactamase gene hydrolisis beta lactum antibiotic, hence this antibiotic fails to bind PBP [22]. Beta-lactamase enzyme is an extra cellular enzyme in Gram-positive bacteria and found in periplasmic membrane in Gram-negative bacteria [23]. More than 200 types of beta-lactamase have been found. The difference among them is only the catalytic efficacy and turns over rate range from 0.004 to 1,200 molecules per second by 1 molecule of enzyme. Beta-lactamase became wide spread via the mechanism of plasmid exchange/insert among the pathogens [24]. The rapid spread and evolution of these enzymes have seriously threatened the present antimicrobial arsenal. Therefore In silico approach in finding out the beta-lactamase inhibitors coupled with beta-lactam antibiotics could be the solution for rapid spread of multi drug resistant bacteria. These enzyme inhibitors would function to inactivate the beta-lactamase in the periplasmic space so that the partner antibiotics can reach its target, penicillin binding protein (PBP), and interrupt the biosynthesis of cell wall causing the death of the bacteria.

It is worth characterizing the physicochemical properties of the protein by using its primary structure or amino acid sequence which aid in finding out whether the protein share any basic physicochemical properties with other proteins that have been studied experimentally. The most important use of this analysis is while qualitatively assessing particular regions of a protein sequence that may have common characteristics with others in alignments with distantly related protein sequences. A typical physicochemical parameter values can be obtained by using this analytical program.

Conclusion:

In the present study attempts were made to find the putative targets of S. saprophyticus for the drug discovery with all the sources available in databases by computational methods. S. saprophyticus was isolated and confirmed by 16s rRNA gene amplification. A total 44 non homologues pathways amino acid sequence were screened for their location in bacterial cell out of which 39 pathway were found to be in cytoplasm, 2 in cell wall and 3 in the cell membrane. As pathways of cell wall were believed to be the most putative targets for the drug discovery, the, proteins involved in the cell wall pathway were further characterized for the determination of physicochemical properties of the protein. Perhaps the knowledge of the present study made the drug discovery easier which have high affinity to the target site. Possible drug discovery to manage bovine mastitis with a help of bioinformatics tool is more significant, specific, reduce the time and complications involved in the clinical trials.

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Supplementary material:

Table 1: Information about Non- homologues pathways and their locations in S. saprophyticus searched against Bos Taurus

| Sl no | Gene id | Pathway                                | Location         | No of Amino acids |
|-------|---------|----------------------------------------|------------------|------------------|
| 1     | SSP1071 | Citrate synthase                       | Cytoplasm        | 372              |
| 2     | SSP0629 | Galactokinase                          | Cytoplasm        | 386              |
| 3     | SSP0068 | UDP-glucose 6-dehydrogenase            | Cytoplasm        | 334              |
| 4     | SSP0324 | 3-hydroxy-3-methylglutaryl-coa synthase| Cytoplasm        | 389              |
| 5     | SSP0146 | Choloylglycine hydratase               | Cytoplasm        | 331              |
| 6     | SSP2380 | NADH dehydrogenase subunit             | Cell membrane    | 509              |
| 7     | SSP1268 | L-asparaginase                         | Cytoplasm        | 324              |
| 8     | SSP1439 | Aspartate kinase                       | Cytoplasm        | 455              |
| 9     | SSP2163 | Branched-chain amino acid aminotransferase | Cytoplasm     | 358              |
| 10    | SSP2423 | Acetyl-coa acetyltransferase           | Cytoplasm        | 394              |
| 11    | SSP0817 | Threonine dehydratase                  | Cytoplasm        | 422              |
| 12    | SSP1325 | 2-oxoglutarate dehydrogenase E1        | Cytoplasm        | 933              |
| 13    | SSP2186 | Beta-lactamase precursor               | Cell wall        | 290              |
| 14    | SSP1531 | Monoxygenase                           | Cytoplasm        | 432              |
| 15    | SSP1325 | 2-oxoglutarate dehydrogenase           | Cytoplasm        | 933              |
| 16    | SSP0933 | Aspartate alpha-decarboxylase          | Cytoplasm        | 077              |
| 17    | SSP2168 | Alkylphosphonate dehydrogenase         | Cytoplasm        | 177              |
| 18    | SSP1645 | Glutamate racemase                     | Cytoplasm        | 269              |
| 19    | SSP1013 | D-alanine aminotransferase             | Cytoplasm        | 281              |
| 20    | SSP1351 | Alanine racemase                       | Cytoplasm        | 357              |
| 21    | SSP1015 | Beta-D-galactosidase                    | Cytoplasm        | 992              |
| 22    | SSP1272 | NAD(P)H-dependent glycerol-3-phosphate dehydrogenase | Cytoplasm | 332              |
| 23    | SSP1455 | Glutathione peroxidase                 | Cytoplasm        | 157              |
| 24    | SSP2389 | Acetate-coa ligase                     | Cytoplasm        | 523              |
| 25    | SSP1531 | Monoxygenase                           | Cytoplasm        | 432              |
| 26    | SSP0824 | Acetolactate synthase large subunit    | Cytoplasm        | 596              |
| 27    | SSP1313 | Dihydrofolate reductase                | Cytoplasm        | 161              |
| 28    | SSP1432 | Catalase                               | Cytoplasm        | 495              |
| 29    | SSP0824 | Acetolactate synthase large subunit    | Cytoplasm        | 596              |
| 30    | SSP1288 | Biotin-acetyl-coa carboxylase ligase   | Cytoplasm        | 323              |
| 31    | SSP1850 | Lipoyl synthase                        | Cytoplasm        | 313              |
| 32    | SSP0592 | Folate biosynthesis                    | Cell wall        | 477              |
| 33    | SSP0762 | Aldehyde dehydrogenase                 | Cytoplasm        | 475              |
| 34    | SSP1218 | Glycine cleavage system aminomethyltransferase T | Cytoplasm | 363              |
| 35    | SSP2401 | Adenyllylsulfate kinase                 | Cytoplasm        | 204              |
| 36    | SSP1530 | Fabg                                   | Cytoplasm        | 250              |
| 37    | SSP1707 | Phosphoenolpyruvate-protein phosphotransferase | Cytoplasm | 571              |
| 38    | SSP1270 | Ribosomal protein S1                   | Cytoplasm        | 393              |
| 39    | SSP0689 | DNA-directed RNA polymerase subunit alpha | Cytoplasm | 314              |
| 40    | SSP0897 | Exonuclease                            | Cytoplasm        | 184              |
| 41    | SSP0683 | Preprotein translocase subunit sey     | Cell membrane    | 430              |
| 42    | SSP1122 | Biprotential preprotein translocase subunit secd/secf | Cell membrane | 758              |
| 43    | SSP2253 | Transcription-repair coupling factor   | Cytoplasm        | 1170             |
| 44    | SSP1465 | DNA mismatch repair protein muts       | Cytoplasm        | 887              |

Table 2: Characterization of alkaline phosphates

| Residue | Number | Mole%  | DayhoffStat |
|---------|--------|--------|-------------|
| A       | 43     | 9.015  | 1.048       |

PEPSTATS of ssp:SSP0592 from 1 to 477
Molecular weight = 51985.09
Average Residue Weight = 108.983
Isoelectric Point = 4.9759
A280 Molar Extinction Coefficient = 35700
A280 Extinction Coefficient 1mg/ml = 0.69
Improbability of expression in inclusion bodies = 0.626
Table 3: Characterization for Beta-lactamase precursor

**PEPSTATS of ssp: SSP2186 from 1 to 290**

- Molecular weight = 32144.37
- Average Residue Weight = 110.843
- Isoelectric Point = 6.3029
- A280 Molar Extinction Coefficient = 21050
- A280 Extinction Coefficient 1mg/ml = 0.65
- Improbability of expression in inclusion bodies = 0.746

| Property   | Residues | Number | Mole%  |
|-------------|----------|--------|--------|
| Tiny        | (A+C+G+S+T) | 146    | 30.608 |
| Small       | (A+B+C+D+G+N+P+S+T+V) | 274    | 57.442 |
| Aliphatic   | (A+I+L+V) | 69     | 14.465 |
| Aromatic    | (F+H+W+Y) | 48     | 10.063 |
| Non-polar   | (A+C+F+G+I+L+M+P+S+T+V+W+Y) | 220    | 46.122 |
| Polar       | (D+E+H+K+N+Q+R+S+T+Z) | 257    | 53.878 |
| Charged     | (B+D+E+H+K+R+Z) | 134    | 28.092 |
| Basic       | (H+K+R) | 64     | 13.417 |
| Acidic      | (B+D+E+Z) | 70     | 14.675 |

**Table 3: Characterization for Beta-lactamase precursor**

| Residue | Number | Mole%  | DayhoffStat |
|---------|--------|--------|-------------|
| A = Ala | 23     | 7.931  | 0.922       |
| B = Asx | 0      | 0.000  | 0.000       |
| C = Cys | 1      | 0.345  | 0.119       |
| D = Asp | 15     | 5.172  | 0.940       |
| E = Glu | 19     | 6.552  | 1.092       |
| F = Phe | 7      | 2.414  | 0.670       |
| G = Gly | 15     | 5.172  | 0.616       |
| H = His | 3      | 1.034  | 0.517       |
| I = Ile | 24     | 8.276  | 1.859       |
| J = --  | 0      | 0.000  | 0.000       |
| K = Lys | 27     | 9.310  | 1.411       |
| L = Leu | 25     | 8.621  | 1.165       |
| M = Met | 8      | 2.759  | 1.623       |
| N = Asn | 25     | 8.621  | 2.005       |
| O = --  | 0      | 0.000  | 0.000       |

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| Property     | Residues | Number | Mole% |
|--------------|----------|--------|-------|
| Tiny         | (A+C+G+S+T) | 82     | 28.276|
| Small        | (A+B+C+D+G+N+P+S+T+V) | 145    | 50.000|
| Aliphatic    | (A+I+L+V)  | 62     | 21.379|
| Aromatic     | (F+H+W+Y)  | 23     | 7.931 |
| Non-polar    | (A+C+F+G+I+L+M+P+V+W+Y) | 139    | 47.931|
| Polar        | (D+E+H+K+N+Q+R+S+T+Z) | 151    | 52.069|
| Charged      | (B+D+E+H+K+R+Z) | 69     | 23.793|
| Basic        | (H+K+R)    | 35     | 12.069|
| Acidic       | (B+D+E+Z)  | 34     | 11.724|

| Property | Residues | Number | Mole% |
|----------|----------|--------|-------|
| P = Pro  | 10       | 3.448  | 0.663 |
| Q = Gln  | 14       | 4.828  | 1.238 |
| R = Arg  | 5        | 1.724  | 0.352 |
| S = Ser  | 23       | 7.931  | 1.133 |
| T = Thr  | 20       | 6.897  | 1.131 |
| U = ---  | 0        | 0.000  | 0.000 |
| V = Val  | 13       | 4.483  | 0.679 |
| W = Trp  | 1        | 0.345  | 0.265 |
| X = Xaa  | 0        | 0.000  | 0.000 |
| Y = Tyr  | 12       | 4.138  | 1.217 |
| Z = Glx  | 0        | 0.000  | 0.000 |