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Isolation and Molecular Identification of Pathogenic Microbes From Hospital Burn Wounds by Polymerase Chain Reaction(Pcr)Technology

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Abstract:

Molecular Diagnostic Techniques are expected to play a significant role in clinical and diagnostic bacteriology. Although their adoption may never replace the conventional methods their efficiency, quality, quickness and their role in the detection of slow growing organisms cannot be overlooked. Infection control programs need to document and report burn wound infections according to the recently established definitions of the classification system. Future studies of burn wound infections should use this standardized burn wound classification system so that clinical outcomes can be compared for burn patients with a specific condition (e.g., burn wound cellulitis). More research is required to determine the best methods for sampling excised and unexcised burn wound areas over the course of a severe deep partial-thickness and/or full-thickness injury. Reproducible standardized methods should be developed so that clinical microbiology laboratories can routinely test burn wound bacterial isolates for susceptibility to the topical antimicrobial agents on formulary at a particular burn center. A rotation program for topical antimicrobial use may also retard the development of resistance. Laboratory surveillance should include the reporting of burn unit-specific antibiograms for topical antimicrobial agents once standardized methods are available for performing susceptibility testing.

INTRODUCTION:

Burn Wounds and Infection:

Infection remains the leading cause of death among patients who are hospitalized for burns. Rapidly emerging nosocomial pathogens and the resistant microbes remain the main cause for the isolation of the microorganisms. The risk of infection is related directly to the extent of the burn and is also related to the failure of the body's immune system due to the disruption of the cell's integrity. Burns are one of the most common and devastating forms of trauma. Patients with serious thermal injury require immediate specialized care in order to minimize morbidity and mortality. Significant thermal injuries induce a state of immunosuppression that predisposes burn patients to infectious complications. Burn injury causes mechanical disruption to the skin, which allows opportunistic pathogens to invade deeper tissues. The usual skin barrier is replaced by a moist layer that helps microbial growth. The burn wound surface is sterile immediately following injury; however, it is repopulated quickly with gram-positive organisms from hair follicles, skin appendages, and the environment during the first 48 hours. More virulent gram-negative organisms replace the gram-positive organisms after 5-7 days. Gram-negative organisms have greater motility, possess many antibiotic resistance mechanisms, and have the ability to secrete collagenases, proteases, lipases, and elastases, enabling them to proliferate and penetrate into the cells. If host defenses are inadequate, invasion of viable tissue occurs. Normally thermal injury has a severe impact on the host's cellular

immune systems. The degree of immune suppression is proportional to the duration and temperature of thermal exposure. Infection is an important cause of mortality in burns. It has been estimated that 75% of all deaths following thermal injuries are related to infections. In various countries, including India, the importance of *Acinetobacter* species, as a rapidly emerging nosocomial pathogen, has been documented and these bacteria are predominantly isolated from ICUs, burn units and surgical wards. In addition, the problem of multi-drug resistance in gram-negative bacilli due to extended spectrum beta lactamases (ESBL) production is becoming a serious threat to the healthcare worker, who is likely to contract the infection, as the therapeutic options to these organisms are limited. This necessitates periodic review of the isolation pattern and antibiogram of the burn ward, which forms the basis for modification of drug regimen strategy. Burn wound infections are rare in partial-thickness burns unless they have been neglected or managed improperly. Invasive wound infections typically occur in patients with full-thickness burns involving more than 30% of their body. Risk of wound infection is increased in wounds that remain open because of a failure of the primary closure modality or because of comorbidities.

INVASIVE INFECTION

Invasive infection occurs in patients with a significant full-thickness burn in which closure is delayed Gauging burn wound sepsis by clinical signs and symptoms is difficult. Patients with extensive burn wounds generally manifest physiologic changes associated with hyper metabolism, including tachycardia, hypothermia or hyperthermia, tachypnea, ileus, glucose intolerance, and mental status changes.

PATHOGENESIS OF BURN WOUND INFECTIONS:

Thermal destruction of the skin barrier and concomitant depression of local and systemic host cellular and humoral immune responses are pivotal factors contributing to infectious complications in patients with severe burns (Alexander,1990). The burn wound surface (in deep partial-thickness and in all full-thickness burns) is a protein-rich environment consisting of avascular necrotic tissue (eschar) that provides a favorable niche for microbial colonization and proliferation (Barret, et al,1999).The avascularity of the eschar results in impaired migration of host immune cells and restricts delivery of systemically administered antimicrobial agents to the area, while toxic substances released by eschar tissue impair local host immune responses Although burn wound surfaces are sterile immediately following thermal injury, these wounds eventually become colonized with microorganisms. The nature and extent of the thermal injury along with the types and amounts of microorganisms colonizing the burn wound appear to influence the future risk of an invasive wound infection . Gram-positive bacteria that survive the thermal insult, such as staphylococci located deep within sweat glands and hair follicles, heavily colonize the wound surface within the first 48 h unless topical antimicrobial agents are used. Eventually after an average of 5 to 7 days), these wounds are subsequently colonized with other microbes, including gram-positive bacteria, gram-negative bacteria, and yeasts derived from the host's normal gastrointestinal and upper respiratory flora and/or from the hospital environment or that are transferred via a health care worker's hands. Over the last several decades, gram-negative organisms have emerged as the most common etiologic agents of invasive infection by virtue of their large repertoire of virulence factors and antimicrobial resistance traits. If the patient's host defenses and therapeutic measures (including excision of necrotic tissue and wound closure) are inadequate or delayed, microbial invasion of viable tissue occurs, which is the hallmark of an invasive burn wound infection.

MICROORGANISMS CAUSING INVASIVE BURN WOUND INFECTIONA

Prior to the antibiotic era, Streptococcus pyogenes (group A beta-hemolytic streptococci) was the predominant pathogen implicated in burn wound infections and was a major cause of death in severely burned patients. Staphylococcus aureus became the principal etiological agent of burn wound infections shortly after the introduction of penicillin G in the early 1950s, Although Staphylococcus aureus remains a common cause of early burn wound infection, Pseudomonas aeruginosa from the patient's endogenous gastrointestinal flora and/or an environmental source is the most common cause of burn wound infections in many centers. The incidence of infections due to less commonly encountered microbes, including other gram-positive and gram-negative bacteria, fungi, and viruses, has also increased steadily in subsequent decades. The emergence worldwide of antimicrobial resistance among a wide variety of human bacterial and fungal burn wound pathogens, particularly nosocomial

isolates, limits the available therapeutic options for effective treatment of burn wound infections.

MOLECULAR DIAGNOSIS OF MEDICALLY IMPORTANT BACTERIAL INFECTIONS

Infectious diseases are common diseases all over the world. Infectious diseases in non-industrialized countries caused 45% in all and 63% of death in early childhood. It is reported that infectious diseases are responsible for more than 17 million deaths worldwide each year, most of which are associated with bacterial infections. In patients with severe burns over more than 40% of the total body surface area (TBSA), 75% of all deaths are currently related to sepsis from burn wound infection or other infection complications and/or inhalation. The ability to control such bacterial infections is largely dependent on the ability to detect these etiological agents in the clinical microbiology laboratory. Molecular biology techniques continue to evolve rapidly, so it has been problematic for many laboratories to decide upon which test to introduce before that technology becomes outdated.

Improved outcomes for severely burned patients have been attributed to medical advances including early identification if the infecting organism, fluid resuscietation, nutritional support, pulmonary and burn wound care, and infection control practices. Presently Molecular Biology offers a wide repertoire of techniques and permutations of these analytical tools. A phylogenetic tree is described as, a branching diagram that shows, for each species, with which other species it shares its most recent common ancestor. The evolutionary tree or cladograms were traditionally used to draw evolutionary relationship among the organism; a more modern version of the same is phylogenetic tree which uses gene / protein sequences to draw the evolutionary relationship. These trees dictate the relationship among the organisms based on the similarity and dissimilarity among the nucleotide or nucleic acid sequences. The tree construction can be done through variety of tree-building methods which include methods based on distances, likelihood and characters. After a phylogenetic tree is constructed, it is important to test its accuracy which refers to the degree to which a tree is close to the true tree. Phylogenetics is the study of evolutionary relationships among organisms or genes. There are several types of data that can be used to build phylogenetic trees: Traditionally, phylogenetic trees were built from morphological features (e.g., beak shapes, presence of feathers, number of legs, etc). Today, we use mostly molecular data like DNA sequences and protein sequences.

UNIVERSAL GENE TARGETS:

In the presence of unknown bacterial organism, employment of amplification of DNA encoding ribosomal RNA genes serve as gene targets. In bacteria there are three genes which make up the rRNA functionality i.e. 5S, 16S, and 23S rRNA.

5’ – 16Sgene – spacer – tRNA – spacer - 23Sgene - spacer – 5S gene – 3’

Since 16S rRNA is highly conserved and has moderate copy number it is commonly employed for the identification purposes. 16S rRNA accumulates mutations at a slow, constant rate over time. Hence they are called “molecular clocks”. To maximize the utility of 16s rRNA gene analysis for species determination it is now routine to amplify almost the entire 16s rRNA gene, which spans ~1500bp and is thus readily sequenced in its entirety through bidirectional sequencing of cloned 16S amplicons. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Although PCR amplifications of 16S sequences have been of enormous value, there are caveats to this approach. For example; the 16S rRNA gene sequence of Nanoarcheota is so divergent that PCR with the “universal primers” failed to detect this species even from cultured organisms. PCR conditions such as temperature or extension time may allow the formation of chimeras or produce amplification bias that skews the representation of each species in cloned libraries.Another Drawback of 16S rRNA gene sequencing is the need for specialized equipment in the form of PCR Cycler and properly trained technicians

MOLECULAR DETECTION AND IDENTIFICATION

Detection involves the presence of a particular target organism within a sample, whilst diagnosis refers to the identification of the nature and the cause of a disease problem. Detection and diagnosis techniques involve certain levels of specificity, sensitivity and speed. In the case of PCR based methods, specificity is determined by primer selection and amplification conditions, while sensitivity depends on the nature of the PCR protocol and sample. Identification is defined as the assignment of an unknown organism into a known

taxonomic group, based on the selected characteristics. PCR- based amplification techniques, for detection and diagnosis of pathogens, involve the use of specific primers to amplify a diagnostic fragment or universal primers that provide a diagnostic genomic fingerprint of the organism. The three-domain system is a biological classification introduced by Carl Woese. That emphasizes his separation of prokaryotes into two groups, originally called Eubacteria and Archaebacteria. The groups were also renamed the Bacteria, Archaea, and Eukarya, further emphasizing the separate identity of the two prokaryote groups.

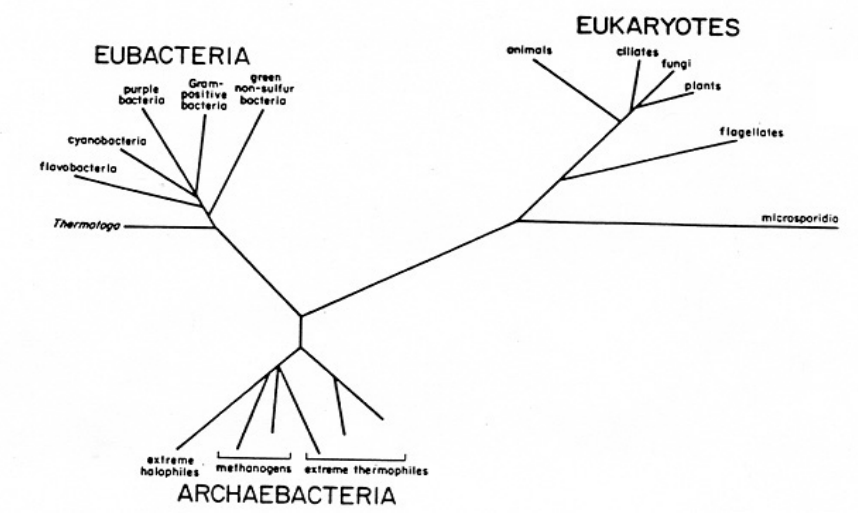

Figure 1: Classification Of Archebacteria

rRNA is useful in taxonomy because:

- Ribosome of ancient origin
- Universally distributed and functionally equivalent
- Changes not extensive, primary structure conserved
- Extreme conservation of some regions coupled with variability makes it useful
- 16S rRNA gene large enough for statistical analysis

TEHNIQUES INVOLVED IN MICROBIAL TAXONOMY

DNA-DNA HYBRIDIZATION STUDIES:

DNA-DNA hybridization generally refers to a molecular biology technique that measures the degree of genetic similarity between pools of DNA sequences. It is usually used to determine the genetic distance between two species. When several species are compared that way, the similarity values allow the species to be arranged in a phylogenetic tree; it is therefore one possible approach to carrying out molecular systematics. GC content is found to be variable with different organisms, the process of which is envisaged to be contributed by variation in selection, mutational bias and biased recombination-associated DNA repair. The species problem in prokaryotic taxonomy has lead to various suggestions in classifying bacteria For example, the Actinobacteria are characterised as "high GC-content bacteria". The GC-content of Yeast (Saccharomyces cerevisiae) is 38%, and that of another common model organism Thale Cress (Arabidopsis thaliana) is 36%.Because of the nature of the genetic code, it is virtually impossible for an organism to have a genome with a GC-content approaching either 0% or 100%. A species with an extremely low GC-content is Plasmodium falciparum (GC% = ~20%) and it is usually common to refer to such examples as being AT-rich instead of GC-poor.

THE ROLE OF 16S rRNA GENE SEQUENCING IN IDENTIFICATION OF MICROORGANISMS MISIDENTIFIED BY CONVENTIONAL METHODS:

Full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant microorganisms. Because phenotypic identification suggested unusual organisms not typically associated with the submitted clinical diagnosis.16S rRNA gene sequencing is a more objective identification tool, unaffected by phenotypic variation or technologist bias, and has the potential to reduce laboratory errors. Most clinical laboratories rely on manual, automated, or semi-automated phenotypic methods and commercial systems for identification of bacterial pathogens.Phenotypic profiles including Gram stain results, colony morphologies, growth requirements, and enzymatic and/or metabolic activities are generated, but these characteristics are not static and can change with stress or evolution. Technologist bias or inexperience with an unusual phenotype or isolate may similarly compromise identification when results of biochemical tests are interpreted to fit expectations. Although not perfect, genotypic identification of microorganisms by 16S rRNA gene sequencing has emerged as a more objective, accurate, and reliable method for bacterial identification, with the added capability of defining taxonomical relationships among bacteria. The initial identification by phenotypic methods was erroneous and genotypic identification by 16S rRNA gene sequencing provided clinicians with a more accurate and meaningful result.

CLASSIFICATION OF MICROORGANISMS BY ANALYSIS OF CHEMICAL COMPOSITION:

The feasibility of utilizing gas chromatography as a sensitive and rapid method for the analysis of lipids as a natural basis for the classification of microorganisms by chemical composition was investigated. The lipids were extracted and trans-esterified to component carboxylic acid methyl esters in a single step, after which , the methyl esters were resolved by gas chromatography to provide distinctive chromatographic elution patterns. Similarities in the lipid carboxylic acid distribution were noted among selected species of the family Enterobacteriaceae, and significant differences were noted among selected families of the class Schizomycetes. Methods for the classification of microorganisms are based upon an analysis of their physical and biochemical characteristics. Although physical characterization has been based largely upon factors, which do not change appreciably upon the death of the organism, this is not true of chemical characterization methods. The chemical methods presently in use (with the exception of staining techniques) require living organisms because products of metabolism, enzymatic or antigenic reactions, or chemically induced changes in growth rate or morphology are analyzed. Wolochow (1959) suggested that microorganisms (both living and nonliving) could be differentiated from higher forms of life on the basis of chemical compounds, which are unique to microorganisms. Extension of this concept to provide for both quantitative and qualitative analysis of several selected compounds could reasonably result in a method capable of differentiating between individual species. The similarities as well as the differences in chemical composition would provide a better theoretical basis for classification than is presently available, because differences in chemical composition would be governed by natural or evolutionary relationships, provided the bacteria are grown under defined conditions. For characterization of chemical composition to be practical as a method for classification, extremely selective, sensitive, and rapid methods would have to be used. To complement present methods, the analysis should, at a minimum, be of such sensitivity that a single normal-sized colony of microorganisms (such as those grown on Millipore filters) would be sufficient. At present, the only analytical method which appears to have the degree of sensitivity, rapidity, and selectivity required for such analyses is gas chromatography.

SAMPLE COLLECTION:

Discarded bandages from infected burn wounds were collected from Osmania General Hospital into polythene bags wearing hand gloves and immediately transferred to the Laboratory.

SERIAL DILUTIONS AND PLATING:

Materials required:

- Distilled water
- Sterile test tubes
- Sterile pipettes
- Samples
- Test tube stands

PROCEDURE:

The first step in making a serial dilution is to take a known volume (usually 1ml) of stock and place it into a known volume of distilled water (usually 9ml). This produces 10ml of dilute solution. The dilute solution has 1ml of extract /10ml. This is a 10-fold dilution. The concentration of stock in each ml of the diluted solution is 1ml. The technique used to make a single dilution is repeated sequentially using a more and more dilute solution as the "stock" solution. At each step 1ml of the previous dilution is added to 9ml of distilled water. Each step results in a 10-fold change in the concentration from the previous concentration (10^{-1} to 10^{-8}). O.5 ml from each dilution was inoculated by pour plate technique in nutrient agar medium and all the plates are incubated at 30 ^{0}C for 24 hrs.

MORPHOLOGICAL CHARACTERIZATION:

Among 35 colonies obtained on 10-5 plate by serial dilution and plating, two isolated colonies with different colony morphology were selected and pure cultures of these isolates were obtained by repeated streaking on nutrient agar slants. Finally, pure cultures were stored on nutrient agar slants at 4^0C in a refrigerator. These cultures were first identified by preliminary morphological characterization as mentioned below:

GRAMS STAINING:
Materials required:

Set up a staining area, away from the Bunsen burner, with the following items:

- Crystal Violet stain.
- Gram's stain.
- Saffranin stain.
- Washing bottles with distilled water.
- 95% Ethanol solution in a washing bottle.

PROCEDURE:

- Smear and heat fix a clean microscope slide with bacterial culture.
- Put 10 to 15 drops of Crystal Violet stain on bacterial smear and leave on for one minute.
- Rinse the Crystal Violet stain off with water (from the washing bottle).
- Put the Grams' stain on the smear and leave it on for one minute, then rinse it off with water from the washing bottle.
- Add 10 to 15 drops of 95% ethanol and leave on for 10 to 15 seconds. Rinse off with water for the washing bottle.
- Place the Saffranin on the slide and leave on for 45 seconds. Rinse off with water from the washing bottle. Add a cover slip.
- Look under the microscope. Determine whether or not bacterial culture stained positive or negative.

OBSERVATION: Presence of violet color indicates Gram Positive bacteria and presence of pink color indicates Gram Negative bacteria.

ENDOSPORE STAINING:
Materials required:

- Malachite green
- Saffranin

PROCEDURE:

- Prepare smears of organisms to be tested for endospores.
- Heat fixes the smears.
- Cover the smears with a piece of absorbent paper cut to fit the slide and place the slide on a wire gauze on a ring stand.
- Saturate the paper with malachite green and holding the Bunsen burner in the hand heat the slide until steam can be seen rising from the surface.
- Remove the heat and reheat the slide as needed to keep the slide steaming for about three minutes. As the paper begins to dry add a drop or two of malachite green to keep it moist, but don't add so much at one time that the temperature appreciably reduced. DO NOT OVERHEAT. The process is steaming and not baking.
- Remove the paper with tweezers and rinse the slide thoroughly with tap water.
- Drain the slide and counter stain 45 seconds with 0.5% Saffranin.
- Wash, blot, and examine.

The appearance of vegetative cells in red color and spores in green color indicates positive test otherwise negative.

MORPHOLOGICAL CHARACTERIZATION:

GRAMS STAINING:

Presence of violet color indicates Gram Positive bacteria and presence of pink color indicates gram negative bacteria.

RESULT: Violet color colonies were observed which indicates GRAM POSITIVE BACTERIA.

ENDOSPORE STAINING:

The appearance of vegetative cells in red color and spores in green color indicates positive test otherwise negative.

RESULT: No spore formation.

CAPSULE STAINING:

White capsule was observed in a black background.

RESULT: The Organism Was Found To Contain A Capsule.

MOTILITY TEST: (Hanging drop method)

If motility is seen on the disc slide the test is Positive otherwise the test is Negative.

RESULT:

Non-Motility Of The Microorganisms Was Observed. Hence the Test Is Negative.

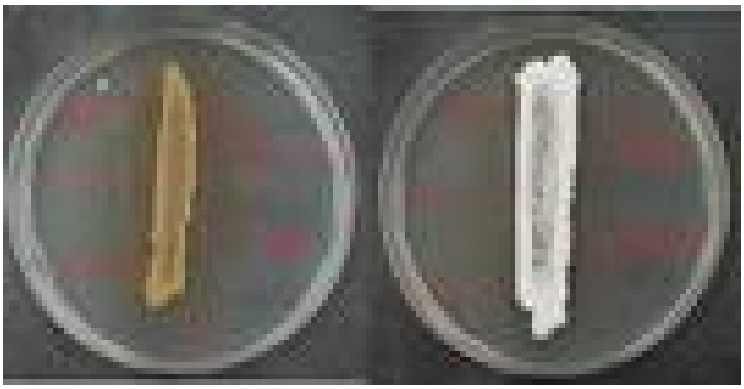

Figure 2: Non-Motility of the Microorganisms

S.NO	TEST	RESULT
1	GRAM'S STAINING	POSITIVE
2	ENDOSPORE STAINING	NEGATIVE
3	CAPSULE STAINING	POSITIVE
4	MOTILITY	NEGATIVE

Table 1: Summary of the Results on Morphological Tests":

BIOCHEMICAL TESTS:

IMVIC TESTS:

INDOLE PRODUCTION TEST:

Appearance of red color ring near the surface indicates Positive test and yellow color ring indicates Negative test.

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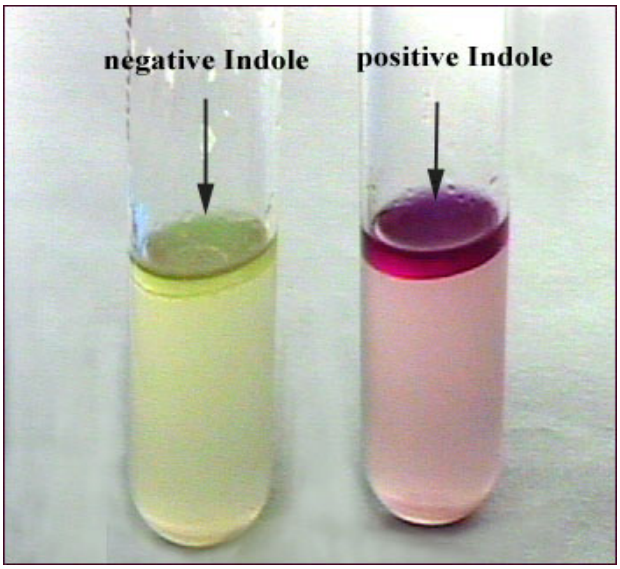


Figure 3: INDOLE PRODUCTION TEST

RESULT: Red color ring was not observed. Hence the test is NEGATIVE.

METHYL RED TEST:

OBSERVATION: Appearance Of Red Color Indicates Positive Test And Yellow Color Indicates Negative Test.

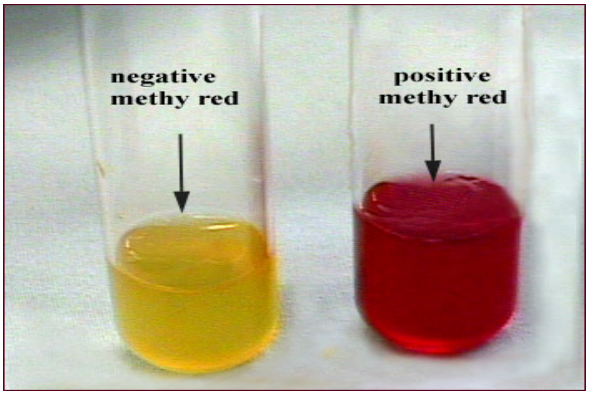


Figure 4: METHYL RED TEST

RESULT: Red color is observed. Hence the test is POSITIVE

VOGES-PROSKAUER TEST:
Color change from pink to crimson indicates Positive test and colorless indicates Negative test.

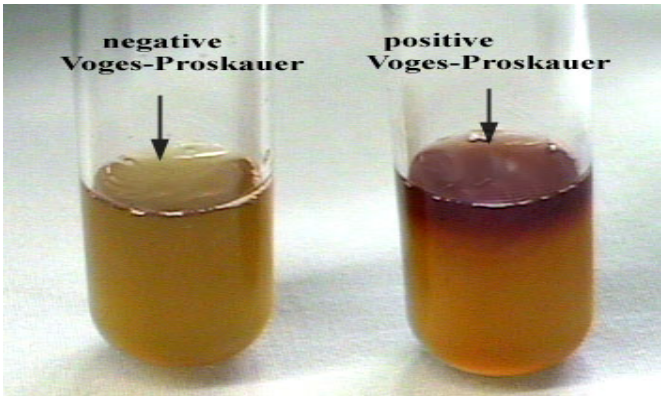


Figure 5: VOGES-PROSKAUER TEST

RESULT: No Color Was Observed. Hence the Test Is NEGATIVE.

CITRATE UTILIZATION TEST:

Change of color to blue indicates Positive test and no color change indicates Negative test.

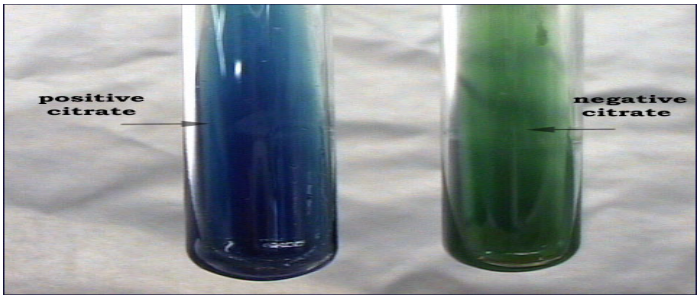


Figure 6: CITRATE UTILIZATION TEST

RESULT: The color did not chang from green to blue. Hence the test is NEGATIVE.

HYDROGEN SULFIDE (H_2S) PRODUCTION TEST:

Appearance of black color indicates Positive test and colorless indicates Negative test.

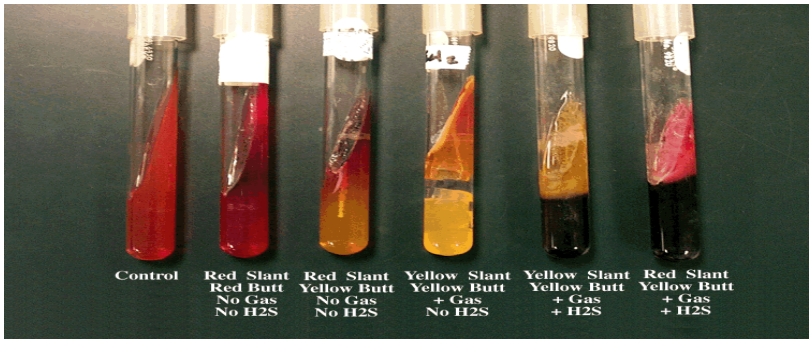


Figure 7: HYDROGEN SULFIDE (H_2S) PRODUCTION TEST

RESULT: Black color was NOT observed. Hence the test is NEGATIVE.

CARBOHYDRATE FERMENTATION

Appearance of air bubbles and yellow color indicates both acid and gas production (Positive test). And absence of air bubbles and appearance of purple color indicates no fermentation and no gas production (Negative test).

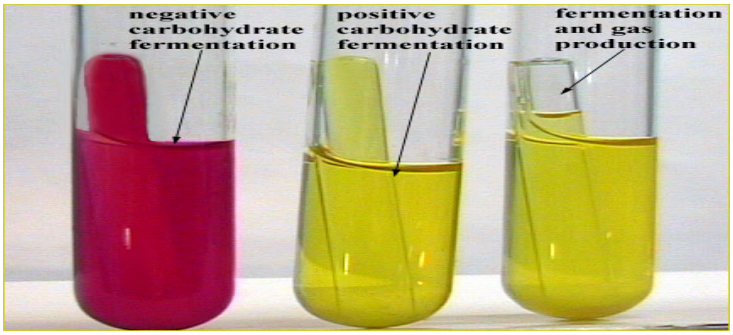


Figure 8: CARBOHYDRATE FERMENTATION

RESULT: Air bubbles were observed which indicates gas production and yellow color was observed which indicates acid production. Hence the test is POSITIVE.

NITRATE REDUCTION TEST:

Presence of Red Color indicates Positive test and absence of color indicates Negative test.

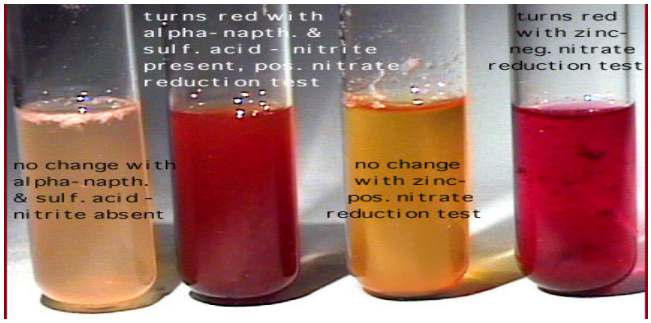


Figure 9: Nitrate Reduction Test

RESULT: Red color was observed. Hence the test is POSITIVE.

CATALASE TEST:

Presence of air bubbles indicates Positive test and absence of air bubbles Indicates Negative test.

Figure 10: CATALASE TEST

RESULT: Air bubbles were observed within 20 seconds. Hence test is POSITIVE.

UREASE TEST:

Appearance of pink color indicates Positive test and pale yellow color indicates Negative test.

Figure 11: UREASE TEST

RESULT: Pink color was not observed. Hence test is NEGATIVE.

SUMMARY OF THE RESULTS ON BIOCHEMICAL TESTS:

S.NO	TEST	RESULT
1	INDOLE PRODUCTION TEST	*NEGATIVE*
2	METHYL RED TEST	POSITIVE
3	VOGES-PROSKAURE	NEGATIVE
4	CITRATE UTILIZATION TEST	NEGATIVE
5	*H_2S PRODUCTION*	NEGATIVE
6	CHO FERMENTATION	POSITIVE
7	NITRATE REDUCTION	POSITIVE
8	CATALASE TEST	POSITIVE
9	UREASE TEST	POSITIVE

Table 2: Summary of the Results on Biochemical Tests

RESULT: The organism isolated from burn wounds was identified as staphylococcus aureus from morphological and biochemical tests according to bergey's manual.

MOLECULAR TESTS:

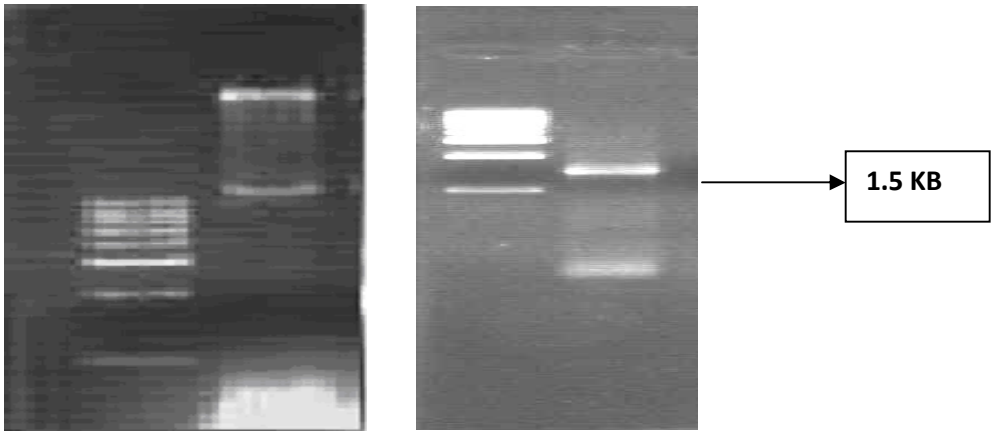
ISOLATION OF BACTERIAL GENOMIC DNA:

OBSERVATION: After running the PCR, we got the product near 1.5kbp region. That is, the fragment size after amplification is found to be1.5kbp.

RESULTS:

Genomic DNA 16s rRNA gene

ladder sample DNA ladder Sample



DNA ladder is of the size 1.0KB.
The arrow (⟶) indicates 1.5 KB, which represents 16S rRNA Gene in the Microorganism.

INSILICO STUDIES

MATERIALS

Bioinformatics is seen as an emerging field with the potential to significantly improve how drugs are discovered, brought to the clinical trials and eventually released to the marketplace. Computer – Aided Drug Design (CADD) is a specialized discipline that uses computational methods to simulate drug – receptor interactions. CADD methods are heavily dependent on bioinformatics tools, applications and databases.

DATABASES

Biological databases are libraries of life sciences information, collected from scientific experiments, published literature, high throughput experiment technology, and computational analyses. They contain information from research areas including genomics, proteomics, metabolomics, microarray gene expression, and phylogenetics. Information contained in biological databases includes gene function, structure, localization (both cellular and chromosomal), clinical effects of mutations as well as similarities of biological sequences and structures. Biological database design, development, and long-term management are a core area of the discipline of Bioinformatics [Bourne P; 2005]. Data contents include gene sequences, textual descriptions, attributes and ontology classifications, citations, and tabular data. These are often described as semi-structured data, and can be represented as tables, key delimited records, and XML structures. Cross-references among databases are common, using database accession numbers.

SWISS-PROT

Swiss-Prot is a manually curated biological database of protein sequences. Swiss-Prot was created in 1986 by Amos Bairoch during his PhD and developed by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute. Swiss-Prot strives to provide reliable protein sequences associated with a high level of annotation (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases. In 2002, the UniProt consortium was created: it is collaboration between the Swiss Institute of Bioinformatics, the European Bioinfomatics Institute and the Protein Information Resource (PIR), funded by the National Institutes of Health. Swiss-Prot and its automatically curated supplement TrEMBL, have joined with the Protein Information Resource protein database to produce the UniProt Knowledgebase, the world's most comprehensive catalogue of information on proteins. As of 3 April 2007, UniProtKB/Swiss-Prot release 52.2 contains 263,525 entries. As of 3 April 2007, the UniProtKB/TrEMBL release 35.2 contains 4,232,122 entries [Apweiler, R et al 2004].

Methodology:

The initial model of 16s RNA was built by using homology-modeling methods and the MODELLER software. The sequence of 16s RNA was obtained from Uniprot. The query sequence from *Staphylococcus aureus* was submitted to domain fishing server for 16s RNA domain prediction. The predicted domain *was* searched to find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) program against PDB (Protein Data bank). Sequence that showed maximum identity with high score and less e-value was aligned and was used as a reference structure to build a 3D model for 16s RNA. The co-ordinates for the structurally conserved regions (SCRs) for 16s RNA were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm. Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved. The final structure obtained was analyzed by Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) and environment profile using ERRAT graph (Structure Evaluation server). This model was used for the identification of active site and for docking of the substrate with the enzyme.

RAMCHANDRAN PLOT ANALYSIS

Ramachandran plot (also known as a Ramachandran map or a Ramachandran diagram), developed by Gopalasamudram Narayana Ramachandran, is a way to visualize dihedral angles φ against ψ of amino acid residues in protein structure. It shows the possible conformations of φ and ψ angles for a polypeptide. The domain of this function is the torus. Hence, the conventional Ramachandran plot is a projection of the torus on the plane, resulting in a distorted view and the presence of discontinuities. One would expect that larger side chains would result in more restrictions and consequently a smaller allowable region in the Ramachandran plot. In practice this does not appear the case; only the methylene group at the β position has an influence. Glycine has a hydrogen atom, with a smaller van der Waals radius, instead of a methyl group at the β position. Hence it is least restricted and this is apparent in the Ramachandran plot for Glycine for which the allowable area is considerably larger. In contrast, the Ramachandran plot for proline shows only a very limited number of possible combinations of ψ and φ.

MOLECULAR DYNAMICS

Molecular dynamics (**MD**) is a form of computer simulation in which atoms and molecules are allowed to interact for a period of time by approximations of known physics, giving a view of the motion of the particles. This kind of simulation is frequently used in the study of proteins and biomolecules, as well as in materials science. It is tempting, though not entirely accurate, to describe the technique as a "virtual microscope" with high temporal and spatial resolution. Whereas it is possible to take "still snapshots" of crystal structures and probe features of the motion of molecules through NMR, no experiment allows access to all the time scales of motion with atomic resolution. Richard Feynman once said that "If we were to name the most powerful assumption of all, which leads one on and on in an attempt to understand life, it is that all things are made of atoms, and that everything that living things do can be understood in terms of the jigglings and wigglings of atoms." Molecular dynamics lets scientists peer into the motion of individual atoms in a way which is not possible in laboratory experiments. Molecular dynamics is a specialized discipline of molecular modeling and computer simulation based on statistical mechanics; the main justification of the MD method is that statistical ensemble averages are equal to time averages of the system, known as the ergodic hypothesis. MD has also been termed "statistical mechanics by numbers" and "Laplace's vision of Newtonian mechanics" of predicting the future by animating nature's forces and allowing insight into molecular motion on an atomic scale. However, long MD simulations are mathematically ill-conditioned, generating cumulative errors in numerical integration that can be minimized with proper selection of algorithms and parameters, but not eliminated entirely. Furthermore, current potential functions are, in many cases, not sufficiently accurate to reproduce the dynamics of molecular systems, so the much more computationally demanding Ab Initio Molecular Dynamics method must be used. Nevertheless, molecular dynamics techniques allow detailed time and space resolution into representative behavior in phase space for carefully selected systems. Before it became possible to simulate molecular dynamics with computers, some undertook the hard work of trying it with physical models such as macroscopic

spheres. The idea was to arrange them to replicate the properties of a liquid. J.D. Bernal said, in 1962: "... I took a number of rubber balls and stuck them together with rods of a selection of different lengths ranging from 2.75 to 4 inches. I tried to do this in the first place as casually as possible, working in my own office, being interrupted every five minutes or so and not remembering what I had done before the interruption." Fortunately, now computers keep track of bonds during a simulation. Because molecular systems generally consist of a vast number of particles, it is in general impossible to find the properties of such complex systems analytically. When the number of particles interacting is higher than two, the result is chaotic motion (see n-body problem). MD simulation circumvents the analytical intractability by using numerical methods. It represents an interface between laboratory experiments and theory, and can be understood as a "virtual experiment". MD probes the relationship between molecular structure, movement and function. Molecular dynamics is a multidisciplinary method. Its laws and theories stem from mathematics, physics, and chemistry, and it employs algorithms from computer science and information theory. It was originally conceived within theoretical physics in the late 1950s and early 1960s, but is applied today mostly in materials science and the modeling of biomolecules.

Energy minimization

Deviations in the protein structure geometry, which have been introduced by the modeling algorithm when joining rigid fragments are regularized in the last modeling step by steepest descent energy minimization using the GROMOS96 force field. Empirical force fields are useful to detect parts of the model with conformational errors. In our own experience and the work of others, energy minimization or molecular dynamics methods are in general not able to improve the accuracy of the models, and are used in SWISS-MODEL only to regularize the structure. However, the successful application of restricted molecular dynamics for improving homology models has recently been reported for a few test cases. To derive more general rules of engagement of molecular dynamics, further systematic experiments have to be conducted.

The four modeling steps:

1. Template superposition,
2. Target-template alignment,
3. Model building
4. Energy minimization have to be implemented in the program ProModII in ANCI.

Phylogenetics studies

Reference proteins of well-established molecular function, representing each of the 16s RNA families investigated, were chosen as query sequences for searches in the *Staphylococcus aureus* genome databases. Searches were made using the TBLASTN tool against Gen Bank database non-redundant (NR), with search specifications. The other databases used were SWISSPROT and Universal Protein resource Uniprot (http://www.ebi.uniprot.org/uniprot-srv/protein/uniProtView). The BLAST server used was that of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov/BLAST/). As selection criteria of BLAST hits for genomic sequences, a cut off e-value of e- 10 was previously set. The genomic sequences found were used to predict putative genes contained within them. Whenever possible, genes were predicted on the basis of sequences generated by the Staphylococcus aureus Genome database, since these sequences present a higher degree of accuracy. To that end, a mixed procedure was adopted combining initio gene prediction algorithms of genomic sequence alignments with similar sequences from expressed genes (ESTs and cDNAs). The prediction algorithms were GenScan. Such expressed sequences were found by BLAST searches against EST and NR databases of Gen Bank, using the genomic sequence as query. The algorithm of choice for the multiple alignments of protein sequences was ClustalX1.8, available through the BCM Search Launcher server (http://searchlauncher.bcm.tmc.edu/multi-align/multialign. html). The multiple alignments were edited with the help of GENEDOC (Free Software Foundation Inc.). All the genes with greater than 30% identity, with at least one of the reference proteins used in the searches, were regarded as functionally similar (homologous) to the reference proteins, receiving the same name. Those sequences that did not conform to this criterion were discarded. Prediction of homology and signature sequences for the putative transporter proteins were carried out with PROSITE and Pfam databases. Sequences were included into families based on homology and

presence of signature sequences. Protein alignments obtained with ClustalX 1.8 were used as starting points for phylogenetic analysis. Unrooted trees were prepared by the neighbor-joining method using either Clustal, PHYLIP, or and 1000 bootstrap replicates were performed. Bold lines on trees indicate protein sequences that were confirmed by cDNA sequencing. In this work, we aimed to reveal phylogenetic distances across the species using experimental values, rather than sequence information in the graphs. Hence we used the data of 16sRNA experimental values. In the relation network, enzymes and genes are represented as nodes, while the substrate and product compounds are represented as edges. The related structural information from the graphs was used for computing phylogenetic distances.

Results and Discussion

Homology Modeling of Ribosomal RNA small subunit methyltransferase A The amino acid sequence of Ribosomal RNA small subunit methyltransferase A was obtained from the protein sequence databank in the Uniprot_KB

QUERY SEQUENCE OF Ribosomal RNA Small Subunit Methyltransferase A

>sp|Q932G1|RSMA_STAAM Ribosomal RNA small subunit methyltransferase A OS=Staphylococcus aureus (strain Mu50 / ATCC 700699) GN=rsmA PE=3 SV=3

MLDNKDIATPSRTRALLDKYGFNFKKSLGQNF
LIDVNIINNIIDASDIDAQTGVIEIGPG
MGSLTEQLARHAKRVLAFEIDQRLIPVLNDTLS
PYDNVTVINEDILKANIKEAVENHLQD
CEKIMVVANLPYYITTPILLNLMQQDIPIDGYV
VMMQKEVGERLNAEVGSKAYGSLSIVV
QYYTETSKVLTVPKSVFMPPPNVDSIVVKLMQ
RTEPLVTVDNEEAFFKLAKAAFAQRRKT
INNNYQNYFKDGKQHKEVILQWLEQAGIDPRR
RGETLSIQDFAKLYEEKKKFPQLEN

Template selection is a process of identifying a suitable protein which shares nearly the same structure of the query protein which doesn't possess the 3D structure. Template selection is very important in comparative protein modeling. Templates can be chosen by various tools such as BLAST, FASTA, Swiss-model, etc. In the case of Blast and Fasta the sequence of protein in fasta format can be uploaded and the templates can be manually selected by considering the score value and the E value. In the case of Swiss-Model server, it automatically chooses the template and models the protein structure.

BLAST SEARCH:

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one-reference protein 1CKJ has a high level of sequence identity and the identity of the reference protein with the **Ribosomal RNA Small Subunit Methyltransferase A** domain are 70%.

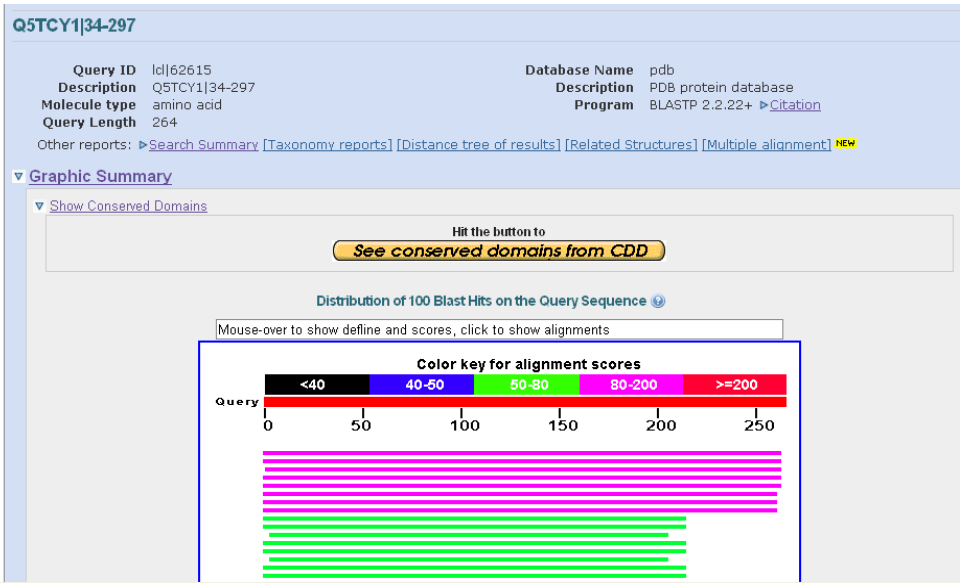


Figure 12: Ribosomal RNA Small Subunit Methyltransferase A

SEQUENCE ALIGNMENT:

In the following study, we have chosen 1CKJ A as a reference structure for modeling **Ribosomal RNA Small Subunit Methyltransferase A**. Coordinates from the reference protein (1CKJ) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. Sequence of the reference structures were extracted from the respective structure files and aligned with the target sequence using the default parameters in ClustalW.

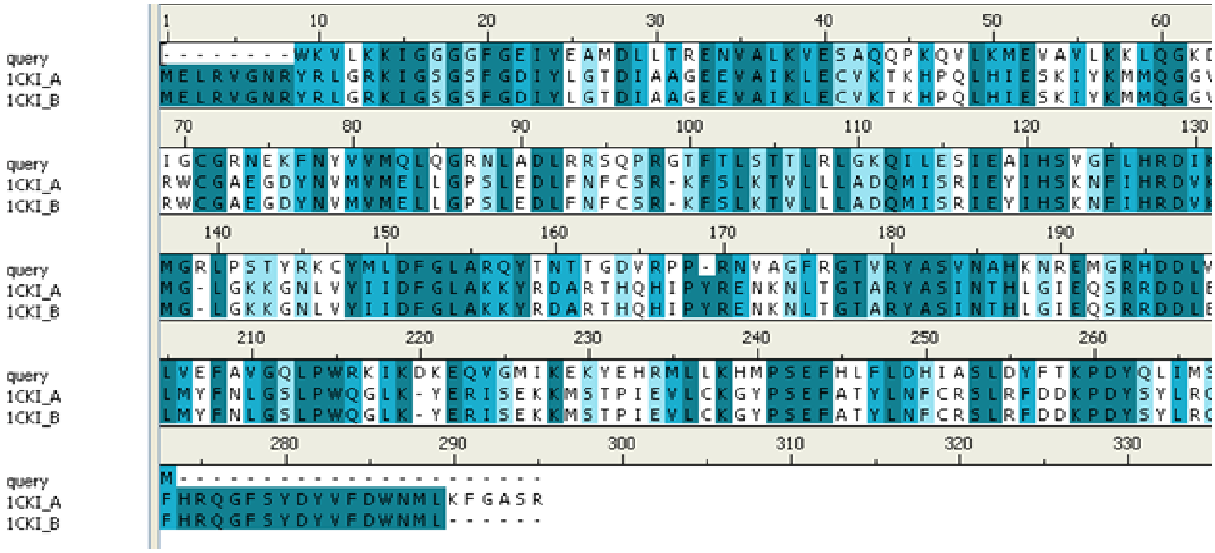


Figure 13: Aligning the query sequence with template

THREE-DIMENSIONAL STRUCTURE OF TAU-PROTEIN KINASE: MOLSOFT

The 1CKJ structure were used as the templates for building the 3D model of the **Ribosomal RNA Small Subunit Methyltransferase A** using Swissmodel.

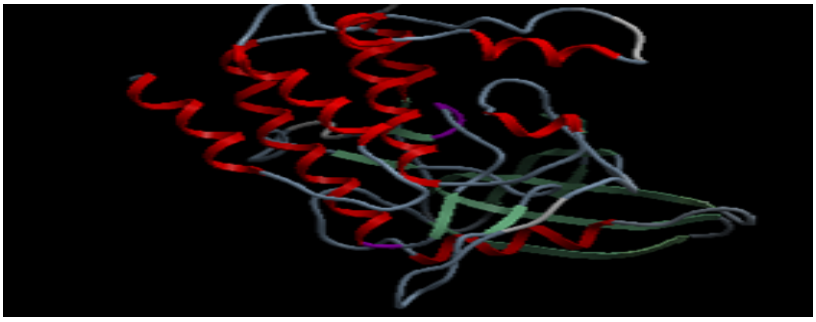


Figure 14: The final stable structure of the **Ribosomal RNA Small Subunit Methyltransferase A** protein

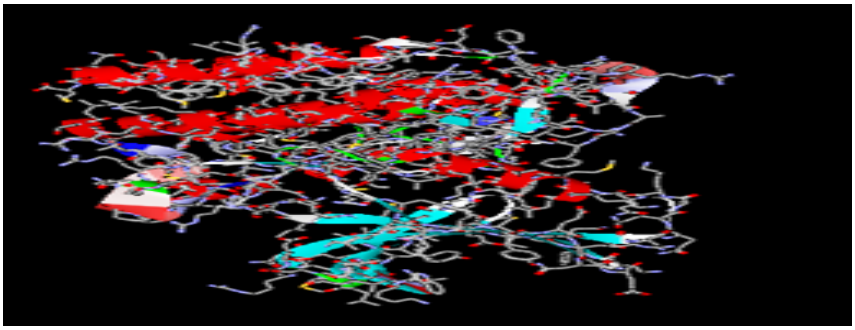


Figure 15: Secondary structure visualization of **Ribosomal RNA Small Subunit Methyltransferase A**

Superimposition Query structure with templates

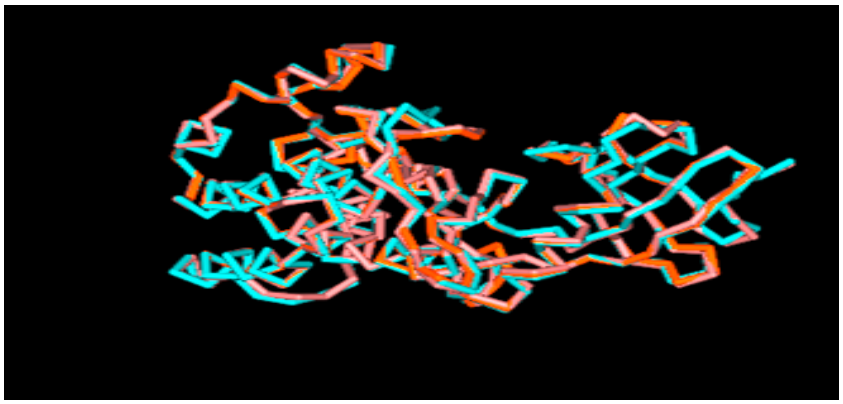
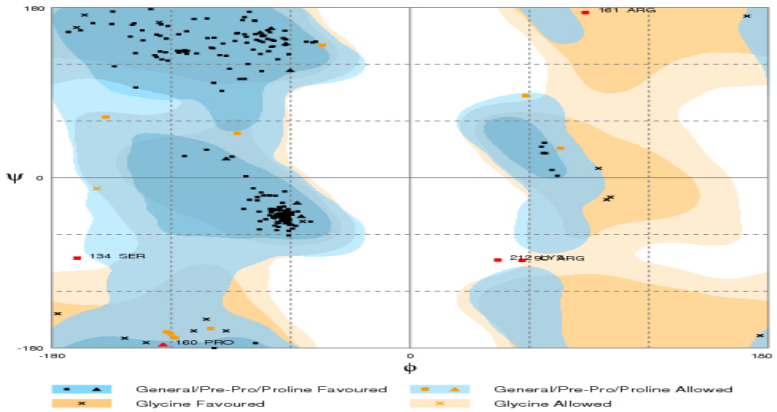


Figure 16: Superimposition of **Ribosomal RNA Small Subunit Methyltransferase A** structure with Template, the RMS deviation is 0.75.

Model Verification: by Ramachandran Plot analysis



Evaluation of residues

Number of residues in favoured region (~98.0% expected) : 247 (94.3%)
Number of residues in allowed region (~2.0% expected) : 10 (3.8%)
Number of residues in outlier region : 5 (1.9%)

Molecular Dynamics Simulation Studies by VMD/NAMD:

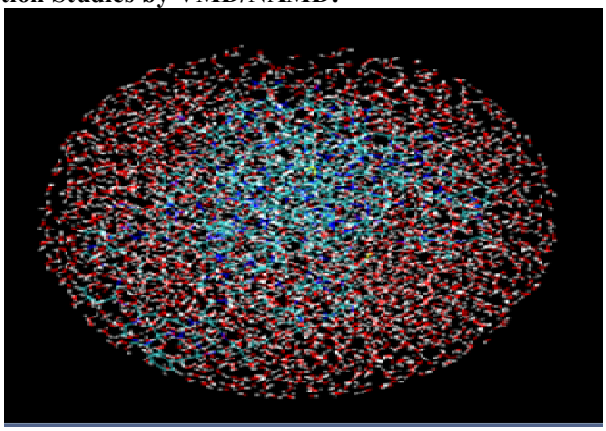


Figure 17: Structure having least energy with low Rmsd which was obtained by NAMD in water molecule (TIP)

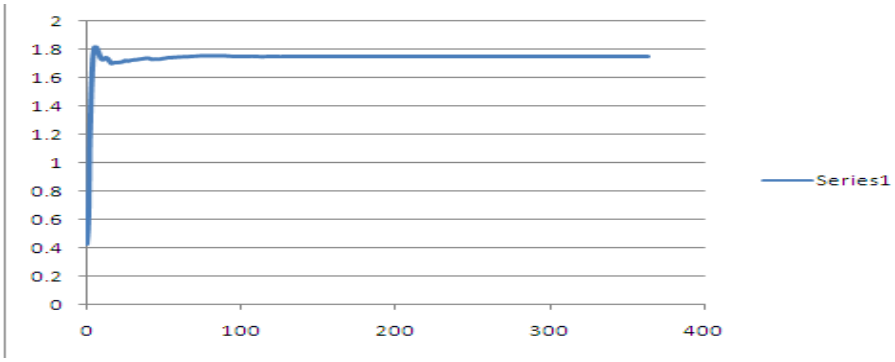


Figure 18: Graphical Representation of RMSD value of Ribosomal RNA Small Subunit Methyltransferase A

Collection of 16sRNA sequence from data base

Using NCBI and UNIPROT, we have collected 16sRNA sequences from *Staphylococcus aureus* different strains for phylogenetic analysis using the algorithm and tree view software's**.** The developed algorithm was used to align the different 16sRNA sequences, and the aligned sequences were converted into phylogenetic tree. With the availability of the data in GenBank, it was possible to construct an overview of 16s RNA in *Staphylococcus aureus*. As a starting point, the protein families in 16s RNA which have positive molecular implications on pain transport, intracellular targeting and storage in *Staphylococcus aureus* strains, were chosen for analysis. Taking specific members of these families as query sequences, searches were carried out for orthologous sequences in GenBank, and Uniprot current databases using TBLASTN. After searching the databanks with TBLASTN sequences, clones having genomic sequences to the related family were taken and converted to amino acid sequences. In each family, similar sequences were removed and the sequences were subjected to PROSITE and Pfam databases to see the presence of signature sequences for the corresponding families. After subjecting the sequences to PROSITE 13 putative genes were predicted in 16s RNA of different strains in Staphyloccus aureus. The percent identity for all the sequences was calculated in each family with the corresponding query sequence using GENEDOC. Phylogenetic analysis of the sequences of transporters revealed that the COX proteins were divergent, showing branches in tree view. The phylogentic analysis shows four branches indicating different transporting function to each family. Some

of the orthologous sequences are available as full-length cDNA clones. The expressed sequence tags were mentioned as accession numbers for the sequences.

SEQUENCE ALIGNMENT

These sequences were aligned using CLUSTALW program. The multiple alignment was used to develop phylogenetic tree.

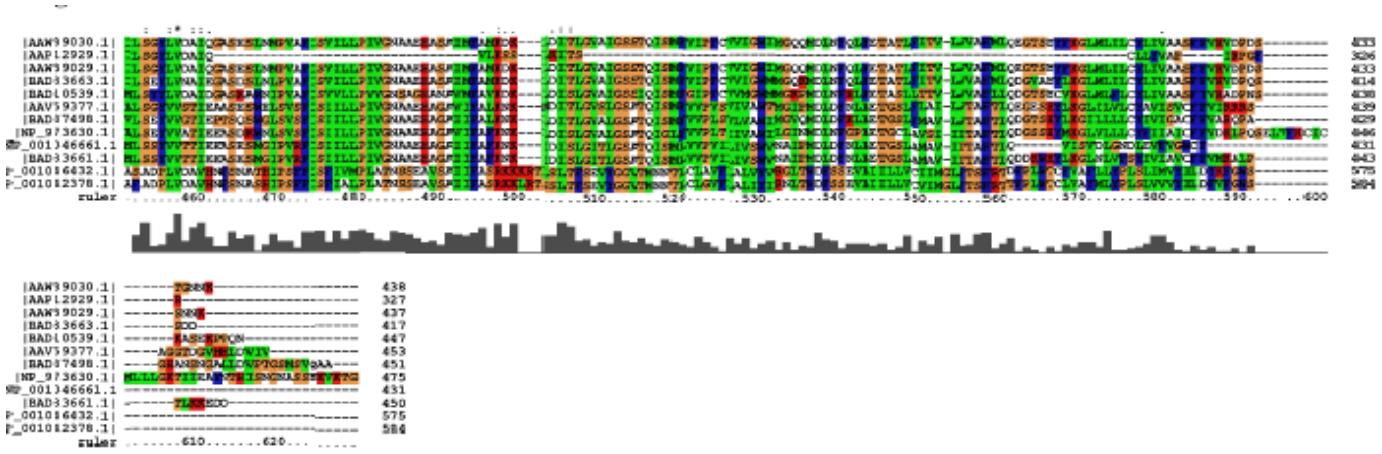


Figure 19: Multiple sequence alignment of 16s RNA of different strains of Staphylococcus aureus

16s RNA family is an important family and these are numbered based on their alignment. The genes, which are showing more than 30% identity with query, are said to be homologous sequences.

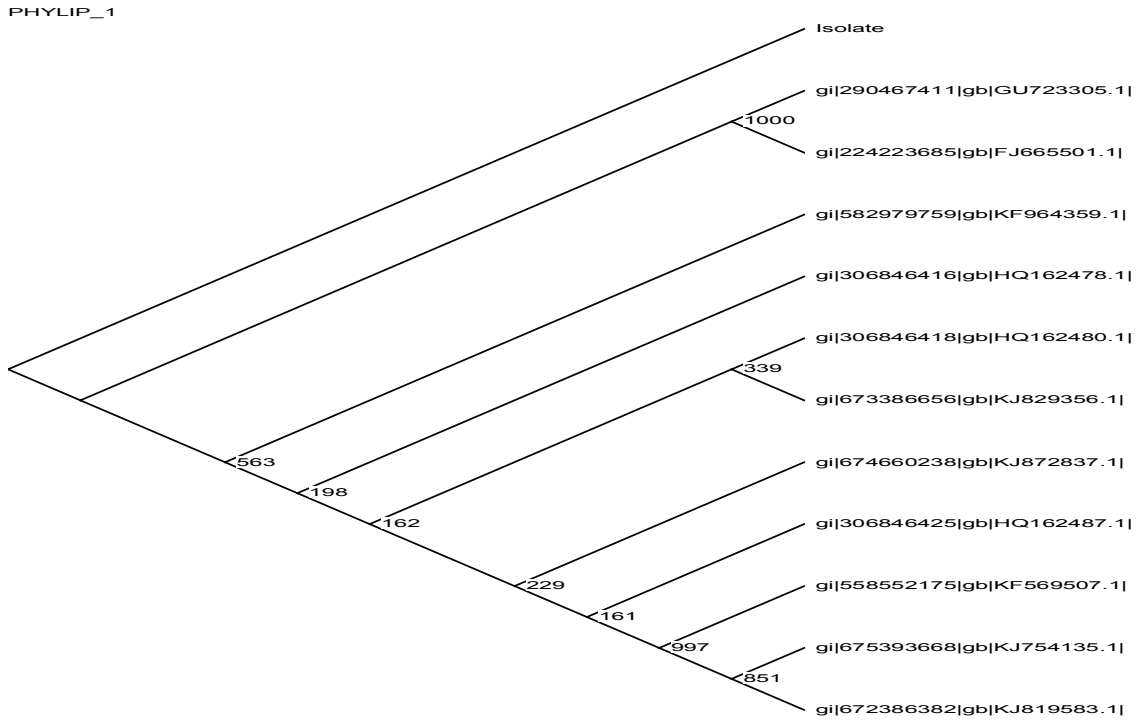


Figure 20: Unrooted Phylogenetic tree of different staphylococcus aureus strains

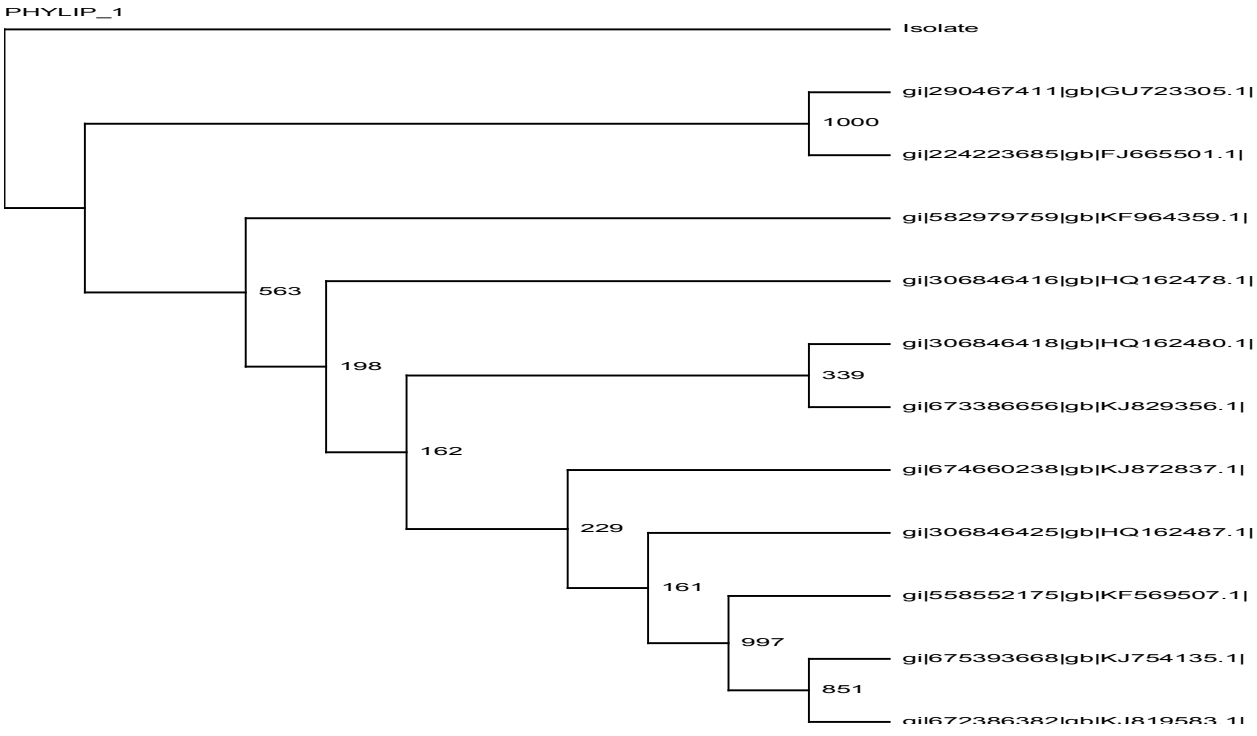


Figure 21: Rooted Phylogenetic Tree with branch length (UPGMA)

DISCUSSION

The organism isolated from infected burn wounds was identified as taphylococcus aureous from morphological and biochemical tests. Staphylococcus aureous was the second most frequent organism isolated in a eight year study of bacterial isolates from burn wound infections in Government Medical College Hospital, Chandigarh. Studies were further extended to amplification of DNA encopding for ribosomal RNA genes (16S rRNA). The 16 S sRNA genes have been the most commonly employed genes for identification purposes in pathogenic bacteria. 16S rRNA genes are highly conserved and are found in all bacteria. Highly variable zones of 16 S rRNA genes sequences provide unique signatures to any bacterium giving useful information about their identity. Molecular detection of pathogenic bacteria have several advantages for their adoption into routine clinican and diagnostic bacteriology. Although perceived to be little expensive , the overall quqlity of the test, and er detection leading to early institution of therapy may overweigh the cost factor.

CONCLUSIONS

Molecular Diagnostic Techniques are expected to play a significant role in clinical and diagnostic bacteriology. Although their adoption may never replace the conventional methods their efficiency, quality, quickness and their role in the detection of slow growing organisms cannot be overlooked. Infection control programs need to document and report burn wound infections according to the recently established definitions of the classification system. Future studies of burn wound infections should use this standardized burn wound classification system so that clinical outcomes can be compared for burn patients with a specific condition (e.g., burn wound cellulitis). More research is required to determine the best methods for sampling excised and unexcised burn wound areas over the course of a severe deep partial-thickness and/or full-thickness injury. Reproducible standardized methods should be developed so that clinical microbiology laboratories can routinely test burn wound bacterial isolates for susceptibility to the topical antimicrobial agents on formulary at a particular burn center. A rotation program for topical antimicrobial use may also retard the development of resistance. Laboratory surveillance should include the reporting of burn unit-specific antibiograms for topical antimicrobial agents once standardized methods are available for performing susceptibility testing. In this work, we have analyzed the phylogenetic relationships of 16s RNA sequences of different Staphylococcus aureus strains. This analysis has focused on 16s RNA families for which initial characterizations have been achieved for individual members. Phylogenetic trees of each family define the evolutionary relationships of the members to each other. These families contain numerous members, indicating diverse functions in vivo. Closely related isoforms and separate subfamilies exist within many of these gene families, indicating possible redundancies and specialized functions. To facilitate their further study, this includes alignment of the analyzed genes.

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