

Isolation and Molecular Identification of *Salmonella Typhi* Using Polymerase Chain Reaction (PCR) Technique

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ABSTRACT

Salmonellosis is a disease condition caused by a large group of bacteria of the genus Salmonella that can affect human being throughout the world. Salmonella infection remains as a serious problem to public health and causes substantial economic losses resulting from mortality, morbidity. Food poisoning with gastroenteritis to human represents a serious problem for the food industry. Traditional methods of identification of Salmonella typhi and its related non-typhoidal food borne pathogens organisms are laborious and time consuming. So there is an immediate need for new, innovative, faster and reliable methods for identification of Salmonella Typhi. Recent developments in the fields of Molecular Biology, have paved the way for such methods and have revolutionized the detection of pathogenic microbes at molecular level. Discovery of Polymerase Chain Reaction Technology was a major step in this direction. The present study aimed at characterization of the isolated Salmonella serovars using Morphological, Biochemical, and Molecular Techniques using Polymerase Chain Reaction Technology. The isolated organism was identified as belonging to Salmonella species by Morphological and Biochemical Tests and confirmed by PCR amplification and matching with specific primers for the species. The results of Agarose Gel Electrophoresis revealed a band of 420bp with HisJ and 466bp with Vi LPS.

INTRODUCTION

Salmonellosis is a disease condition caused by a large group of bacteria of the genus Salmonella that can affect human being throughout the world. Salmonella infection remains as a serious problem to public health significance in world wide and causes substantial economic loss resulting from mortality, morbidity and poor growth with hazard of

transmitting food poisoning with gastroenteritis to human and represents a serious problem for the food industry. Human spreads Salmonella mainly through the stool. Food borne illness among the people and transmission can occur when food and water are contaminated with stool or through direct fecal-oral route. Human stool acts as an important reservoir of Salmonella serovars that are the grouping of micro organisms based on their cell surface antigen. Species isolated from human stool are Salmonella typhi, S. paratyphi A, S. typhimurium, S. wrothington and S. enteritidis. The importance of Salmonellosis in public health sector is a growing concern day by day throughout the world and over the last several decades there have been significant Shift in predominant Salmonella serovars associated with human infections. Salmonellosis in the past has caused tremendous loss to society in many countries around the world. Two to four million of cases have been reported annually and yet a significant number of cases have been unreported worldwide. Non-typhoidal Salmonella is the leading cause of food borne illness and its increasing antimicrobial resistance is associated with higher risks of hospitalization in Bangladesh (ICDDR,B). Non-typhi Salmonella was found responsible for 66% cases of food borne illness in Bangladesh. The highest proportion (15%) was isolated in 1998 followed by in 1995 (13%) while it was less than 10% for other years. Thirty six percent were isolated during the summer while 28% were in the fall. There is lack of sufficient studies emphasizing isolation and characterization of Salmonella serovars considering human stool in Bangladesh. Moreover, knowledge of manifestation of pathogenicity as well as antigenic mosaic of these species would certainly help in suggesting prophylactic measures thereof. This, in turn, will go a long way to avoid much of our loss and inconvenience concerned in public health.

Sortase enzyme catalyzes transpeptidation reactions on the bacterial surface, utilizing protein

precursors with C-terminal sorting signals as substrates. Staphylococcus aureus sortase A (SrtA), the prototypic transpeptidase of this class of enzyme, cleaves LPXTG motif-type sorting signals between the threonine (T) and the glycine (G) residues to generate an acyl enzyme intermediate. Nucleophilic attack of the amino group of cell wall crossbridges resolves the acyl enzyme, forming an amide bond between the carboxyl group of the C-terminal threonine of surface proteins and the cell wall crossbridge of lipid II precursor molecules. The product of this reaction, surface protein linked to lipid II, is then incorporated into the cell wall envelope via the transpeptidation and transglycosylation reactions of peptidoglycan biosynthesis. Twenty different surface proteins with LPXTG motif-type sorting signals have been identified in the staphylococcal genome sequence, and deletion of the *srtA* gene abolishes the cell wall anchoring and surface display of all sortase substrates. As a result, staphylococcal *srtA* mutants display significant defects in the pathogenesis of murine organ abscesses, infectious arthritis, or endocarditis. Perhaps the most astonishing sortase-catalyzed reaction is the assembly of pili on the surface of corynebacteria, actinomycetes, enterococci, group B streptococci, and pneumococci. For example, corynebacterial sortases cleave precursor proteins in a manner that leads to the assembly of pili, high-molecular-weight polymerization products several microns long on the bacterial surface. Two domains of pilus surface proteins, the sorting signal and the pilin motif, are required for this reaction, which occurs in a sortase-specific manner.

CULTURAL CHARACTERISTICS AND MORPHOLOGY:

Salmonella enterica serovar Typhi is a gram negative bacterium, facultative anaerobic and flagellated bacilli from the family of Enterobacteriaceae). Salmonella Typhi measures 2-4 μm long and 0.6 μm wide. S. Typhi is a non-acid fast and motile bacterium with peritrichous flagella. These organisms are non-spore forming, facultative anaerobic bacilli, which produce acid on glucose fermentation and reduce nitrates. Salmonella Typhi is a non-fastidious organism which can grow in basal media like nutrient agar. On MacConkey agar, these organisms produce colorless colonies since S. Typhi is a non-lactose fermentor. Low selective media such as MacConkey and deoxycholate agar and intermediate selective agar such as Salmonella - Shigella agar are widely used in laboratories to

cultivate the organism. High selective media such as brilliantgreen and bismuth sulfite agar can also be used in the detection of Salmonella Typhi. Strontium selenite and selenite F broth were the enrichment broth for the organism

EPIDEMIOLOGY OF TYPHOID FEVER:

Typhoid fever, also known as enteric fever, is a potentially fatal multisystemic illness caused primarily by Salmonella enterica, subspecies enterica serovar typhi and, to a lesser extent, related serovars paratyphi A, B, and C. The protean manifestations of typhoid fever make this disease a true diagnostic challenge. The classic presentation includes fever, malaise, diffuse abdominal pain, and constipation. Untreated, typhoid fever is a grueling illness that may progress to delirium, obtundation, intestinal hemorrhage, bowel perforation, and death within 1 month of onset. Survivors may be left with long-term or permanent neuropsychiatric complications.

Salmonella typhi has been a major human pathogen for thousands of years, thriving in conditions of poor sanitation, crowding, and social chaos. It may have been responsible for the Great Plague of Athens at the end of the Peloponnesian War. The name S typhi is derived from the ancient Greek typhos, an ethereal smoke or cloud that was believed to cause disease and madness. In the advanced stages of typhoid fever, the patient's level of consciousness is truly clouded. Although antibiotics have markedly reduced the frequency of typhoid fever in the developed world, it remains endemic in developing countries. Salmonella paratyphi causes the same syndrome but appears to be a relative newcomer. It may be taking over the typhi niche, in part, because of immunological naivete among the population and incomplete coverage by vaccines that target typhi. Note that some writers refer to the typhoid and paratyphoid fever as distinct syndromes caused by the typhi versus paratyphi serovars, while others use the term typhoid fever for a disease caused by either one. We use the latter terminology. We refer to these serovars collectively as typhoidal salmonella

TYPHOID FEVER ASIAN SCENARIO:

Asia reports high incidences (>100/ 100 000 cases/year) of typhoid, accounting for almost 80% of world's population (Puran, 2009). It is estimated that in Asia, the crude annual typhoid incidence rate was 274 per 100,000 persons in 2000 (Crump, 2004). Epidemic typhoid is a common disease in most of Africa, Asia and central South America. In the developing countries, usually the modern parts of the

cities and towns are almost free of typhoid, general hospitals derive a large majority of their typhoid patients from slums, peripheral shabby towns, and rural districts where sanitation and the water supply are rudimentary. It is prevalent in developing countries where it remains a major health problem, (Arora et al., 1992) and exists both in endemic and epidemic forms (Saha, 2003).

PATHOGENICITY OF SALMONELLA TYPHI:

Typhoid fever starts as an infection of the gastrointestinal tract and develops into a systemic illness. Only 8-10 organisms/ml can cause severe disease. The bacteria find their way to food, drinks and water through house-flies and other insects. These contaminated food or drinks, when consumed, causes typhoid fever. The bacteria are disseminated by typhoid patients and carriers in large quantities through stools and vomit. Almost all Salmonella Typhi are transmitted through oral ingestion of bacteria. The infective dose of bacteria really depends on the host defenses such as gastric acidity, the inhibitory effects of the normal intestinal flora and the gut peristaltic movements. About 10⁵ to 10⁶ S. Typhi bacteria are required for initial infection. Following ingestion, Salmonella Typhi penetrates the intestinal mucous layer at the distal ileum of the small intestine and the proximal large bowel and become localized in the Peyer's patches. M cells, which are specialized ileal epithelial cells lying at the Peyer's patches, are thought to be the site for penetration of S. Typhi. Following penetration of the mucosa the organisms replicate within the macrophages of the Peyer's patches and spread to the mesenteric lymph nodes.

In the case of gastroenteritis, the bacteria do not further penetrate the gut and the gut-associated lymphoid tissue. However, it is common for S. Typhi to penetrate, and the bacteria may spend four to seven days spreading via thoracic duct to systemic circulation (transient primary bacteremia). The bacteria are removed from blood by macrophages that are located in the sinusoids of the liver, spleen and bone marrow and the bacteria can replicate again and re-enter the blood (second bacteremia). The bacteria removed by the liver may infect the gall bladder, which, in turn, may lead to re-infection of the intestinal tract (second exposure of Peyer's patches) and cause inflammation, ulceration and necrosis. However, the common causes of death in typhoid fever are peritonitis and septicemia that occur in the third week of illness, or the perforation of Peyer's patches. Salmonella Typhi continues to cause severe disease. Its most learned complication being

perforation of ulcerated peyer's patches within the small intestine, leading to peritonitis with associated mortality. The pathogenesis of this process is not well understood.

PHENOTYPIC TYPING METHODS:

The genus Salmonella includes more than 2,400 different serotypes. The Kauffmann-White scheme that is used to serotype salmonellae is based on antigenic polymorphisms of the lipopolysaccharides (LPS) (O) and the flagella (H). A second level of characterization is based on phage typing. By use of 37 different phages, serotype Typhimurium can be divided in more than 210 phage types. Besides serotyping and phage typing, powerful bacterial molecular typing methods, such as plasmid profiling, pulsed-field gel electrophoresis (PFGE), IS200 typing, ribotyping, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism, are used for epidemiological investigation of salmonellae. These techniques are useful for defining clonal relationships between strains and for assessing the distribution of Salmonella strains within food-processing environments. Within Salmonella and especially Salmonella enterica serotype Typhimurium, multiple-antibiotic-resistant strains are isolated with increased frequency. Serotype Typhimurium definitive type 104 (DT104) and serotype Typhimurium DT204b are virulent pathogens for humans and animals, with many strains showing multiple drug resistance characteristics. These multiple-antibiotic-resistant serotype Typhimurium strains cause particular concern because of their increasing prevalence in humans. Most of the strains typically carry resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R type), but resistance to 9 or 10 different antibiotics also occurs. The selective pressure created by widespread use of antimicrobial agents in pigs during rearing may have contributed to the dissemination of these multidrug-resistant bacterial strains. It is well established that the distribution of antimicrobial resistance is often plasmid and/or transposon mediated. Integrons, a novel group of mobile DNA elements, have the potential to incorporate several antibiotic resistance genes by site-specific recombination.

In a previous study the prevalences of salmonellae in several slaughterhouses were determined. Salmonellae were isolated from 37% of the carcass samples collected during eight slaughterhouse visits. Overall 28% of the animals carried salmonellae in the feces and/or mesenteric lymph nodes. Swanenburg found only 1.4% of the

carcasses to be positive whereas 25.6% of the fecal samples were positive. At the slaughterhouse, two major contamination parameters are important: the status of the pigs supplied and slaughterhouse hygiene. Berends et al. and Borch et al. considered infected pigs to be the initial source of carcass contamination (calculated at 70%) and estimated that cross-contamination accounted for 30%. In this study *Salmonella* strains, isolated from pigs and carcasses during a whole slaughtering day and from the slaughterhouse environment, were characterized. By using PFGE (serotype Typhimurium, serotype Ohio, and serotype Derby isolates) and phage typing (serotype Typhimurium isolates), the origin of *Salmonella* contamination in the carcasses and in the slaughterhouse environment was determined. Also, antibiotic resistance profiles (ARPs) for six antibiotics were determined in order to monitor the spread of multidrug-resistant strains in the feces of the pigs, on the carcasses, and in the slaughterhouse environment.

TUBE AGGLUTINATION TEST:

Agglutination reaction performed in tubes with different antigens (O, H, Vi) and their respective antibodies raised, showed variable nature of agglutination reactions. Somatic (O) antigen showed fine agglutination whereas, H and Vi showed coarse and very fine agglutination reactions respectively. Although the mainstay of diagnosing typhoid fever is a positive blood culture, the test is positive in only 40-60% of cases, usually early in the course of the disease. Stool and urine cultures become positive after the first week of infection. In much of the developing world, widespread antibiotic availability and indiscriminate prescribing is another reason for the low sensitivity of blood cultures. Although bone marrow cultures are more sensitive, they are difficult to obtain, relatively invasive, and of little use in public health settings. Other haematological

investigations are non-specific. Blood leukocyte counts are often low in relation to the fever and toxicity, but the range is wide. In younger children leucocytosis is a common association and may reach 20000-25000/mm³. Thrombocytopenia may be a marker of severe illness and accompany disseminated intravascular coagulation. Liver function test results may be imbalanced but significant hepatic dysfunction is rare. The value of the *Salmonella* Typhi agglutination tests has declined as the incidence of typhoid fever has decreased, at least in the West, the general use of vaccines has increased, and ever increasing numbers of antigenically related serotypes of *Salmonella* Typhi have been recognized. Widal test is often used when isolation of *Salmonella* Typhi is not feasible, but produces false negative and false positive reactions and does not provide a definitive diagnosis. The Widal test, which detects agglutinating antibodies to lipopolysaccharide (LPS) (TO) and flagella (TH), was introduced over a century ago and is widely used for the serological diagnosis of typhoid fever. In the original format, the Widal test required acute and convalescent phase serum samples taken approximately 10 days apart. Most recently, the test has been adapted for use with a single, acute phase serum sample. ELISA has been considered an alternative approach for the diagnosis of typhoid fever. For the most part, these assays have been based on the detection of anti-LPS antibodies and have been reported to be more sensitive than the Widal test. Newer diagnostic tests have been developed such as the typhidot or tubex, which directly detect IgM antibodies against a host of specific *S. Typhi* antigens but these have not proved to be sufficiently robust in large scale evaluations in community settings. A nested polymerase chain reaction using H1-d primers has been used to amplify specific genes of *S. Typhi* in the blood of patients and is a promising means of making a rapid diagnosis.

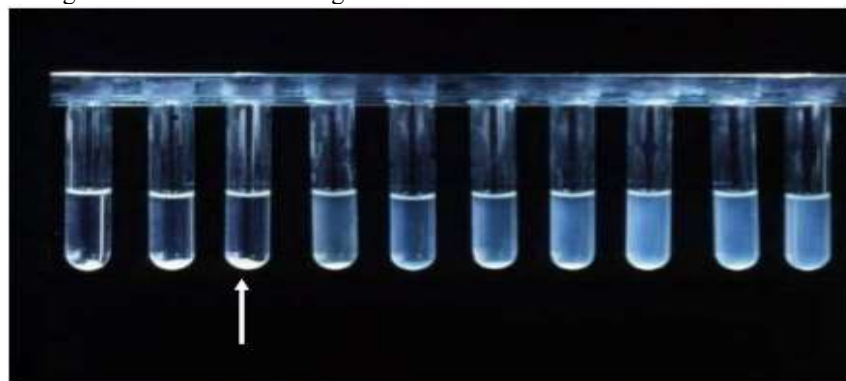


Figure 1: TUBE AGGLUTINATION TEST

ISOLATION OF SALMONELLA TYPHI IN URINE SAMPLE:

Salmonella Typhi can be detected by its antigens present over the surface of the bacteria and by the flagellar H antigen and Vi antigen. A study in Jakarta showed that detection of Salmonella Typhi using group D9 antigen from urine. Since typhoid fever is still a major cause of illness in many developing countries, there is a clear need for a sensitive and specific test that will permit rapid laboratory diagnosis of the disease. A study was carried out both in laboratory and in a clinical situation, for its ability to detect Vi antigen in urine. The work showed that ELISA was capable of detecting as little as 1 ng of purified Vi antigen per ml in urine, compared with 100 ng/ml detectable by co-agglutination method. Another study using Vi antigen in diagnosis of typhoid fever, concluded that using a cut-off value that maximally separated typhoid patients from controls, the ELISA was positive in 62.4% of 141 patients with culture proven

typhoid infections and in 13.2% of 159 afebrile controls. Monovalent antisera coupled to protein A rich staphylococci used in slide coagglutination method for the detection of Salmonella Typhi D, Vi and d antigens in the urine. These antigens were detected in the urine of 59 out of 61 (97%) bacteriologically confirmed typhoid fever. The results suggest that the method of slide coagglutination of urine can be used to screen suspected patient with high degree of reliability. In any infection or disease rapid diagnosis and cost are the main concern. While evaluating 3 immunological kits for the serological diagnosis of typhoid fever in Vietnam it was found that sensitivity and specificity findings were 89 and 53% for multi test dip stick, 79 and 89% for Typhidot, 78 and 89% for TUBEX and 64 and 76% for Widal testing in hospitals and 61% and 100% for Widal testing at the Pasteur Institute. The Widal test was insensitive and displayed inter-operator variability. Two rapid kits Typhidot and TUBEX demonstrated promising results.

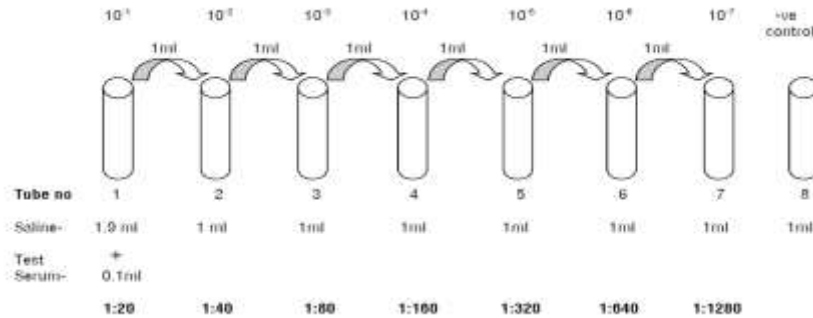


Figure 2: WIDAL TESTING FOR SALEMONELLA

MATERIALS AND METHODS:

SAMPLE COLLECTION AND PROCESSING:

5 ml of patient blood was collected from the suspected Typhoid patient and added to blood culture medium (biphasic tryptic soy agar and brain heart infusion broth). The blood culture bottle was then incubated at 37°C for 24 h. 1 ml of the broth culture was serially diluted with known volume of sterile normal saline usually 9 ml from 10⁻¹ to 10⁻⁸ dilutions. The diluted culture sample was cultured on to nutrient agar plates and incubated at 37°C for 24 hours. The colonies were subcultured on to specific media such as MacConkey agar, Eosin methylene Blue agar and Brilliant green agar plates and slants. The Salmonella Typhi isolates obtained from laboratories in slants were again confirmed in SRU biotechnology by subculture onto MacConkey agar, SS agar and BHI agar and by a series of biochemical tests, as per the recommendations of Murray et al. (2003).

BIOCHEMICAL TESTS:

INDOLE TEST:

Indole tests look for the presence or absence of tryptophanase enzyme production of the bacteria. If the enzyme is present, it will degrade the amino acid tryptophan in the media and will produce Indole, ammonia and pyruvic acid. Indole will react with Kovac's reagent to produce a cherry red complex, which indicates a positive indole test. The absence of red color is indicative of tryptophan hydrolysis due to the lack of tryptophanase enzyme.



Figure 3: INDOLE TEST

3.5.2 METHYL RED TEST:



Figure 4: METHYL RED TEST

VOGES PROSKAUER TEST:

This test determines the ability of microorganism to ferment glucose. The end products of glucose metabolism, pyruvic acid, is further metabolized by using Butylene glucol pathway to produce neutral end such as acetoin and 2, 3 butanediol. When Barrit's reagent A (40% KOH) and Barrit's reagent B (5% solution of alpha naphthol) is added it will detect the presence of acetoin, the precursor in the 2, 3- butanediol synthesis. Acetoin in the presence of Oxygen and Barrit's reagent is oxidized to diacetyl, where alpha naphtholact as a catalyst. Diacetyl then reacts with guanidine components of peptone to produce a cherry red colour .

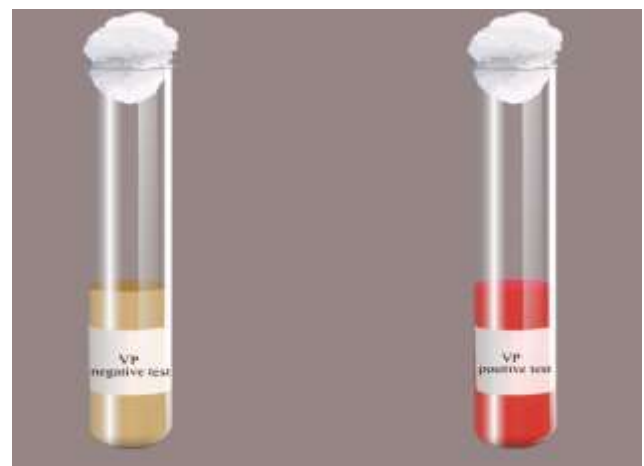


Figure 5: VOGES PROSKAUER TEST

3.5.4 CITRATE UTILIZATION TEST:

This test determines the ability of microorganism to utilize Citrate. Some bacteria have the capability to convert the salts of organic acids, for example, Sodium citrate to alkaline carbonates. Sodium citrate is one of the important metabolite of Krebs's cycle. Certain bacteria use citrate as the sole carbon source. Citrate utilization requires a specific membrane transporter and citrate lyase activity. Citrate is converted to Oxalo acetic acid by citrate lyase and oxaloacetate decarboxylase activity will convert oxaloacetate to pyruvate with the release of carbon dioxide. The other products of the reaction are acetate, Lactic acid, formic acid etc. The carbon dioxide reacts with sodium and water to form sodium carbonate.



Figure 6: CITRATE UTILIZATION TEST

MOLECULAR METHODS:

DNA extraction was conducted from 200 µl of whole blood using the Qiagen DNeasy blood and tissue extraction kit, according to the manufacturer's instructions for extraction from blood samples.

POLYMERASE CHAIN REACTION :

For molecular characterization, bacterial genomic DNA was isolated according to the method of Chen and Kuo with little modifications. PCR amplification of Histidine-binding periplasmic protein gene sequences was carried out using Eppendorph Cycler.

Forward primer (5' GCGCTACCGCAGCATTTGCC 3') and

Reverse primer (5'CTTTCGGCGCCAGTGCTCA 3')

Similarly amplification of Vi exopolysaccharide LPS gene was carried out using

forward primer (5' AGCGCGGCTCACGTA CTCCA 3') and

reverse primer (5'TTCGGGGTTGGA GCTGCTGG 3')

In a reaction volume of 100 µl under the following conditions (initial denaturation 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 90 sec and final extension at 72°C for 7 min). Positive standard strains were included in every reaction of PCRs. A negative control (without DNA template) was included in all the PCR reactions. The PCR products obtained were resolved on 2% agarose gel electrophoresis. Gels were visualized and photographed in UV gel image camera.

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 Bioinformatics 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.

PDB: PDB Founded in 1971 by Drs. Edgar Meyer and Walter Hamilton Brookhaven National Laboratory. This server consists of the 3D structure of the proteins. The Protein Data Bank (PDB) is a repository for 3-D structural data of proteins and nucleic acids. These data, typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are released into the public domain, and can be accessed for free. The structural data can be used to visualize the biomolecules with appropriate software, such as VMD, RasMol, PyMOL, Jmol, MDL Chime, QuteMol, web browser VRML plugin or any web-based software designed to visualize and analyse the protein structures such as STING. A recent desktop software addition is Sirius. The RCSB PDB website also contains resources for education, structural genomics, and related software.

CASTp: Computed Atlas of Surface Topography of proteins (CASTp) provides an online resource for locating, delineating and measuring concave surface regions on three-dimensional structures of proteins. These include pockets located on protein surfaces and voids buried in the interior of proteins. The measurement includes the area and volume of pocket or void by solvent accessible surface model (Richards' surface) and by molecular surface model (Connolly's surface), all calculated analytically. CASTp can be used to study surface features and functional regions of proteins. CASTp includes a graphical user interface, flexible interactive visualization, as well as on-the-fly calculation for user uploaded structures.

DRUG BANK: The DrugBank database available at the University of Alberta is a unique bioinformatics and cheminformatics resource that combines detailed drug (i.e. chemical, pharmacological and pharmaceutical) data with comprehensive drug target (i.e. sequence, structure, pathway) information. The database contains nearly 4800 drug entries including more than 1480 FDA-approved small molecule drugs, 128 FDA-approved biotech (protein/peptide) drugs, more than 71 nutraceuticals and more than 3200 experimental drugs. Additionally, more than 2500 protein (i.e. drug target, non-redundant)

sequences are linked to these drug entries. Each DrugCard entry contains more than 100 data fields with half of the information being devoted to drug/chemical data and the other half devoted to drug target or protein data. Users may query DrugBank in any number of ways. It is maintained by David Wishart and Craig Knox.

CHEM SKETCH:

ACD/ChemSketch is an integrated software package from Advanced Chemistry Development Inc. for drawing chemical structures, reactions, schematic diagrams and designing other chemistry-related reports and presentations. Structure mode for drawing chemical structures and calculating their properties.

RESULTS

MORPHOLOGICAL:

GRAM STAINING:

Pink coloured cells in rod shaped were observed which indicates GRAM NEGATIVE BACTERIA.



Figure 8: Gram Staining In Salmonella Typhimurium

CAPSULE STAINING: Upon capsule staining Salmonella appears as blue coloured cells covering with the light blue coloured capsule.



Figure 9: CAPSULE STAINING IN SALMONELLA TYPHIMURIAM

ENDOSPORE STAINING: Salmonella are non-spore forming bacteria and observed as individual cells upon endospore staining.

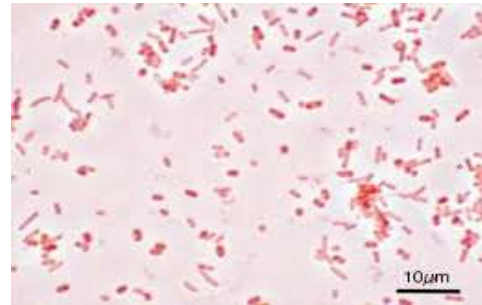


Figure 10: ENDOSPORE STAINING IN SALMONELLA TYPHE

MOTILITY TEST: Salmonella are true motile organisms with the peritrichous flagella.

S.No	Test	Inference
1.	Gram Staining	Gram negative
2.	Morphology	Rods
3.	Capsule Staining	Capsule is present
4.	Endospore Staining	Nonspore forming
5.	Motility	Motile

Table 1: Showing the Results of Morphology & Motility Tests.

BIOCHEMICAL TESTS:

INDOLE TEST: Salmonella typhi species are INDOLE NEGATIVE with no utilization of tryptophan.

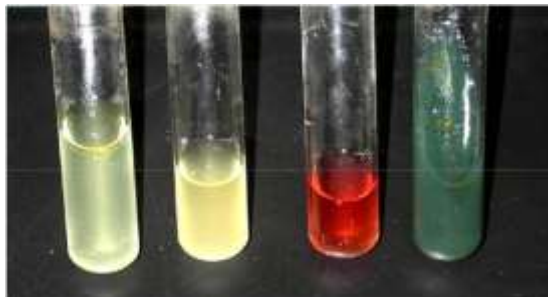


Figure 11: INDOLE TEST OF SALMONELLA TYPHE

VOGUS PROSKAUR TEST: Salmonella species are VOGUS PROSKAUR NEGATIVE.

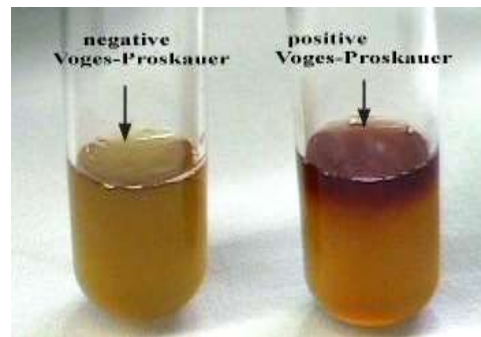


Figure 12: VOGUS PROSKAYR TESTV IN SALMONELLA TYPHE

METHYL RED TEST: Salmonella are METHYL RED POSITIVE with the utilization of sugar provided in the medium.

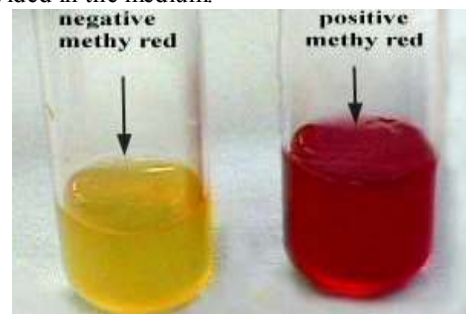


Figure 13: METHUL RED IN SALMONELLA TYPHE

CITRATE TEST: Salmonella is CITRATE
 NEGATIVE organism.



Figure 14 : CITRATE TEST OF SALEMONELLA

S.NO	TEST	INFERENCE
1.	Indole test	Negative
2.	Vogus Proskaur Test	Negative
3.	Methyl Red	Positive
4.	Citrate Test	Negative
5.	Catalase test	Negative

TABLE 2: SHOWING THE RESULTS OF SALMONELLA BIOCHEMICAL REACTIONS

MOLECULAR:

For detection of Salmonella several target genes and different molecular methods have been used by researchers across the globe for detection of salmonella from different clinical and food samples. They used multiplex PCR on rfbE, fliC, invA, virA, spvC, invA, inT and fliO as target genes, uniplex PCR on rRNA gene and Nested PCR over fellagelingene [6-9]. Similaly Soltani Banavandi et al developed a multiplex PCR method which involves in amplification of invA, prt and tyv genes of Salmonella spp.

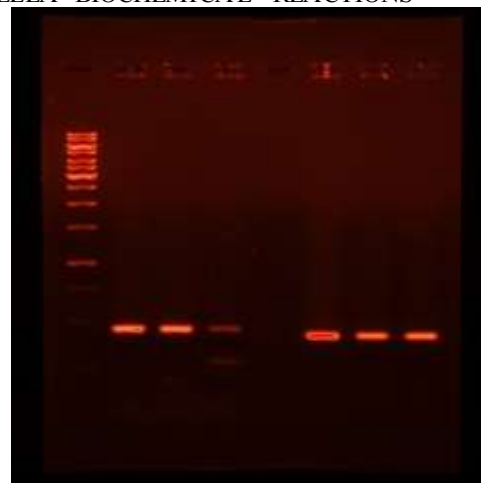


Figure 15 : PCR AMPLIFIED DNA

The isolated organism was identified as belonging to Salmonella species my Morphological and Biochemical Tests and confirmed by PCR amplification and matching with specific primers for the species. The PCR method used in the present experiment was based on amplification of histidine periplasmic gene HisJ and d exopolysaccharide ViLPS gene which differentiate

Salmonella entericaserovar Typhi from other salmonella sp. Salmonella enterica serovar Typhi has a 134-kb island of DNA identified as salmonella pathogenicity island 7 (SPI7), inserted between pheU and 'pheU(truncated), two genes for tRNA^{Phe}. SPI7 has genes for Viexopolysaccharide, for type IVB pili, for putative conjugal transfer, and for sopE bacteriophage. The results of Agarose Gel Electrophoresis revealed a band of 420bp with HisJ and 466bp with Vi LP

BIOINFORMATECES METHODOLOGY:

3D MODEL BUILDING:

The initial model of Sortase from Salmonella typhi was built by using homology-modeling methods and the MODELLER. The query sequence from Salmonella typhi was submitted to domain fishing server for Sortase 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) (Altschul, et al, 1990, 1997) program against PDB (Protein Data bank). Sequence that showed maximum identity with high score and less e-value were aligned (Figure 1) and was used as a reference structure to build a 3D model for Sortase. The sequence of Sortase was obtained from NCBI. The co-ordinates for the structurally conserved regions (SCRs) for Sortase were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm. The structure having the least modeller objective function, obtained from the modeller was improved by molecular dynamics and equilibration methods using NAMD 2.5 software (Kale, et al,1999) using CHARMM27 force field for lipids and proteins along with the TIP3P model for water (Figure 2B) (Jorgensen , et al,1983) The energy of the structure was minimized with 1,00,00 steps. A cutoff of 12 Å (switching function starting at 10 Å) for van der Waals interactions was assumed. No periodic boundary conditions were included in this study. An integration time step of 2 ps was used, permitting a multiple time-stepping algorithm (Grubmuller H,et al,1991) to be employed in which interactions involving covalent bonds were computed every time step, short-range nonbonded interactions were computed every two time steps, and long-range electrostatic forces were computed every four time steps. The pair list of the nonbonded interaction was recalculated every ten time steps with a pair list distance of 13.5 Å. The short-range nonbonded interactions were defined as van der Waals and electrostatics interactions between particles within 12 Å. A smoothing function was employed for the van

der Waals interactions at a distance of 10 Å. CHARMM27 [force-field parameters were used in all simulations in this study. The equilibrated system was simulated for 1 ps with a 500 kcal/mol/Å² restraint on the protein backbone under 1 atm constant pressure and 310 K constant temperature (NPT) and the Langevin damping coefficient was set to 5 ps unless otherwise stated (Mackkerelle ,et al,1992). Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies (Figure 2A). 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) (Brunger A,1992) and environment profile using ERRAT graph (Structure Evaluation server) (Laskoswki,et al,1993). This model was used for the identification of active site and for docking of the substrate with the enzyme.

DOCKING METHOD:

Docking was carried out using GOLD (Genetic Optimization of Ligand Docking) software which is based on genetic algorithm (GA). This method allows as partial flexibility of protein and full flexibility of ligand. The compounds are docked to the active site of the **Sortase**. The interaction of these compounds with the active site residues are thoroughly studied using molecular mechanics calculations. The parameters used for GA were population size (100), selection pressure (1.1), number of operations (10,000), number of island (1) and niche size (2). Operator parameters for crossover, mutation and migration were set to 100, 100 and 10 respectively. Default cutoff values of 3.0 Å^o (dH-X) for hydrogen bonds and 6.0 Å^o for vanderwaals were employed. During docking, the default algorithm speed was selected and the ligand binding site in the **Sortase** was defined within a 10 Å^o radius with the centroid as CE atom of ALA220. The number of poses for each inhibitor was set 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5Å^o RMSD. After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each ligand was selected.

GOLD SCORE FITNEES FUNCTION :

Gold Score performs a force field based scoring function and is made up of four components: 1. Protein-ligand hydrogen bond energy (external H-bond); 2. Protein-ligand vander Waals energy

(external vdw); 3. Ligand internal vander Waals energy (internal vdw); 4. Ligand intramolecular hydrogen bond energy (internal- H- bond). The external vdw score is multiplied by a factor of 1.375 when the total fitness score is computed. This is an empirical correction to encourage protein-ligand hydrophobic contact. The fitness function has been optimized for the prediction of ligand binding positions.

$$\text{GoldScore} = S(\text{hb_ext}) + S(\text{vdw_ext}) + S(\text{hb_int}) + S(\text{vdw_int})$$

Where S (hb_ext) is the protein-ligand hydrogen bond score, S (vdw_ext) is the protein-ligand van der Waals score, S (hb_int) is the score from intramolecular hydrogen bond in the ligand and S (vdw_int) is the score from intramolecular strain in the ligand

RESULTS AND DISCUSSION:

LINOLEIC ACID :

Molecular Formula	=	C18H32O2
Formula Weight	=	280.44548
Composition	=	C(77.09%)
H(11.50%) O(11.41%)		

Molar Refractivity	=	87.12 ± 0.3 cm ³
Molar Volume	=	307.5 ± 3.0 cm ³
Parachor	=	744.4 ± 4.0 cm ³
Index of Refraction	=	1.478 ± 0.02
Surface Tension	=	34.3 ± 3.0 dyne/cm
Density	=	0.911 ± 0.06 g/cm ³
Dielectric Constant	=	Not available
Polarizability	=	34.53 ± 0.5 10-24cm ³
Monoisotopic Mass	=	280.24023 Da
Nominal Mass	=	280 Da
Average Mass	=	280.4455 Da
TLC – R _f (3:7 ethylacetate-hexane)-		0.6
Melting point –		85-90 °C
Yield –		95 %
Mass (m/z) –		154 (M ⁺)
IR (KBr) cm ⁻¹ -		3302 (N-H str), 3019 (Ar C-H str), 1897 (C=O str), 1661 (C=Cstr), 1615 (N-H def), 1301(C-N str), 1241-1118(C-F str), 992,887 (C-H def)
¹ H NMR (300 MHz, CDCl ₃) δ –		3.47 [s N-H], 7.05-7.16 [m Ar-H], 7.25 [s Ar-H], 7.71-7.81 [m Ar-H].

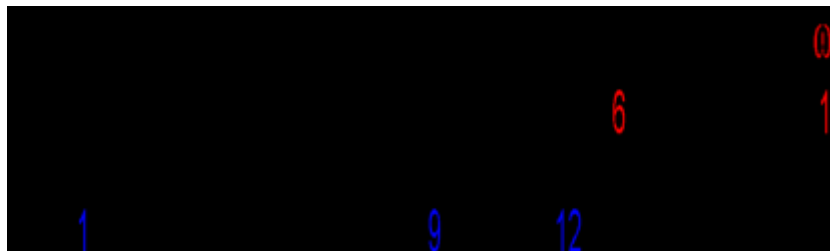


Figure 16: LINOLEIC ACID STRUCTURE

THYMOQUINONE:

Molecular Formula	=	C10H12O2
Formula Weight	=	164.20108
Composition	=	C(73.15%) H(7.37%) O(19.49%)
Molar Refractivity	=	45.70 ± 0.3 cm ³
Molar Volume	=	154.1 ± 3.0 cm ³
Parachor	=	377.0 ± 6.0 cm ³
Index of Refraction	=	1.504 ± 0.02
Surface Tension	=	35.8 ± 3.0 dyne/cm
Density	=	1.065 ± 0.06 g/cm ³
Dielectric Constant	=	Not available
Polarizability	=	18.11 ± 0.5 10-24cm ³
Monoisotopic Mass	=	164.08373 Da
Nominal Mass	=	164 Da
Average Mass	=	164.2011 Da
TLC – R _f (3:7 ethylacetate-hexane)-		0.53
Melting point –		180-190 °C

Yield – 83 %

Mass (m/z) – 196 (M⁺)

IR (KBr) cm⁻¹ - 3066(N-H str), 3066 (Ar C-H str), 1610 (C=Cstr), 1510 (N-H def), 1415 (C=Nstr), 1348 (C-S str), 1291(C-N str), 1185(C-O-C), 1156 (C-F str), 969,941 (C-H def)

¹H NMR (300 MHz, CDCl₃) δ – 2.03 [s SH], 7.12-7.39 [m Ar-H], 7.96 [s Ar-H] .

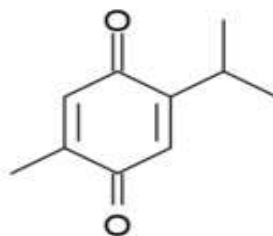


Figure 17: THYMOQUINONE STRUCTURE

DITHYMOQUINONE:

Molecular Formula	=	C ₂₁ H ₂₈ O ₄
Formula Weight	=	344.44462
Composition	=	C (73.23%) H(8.19%) O(18.58%)
Molar Refractivity	=	Not available
Molar Volume	=	Not available
Parachor	=	Not available
Index of Refraction	=	Not available
Surface Tension	=	Not available
Density	=	Not available
Dielectric Constant	=	Not available
Polarizability	=	Not available
Monoisotopic Mass	=	344.198759 Da
Nominal Mass	=	344 Da
Average Mass	=	344.4446 Da

TLC – R_f (4:6 ethylacetate-hexane)- 0.52

Melting point – 65-72 °C

Yield – 78 %

Mass (m/z) – 273 (M⁺)

IR (KBr) cm⁻¹ - 3447(N-H str), 2958 (Ar C-H str), 1606 (C=Cstr), 1501 (N-H def), 1468 (C=Nstr), 1411(C=C), 1338 (C-S str), 1286(C-N str), 1186(C-O-C), 1152 (C-F str), 969,941 (C-H def), 770,728(C-Cl).

¹H NMR (300 MHz, CDCl₃) δ – 2.32-2.44[m CH₂], 3.46[t,CH₂,J=6.79], 3.71[t,CH₂,J=6.043], 7.18 [t,CH₂,J=8.5], 7.96-8.05 [m,Ar H].

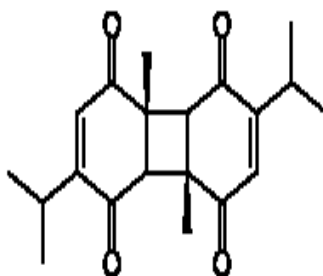


Figure 18: DITHYMOQUINONE STRUCTURE

DAMASCENINE:

Molecular Formula	=	C10H13NO3
Formula Weight	=	195.21512
Composition	=	C(61.53%) H(6.71%) N(7.18%) O(24.59%)
Molar Refractivity	=	54.31 ± 0.3 cm ³
Molar Volume	=	170.7 ± 3.0 cm ³
Parachor	=	427.6 ± 4.0 cm ³
Index of Refraction	=	1.548 ± 0.02
Surface Tension	=	39.3 ± 3.0 dyne/cm
Density	=	1.143 ± 0.06 g/cm ³
Dielectric Constant	=	Not available
Polarizability	=	21.53 ± 0.5 10 ⁻²⁴ cm ³
Monoisotopic Mass	=	195.089543 Da
Nominal Mass	=	195 Da
Average Mass	=	195.2151 Da

TLC – R_f (6:4 ethylacetate-hexane)- 0.50

Melting point – 63-69 °C

Yield – 65 %

Mass (m/z) – 407 (M⁺)

IR (KBr) cm⁻¹ - 3275(N-H str), 2923 (Ar C-H str), 1604 (C=C str), 1502 (N-H def), 1465 (C=N str), 1428(C=C str), 1319 (C-S str), 1223(C-N str), 1159(C-O-C), 1067 (C-F str), 951 (C-H def), 664(C-H def).

¹H NMR (300 MHz, CDCl₃) δ – 1.86-1.97 [m,CH₂], 2.35-2.43 [m,CH₂], 2.49-2.59[m,CH₂], 2.88-2.99 [m,CH₂], 3.27 [s,CH₃], 7.28-7.36 [m,Ar H], 7.65-7.71 [m,Ar H], 7.98-8.05 [m,Ar H].

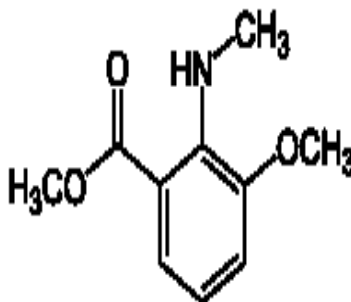


Figure 19: DAMASCENINE STRUCTURE

TANINNS :

Molecular Formula	=	C40H44O18
Formula Weight	=	812.76656
Composition	=	C(59.11%) H(5.46%) O(35.43%)
Molar Refractivity	=	Not available
Molar Volume	=	Not available
Parachor	=	Not available
Index of Refraction	=	Not available
Surface Tension	=	Not available
Density	=	Not available
Dielectric Constant	=	Not available
Polarizability	=	Not available
Monoisotopic Mass	=	812.252765 Da
Nominal Mass	=	812 Da
Average Mass	=	812.7666 Da

TLC – R_f (6:4 ethylacetate-hexane)- 0.51

Melting point – 65-70 °C

Yield – 62 %

Mass (m/z) – 470 (M+1)

IR (KBr) cm^{-1} - 2921 (Ar C-H str), 1713 (C=C str), 1460 (C=N str), 1663 (C=C str), 1375 (C-S str), 1159 (C-O-C), 965 (C-H def), 552 (C-Br str).

$^1\text{H NMR}$ (300 MHz, CDCl_3) δ - 1.39-1.54 [m, CH_2], 2.40 [s, CH_3], 3.06-3.12 [m, CH_2], 3.36 [t, ArH_2 , $J=6.922$], 7.64 [d, ArH_2 , $J=8.99$], 7.74 [d, ArH_2 , $J=7.911$], 7.79 [d, ArH_2 , $J=7.911$], 7.86 [d, ArH_2 , $J=8.99$]

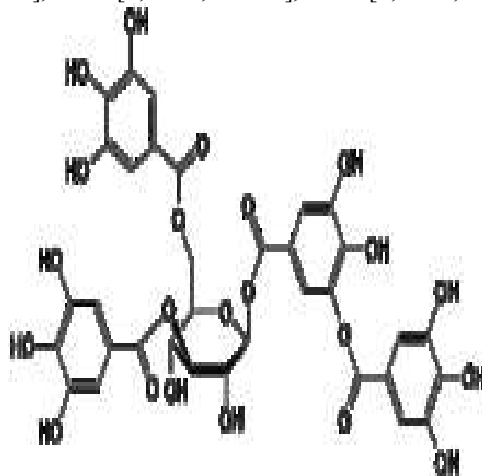


Figure 20: TANINNS STRUCTURE

HOMOLOGY MODELLING OF SORTASE DOMAIN:

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 the 2QLY which has a high level of sequence identity with the Sortase A domain. Structurally conserved regions (SCRs) for the model and the template were determined by superimposition of the two structures and multiple sequence alignment. In the following study, we have chosen 2QLY as a reference structure for modeling Sortase A domain. Coordinates from the reference protein (2QLY) 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. In the modeller we will get a 20 PDB out of which we select a least energy. The energy unit will be in kilo joule. All side chains of the model protein were set by rotamers. The final stable structure of the Sortase A protein obtained.



Figure 21 : MODELLED PROTIEIN WITH SIDE CHAIN

The structure having the least energy with low RMSD (Root Mean Square Deviation) which was obtained by the NAMD is in water molecule (TIP3).

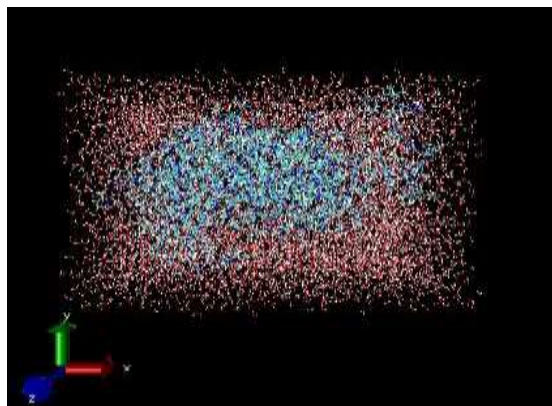


Figure 22: ENERGY MINIMISATION USING NAMD/VMD

The final structure was further checked by verify3D graph and the results have been shown in (Figure 23)

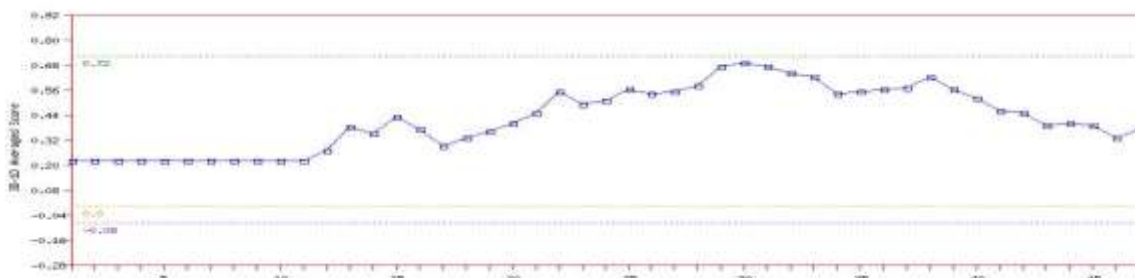


Figure 23: GRAPHICAL REPRESENTATION FOR VERIFY 3D

VALIDATION OF SORTASE A DOMAIN:

After the refinement process, validation of the model was carried out using Ramachandran plot calculations computed with the RAMPAGE Server.

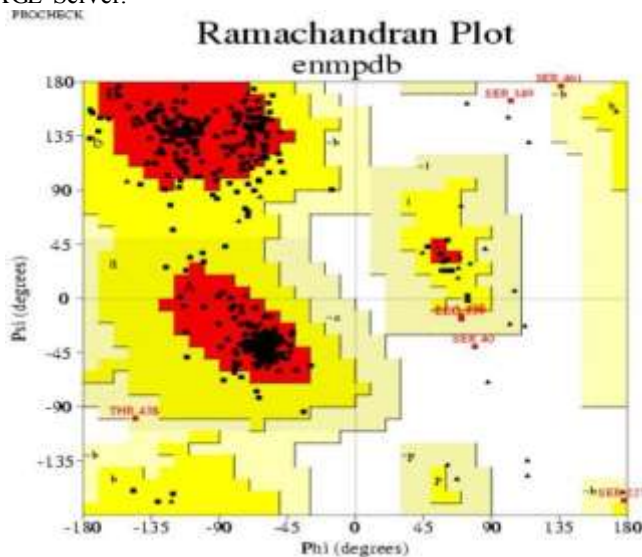


Figure 24: RAMACHANDRAN PLOT USING RAMPAGE SERVER

% of residue in most favored regions	86.6
% of residue in the additionally allowed zones	11.7
% of residue in the generously regions	1.2
% of residue in disallowed regions	0.5
Total no of Residues	100

TABLE 3: % OF RESIDUE FALLING IN THE CORE REGION OF THE RAMACHANDRAN'S PLOT

ACTIVE SITE IDENTIFICATION OF SORTASE A:

After the final model was built, the possible binding sites of Sortase A was searched based on the structural comparison of template and the model build and also with CASTP server and was shown in (Figure 25). Infact from the final refined model of Sortase A domain using SPDBV program, it was found that secondary structures are highly conserved and the residues, ARG130, ASP132, SER133, VAL 134, ASP135, PHE136, GLN152, GLU242, THR243, THR244

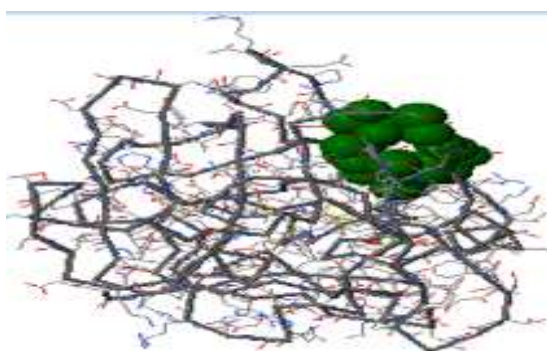


Figure 25: ACTIVE SITE OF SORTASE

DOCKING OF INHIBITORS WITH THE ACTIVE SITE :

Docking of the inhibitors with Sortase A domain was performed using GOLD 3.0.1, which is based on genetic algorithm. This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a cocrystallized ligand by 4Å. This dimension was considered here appropriate to allow, for instance, compounds larger than the cocrystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds used for docking was converted in 3D with SILVER. To this set, the substrate corresponding to the modeled protein were added. Docking of best inhibitor with the active site of protein showed the activity of the molecule on protein function .

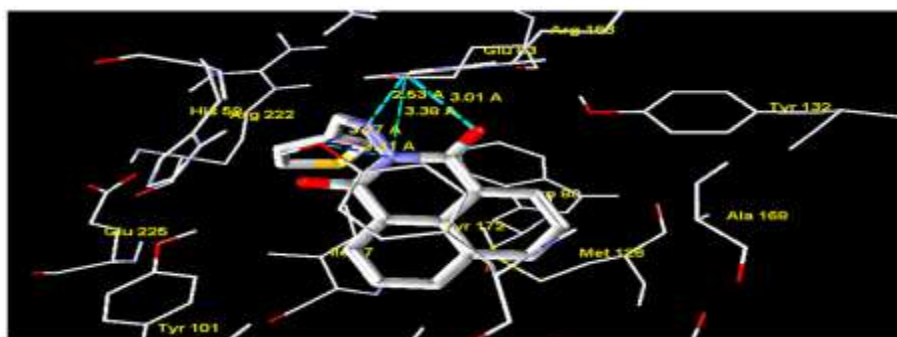


Figure 26 : DOCKING STUDIES OF NIGELLA SATIVA COMPOUNDS

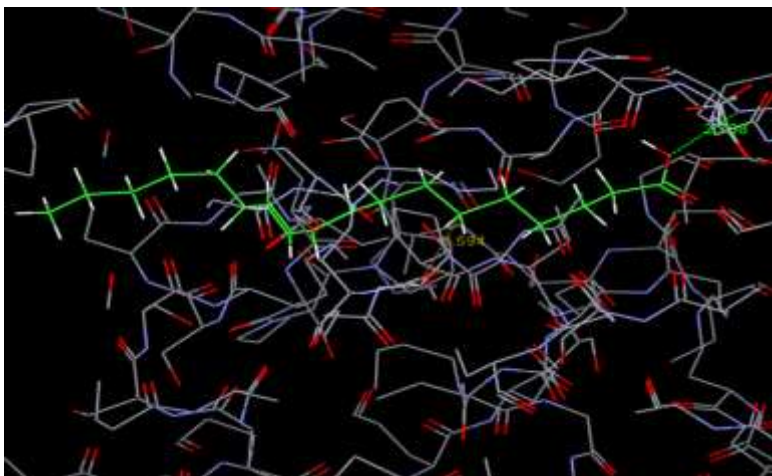


Figure 27 : DOCKING OF CONJUGATED FATTYACID

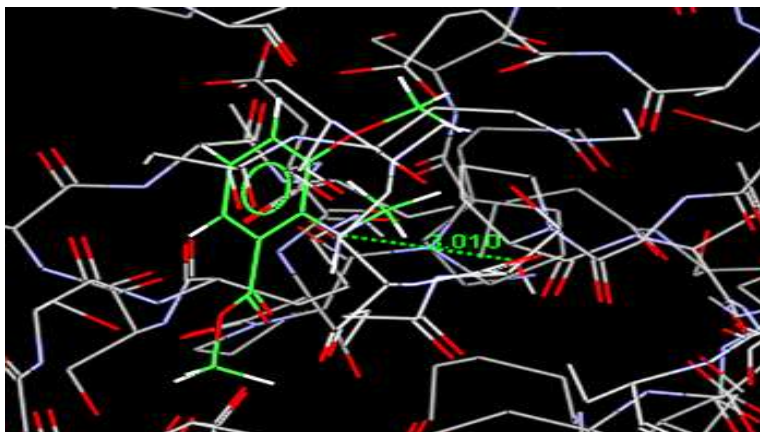


Figure 28 : DOCKING STUDY OF DAMASCENINE

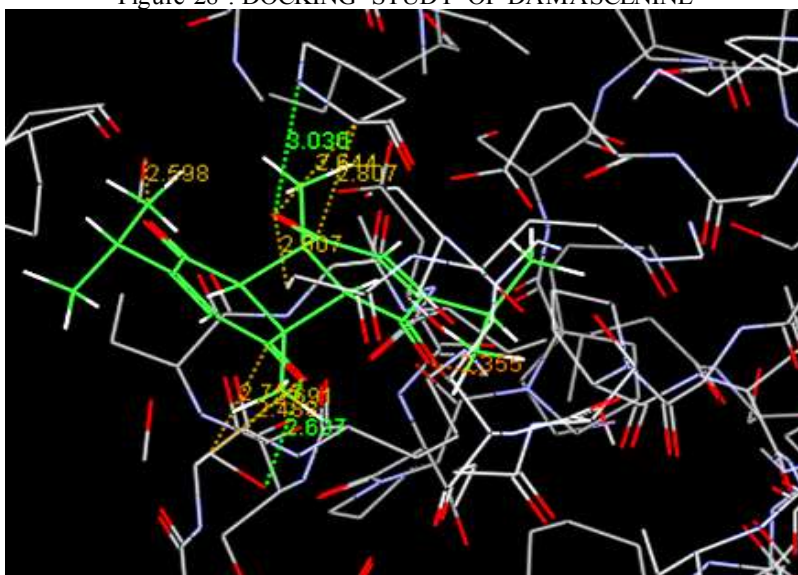


Figure 29 : DOCKING STUDY OF DITHYMOQUINON

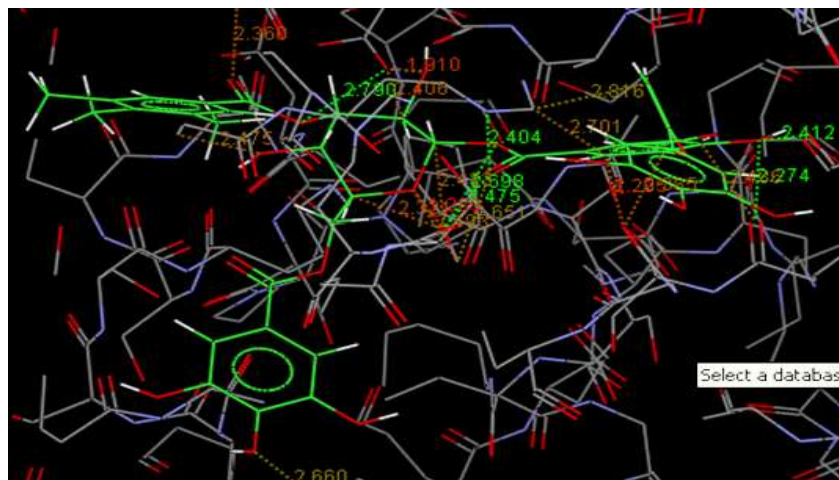


Figure 30 : DOCKING STUDY OF TANNIN - A

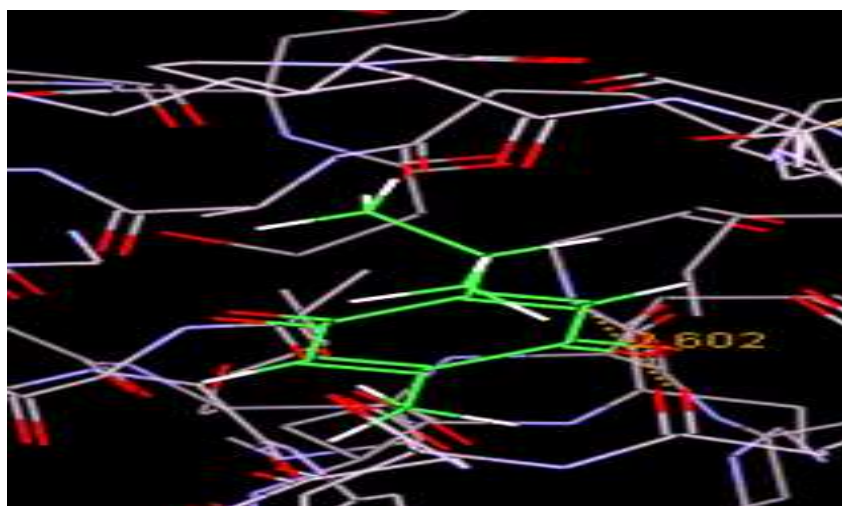


Figure 31 : DOCKING STUDY OF TANNIN - B

Fitness	S(hb_ext)	S(vdw_ext)	S(hb_int)	S(int)	Ligand name
29.62	0.00	36.06	0.00	-15.36	conjugated fattyacid
23.23	0.00	25.00	0.00	-11.14	Damascenine
32.62	0.00	29.61	0.00	-8.09	Dithymoquinon
24.97	4.24	46.88	0.00	-43.72	Tannin
27.77	0.00	24.24	0.00	-5.56	Thymoquinone

Table 4: DOCKING STUDIES OF COMPOUND WITH SORTASE

CONCLUSIONS:

Although recently there has been more focus on these more advanced molecular methods, in our experience, conventional PCR gives results that are

as good, with the advantage of simplicity and a lesser requirement for expertise. Our observations have been consistent with previous studies, which inferred that various PCR methods have a better sensitivity

than blood culture for the diagnosis of typhoid. The efficacy of blood culture decreases with the duration of illness and the use of antibiotics before collection of blood samples severely affects the isolation rate. Our PCR results were ready in less than 10 h, compared with 3–5 days for blood culture confirmation. Our results suggest that PCR-based diagnosis is particularly useful for all clinically suspected cases of typhoid fever caused by *Salmonella Typhi* and *paratyphi*, mainly those with problematic and varying clinical manifestations. Moreover, several additional cases of typhoid fever, not detected in culture, were also identified by PCR. The follow-up of PCR positive/culture negative cases revealed a good therapeutic response to specific antibiotic therapy as reported by clinicians. The technique therefore seems reliable and simple, and enables early and accurate detection of *S. Typhi* in human clinical samples. In this work, we have collected 3D model of Sortase domain, from *Salmonella typhi* using the SPDBV software and obtained a refined model after energy minimization. The final refined model was further assessed by ERRAT & PROCHECK program, and the results show that this model is reliable. The stable structure is further used for docking of substrate with the compounds of *Nigella sativa*. Docking results indicate that conserved amino-acid residues in Sortase main play an important role in maintaining a functional conformation and are directly involved in donor substrate binding. The interaction between the domain and the inhibitors proposed in this study are useful for understanding the potential mechanism of domain and the inhibitor binding. As is well known, hydrogen bonds play important role for the structure and function of biological molecules. The extracts of *Nigella sativa* seeds proved excellent antimicrobial activity.

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