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PROTEIN MARKER IDENTIFICATION OF VERATOXIN FROM CLINICALLY ISOLATED ESCHERICHIA COLI

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ABSTRACT

This investigation was carried out to identify the protein marker for veratoxin from clinically isolated *E.coli* samples. The *E.coli* samples were isolated from clinical samples like blood, urine and stool of infected patients. The *E.coli* samples were conformed by the growth of the bacteria on EMB agar medium where they showed metallic sheath. The *E.coli* samples were grown on nutrient broth for the isolation of the protein. The proteins were precipitated using ammonium sulphate. The proteins got precipitated at 50% saturation of ammonium sulphate. The eluted proteins were then identified using SDS-PAGE with standard protein marker. Three *E.coli* samples isolated showed the band with 32,000KDa determing the presence of veratoxin protein in the *E. coli* samples.

KEY WORDS: *E.coli,* Clinical samples, Veratoxin protein, SDS-PAGE.

INTRODUCTION

E.coli is a Gram-negative, rod-shaped, facultatively anaerobic bacterium belongs to the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms [1]. Most of the *E. coli* strains are harmless, but some of the serotypes can cause serious food poisoning in their hosts, and some *E.coli* strains are occasionally responsible for product recalls due to food contamination [2]. The strains of *E.coli* which are harmless they are part of normal flora of the gut, and it can be beneficial to their hosts by producing vitamin K_2 [3],

and preventing colonization of the intestine with pathogenic bacteria [4,5]. About 0.1% of gut flora consists of *E. coli* and other facultative anaerobes [6], and fecal to oral transmission is the major route through which pathogenic strains can enter and bacterium can cause disease. For a limited period of time cells are able to survive outside the body, which makes them ideal organisms to test the environmental samples for fecal contamination. However, a growing body of research that has been examined that environmentally persistent *E. coli* which can survive for long periods outside of the host [7].

E. coli is the most widely studied and it is used as a model organism in the fields of biotechnology , microbiology and recombinant DNA technology. *E.coli* encompasses a vast population of bacteria that exhibit a very high degree of both phenotypic and genetic diversity. The genome sequencing of enormous number of isolates of *E. coli* and other related bacteria shows that a taxonomic reclassification would be desirable. However, due to its medical importance this not has been done largely [8] and *E. coli* remain one of the most diverse bacterial species: only 20% of the genome is common to all strains [9].

Indeed, from the evolutionary point of view, the members of genus Shigella (S. boydii, S. flexneri, S. dysenteriae, S. sonnei) should be classified as *E. coli* strains, an aspect termed taxa in disguise [10]. Similarly, other strains of *E. coli* (e.g. the K-12 strain commonly used in the recombinant DNA work) are sufficiently different that they would merit reclassification.

Proteomics of E.coli

Proteome: Several studies have been investigated and reported the proteome of *E. coli*. By 2006, 1,627 (38%) of the 4,237 open reading frames (ORFs) had been identified experimentally [11].

Interactome: The interactome of *E. coli* has been studied by mass spectrometry and affinity purification (MS/AP) and by analyzing the binary interactions among its proteins.

Protein Complexes: In 2006 study, about 4,339 proteins have been purified from the cultures of strain K-12 and found interacting partners for 2,667 proteins; most of the functions are unknown at the time [12]. And in 2009 studies they have found 5,993 interactions between proteins of the same *E. coli* strain though this data showed a little overlap with the 2006 publication [13].

Binary Interactions: Rajagopala *et al*. has been carried out the systematic yeast two-hybrid screens with most of *E. coli* proteins and found a total of 2,234 protein-protein interactions [14].This study also integrated protein structures, genetic interactions and mapped 458 interactions within 227 protein complexes.

Shiga-like toxin or Verotoxin: Shiga-like toxin is also known as verotoxin [15, 16]. This toxin is generated by only some specific strains of *Escherichia coli* [17]. It is named for its similarity to the AB5-type Shiga toxin produced by the bacteria Shigella dysenteriae. There are two types of toxins SLT1 and SLT2 [18].

Structure and Mechanism: The toxin is a multisubunit protein made up one molecule of A subunit (32,000 molecular weight) and five molecules of the B subunit which has molecular weight of 7,700. A subunit is responsible for the toxic action of the protein and B subunit is responsible for binding to a specific cell type.

This toxin acts on the lining of the blood vessels. The B subunits of the toxin bind to an integral part of the cell membrane known as glycolipid globotriaosyl ceramide (Gb3) [19]. When B subunit binds to Gb3 causes induction of narrow tubular membrane invaginations, which drives and form an inward membrane tubules for the bacterial uptake into the cell. These tubules play an essential role for uptake into the host cell [20].Due to the protein which is present inside the cell, A subunit interacts with the ribosome's to inactivate them. The A subunit of the toxin is an N-glycosidase that modifies the RNA component of the ribosome to inactivate it and so bring a halt to protein synthesis leading to the death of the cell. The vascular endothelium has to continuously renew itself, so killing of this cells leads to a breakdown of the lining and then to hemorrhage. The first response is usually a bloody diarrhea. This is because of Shiga toxin that usually taken in with the contaminated water or food. The toxin is very effective against small blood vessels, such as found in the digestive tract, lungs and the kidney, but not against large blood vessels such as the major veins or arteries. A specific target for the toxin emerges to the vascular endothelium of the glomerulus. Glomerulus is the filtering structure that is a key to the function of the kidney. Destroying of these structures may lead to kidney failure and the development of the often deadly and frequently debilitating hemolytic uremic syndrome. Food poisoning with this toxin may often show severe effects on the nervous system and lungs. Source of toxin gene: It has been suggested by some the researchers that the gene coding for Shiga-like toxin comes

from a toxin-converting lambdoid prophage, such as 933W or H-19B, which is inserted into the bacteria's chromosome via transduction [21].

MATERIALS AND METHODS

Sample Collection: Strains of *E.coli were* isolated from different clinical sources like blood and urine samples of infected patients.

Isolation of E. coli: The samples were streaked on Nutrient agar plates and incubated at 37 ºC for 24 – 48 hours. After incubation the colonies which are isolated were characterized by their morphology of colonies and Gram's staining. The colonies having similar characteristic to *E.coli* were isolated individually by quadrant streaking on nutrient agar plate. These cultures were then maintained in nutrient agar slants for further studies.

Staining Techniques & Biochemical Tests

Staining procedure makes differences between bacterial cells by imparting different colors to different bacteria are termed as staining techniques. According to Bergey's Manual different staining techniques like (Gram staining; Acid fast staining and Bacterial spore staining of bacteria) have been performed for identification of *E.coli.* And also different Biochemical tests like (Indole production test, Methyl red test, Citrate utilization test, Voges – Proskauer test Catalase test, Starch hydrolysis, Urease test, Nitrate reduction test, Casein hydrolysis, Glucose fermentation) have been performed for identification of *Lactobacillus spp*.

Purification of Intracellular Enzyme

Ammonium Sulphate Precipitation

The various steps of protein purification were carried out at 4ºC. The precipitation of the proteins was carried out according to the chart of Gomori (1955). 30ml of the broth centrifuged at 10000rpm for 20minutes and the pellet was lysed using HEPES buffer. The sample was then brought to 50% (w/v) saturation with solid ammonium sulphate; keep that for overnight at 4ºC. And then precipitate was collected by centrifugation in an ultracentrifuge at 10,000 rpm for 20 minutes. The obtained pellet was dissolved in 1ml of 0.1M TrisHcl buffer for further purification through dialysis.

Dialysis

The resultant ammonium sulphate precipitate (in solution) was introduced to a special plastic bag called the dialysis tube. Dialysis was carried out to remove all the traces of the

ammonium sulphate. 1ml of the sample was loaded in the dialysis tube and was kept in an inverted position in a 500 ml beaker containing. It was then kept on a magnetic stirrer for 24h at room temperature and after every 6h the Tris HCl buffer in the beaker was changed.

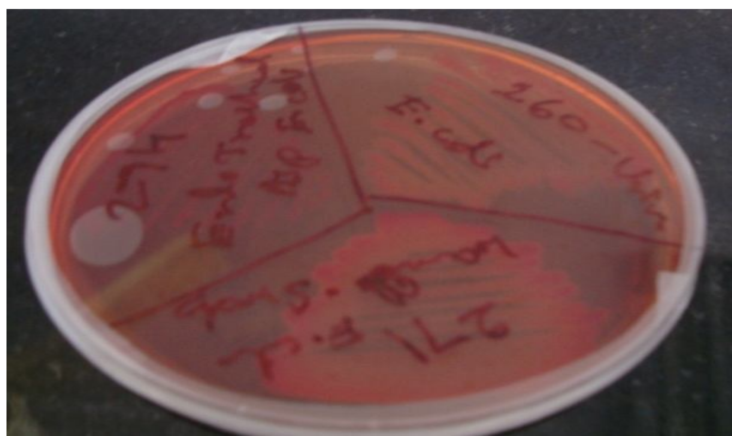
SDS PAGE of Protein Extract

One dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Concentration of 10% separating gel and 5% stacking gel were prepared, in eppendorf tube 35µl of each crude sample were mixed with 30µl of sample loading buffer. The samples containing with loading buffer were heated in the dry bath at 95˚C for 5 minutes for denaturation. The denatured protein samples were centrifuged briefly at 5000rpm for 5 minutes to precipitate out the debris. 35µl from each sample and 20µl of standard marker were loaded into the gel wells. The samples were run at 120V with 25mA in 1X running buffer according to standard protocol. After dye reaches the bottom of gel, it took it out from the electrophoresis apparatus and the gel was fixed with 10% TCA. Protein bands were visualized by overnight staining with 0.25% CBB-250. Protein bands were observed after destaining the gel in the next day using destaining solution.

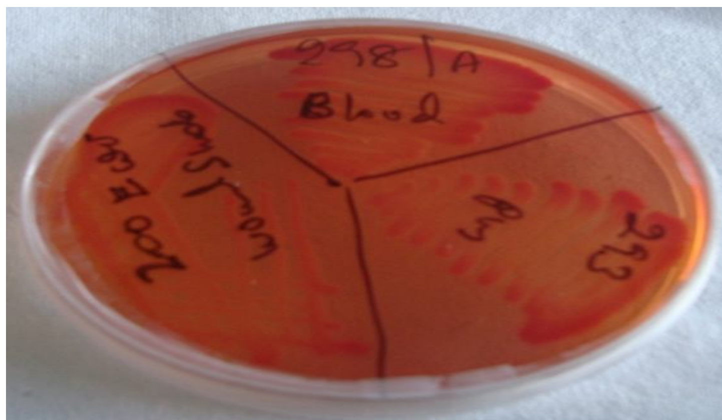
RESULTS AND DISCUSSION

Isolation of *E. coli* from Clinical Samples

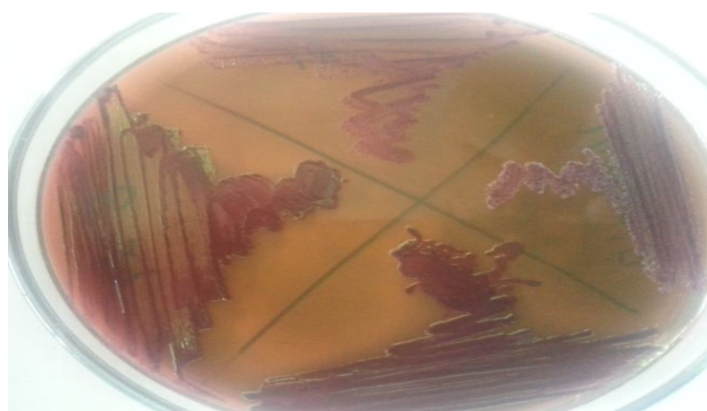
The colonies of following characteristics were isolated on selective medium such as Eeosin metylene blue agar. Five *E. coli* cultures were isolated from clinical sample and were maintained on nutrient agar slants for further use.



Escherichia Coli Clinical Sample 1



Escherichia Coli Clinical Sample 2



***E.coli* on EMB Plate**

Gram Staining: Upon gram staining, it was observed that the selected colonies were gram negative rods.

Biochemical Test Analysis

Indole Production Test – Fig.1 shows absence of the cherry red ring formation indicated that all strains were negative for Indole test.

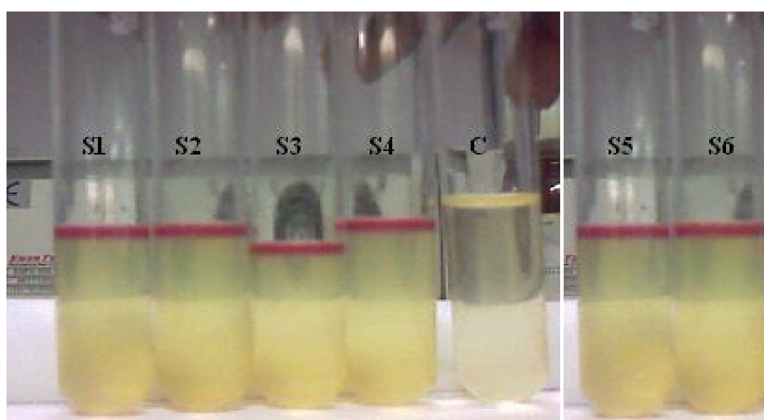
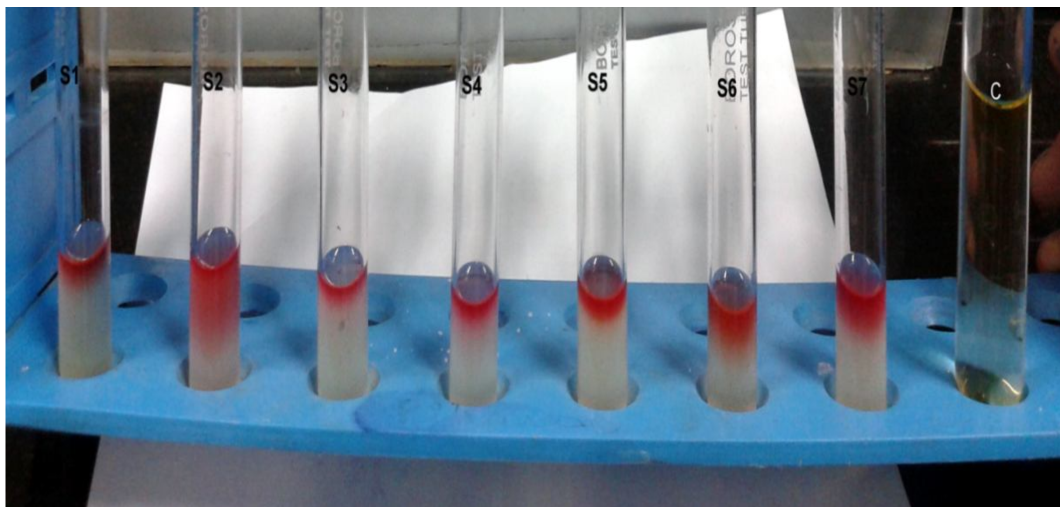


Fig.1 Indole test

Methyl Red Test- Fig.2 Shows Development of Red Colouration Upon The Addition MR Reagent Indicated Positive Test For All The Strains.



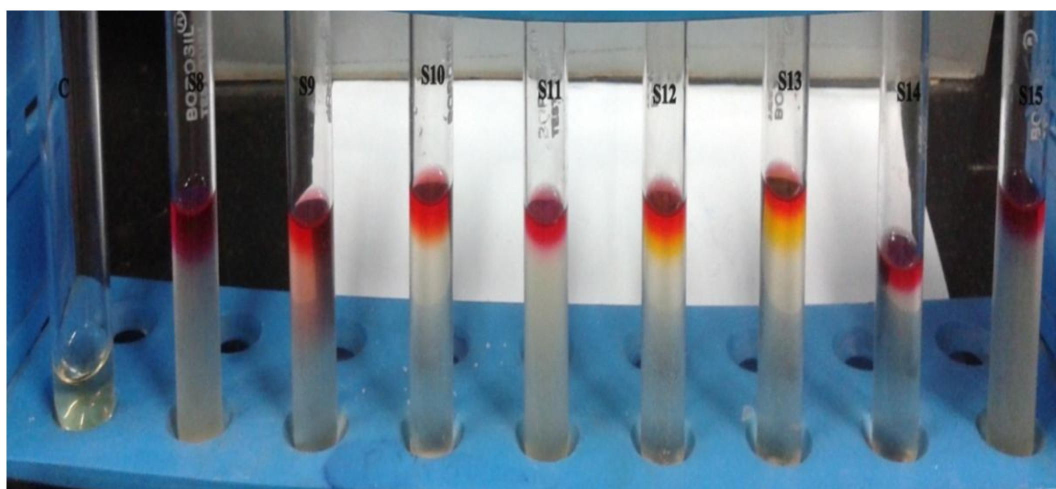
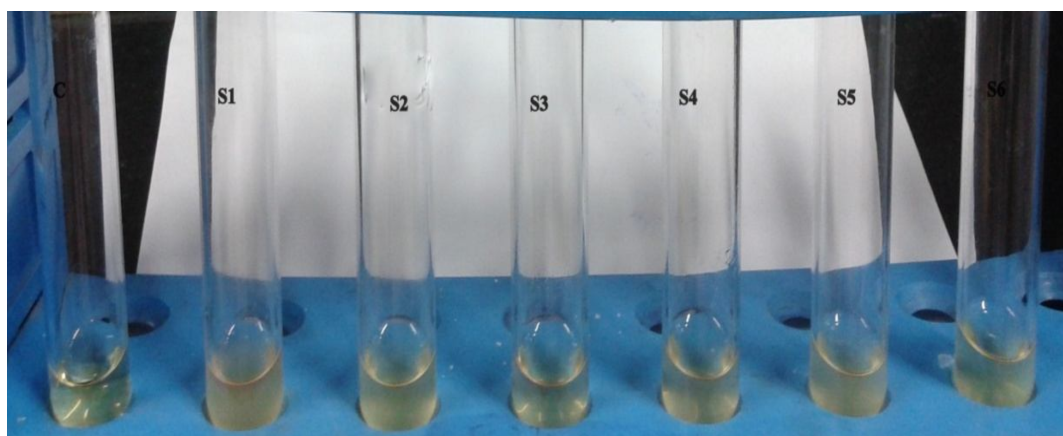


Fig.2 Methyl red test

Voges-Proskauer Test – Fig.3 Shows Development of Brown Ring Upon The Addition of VP Reagent Indicated A Positive Test For All The Strains.



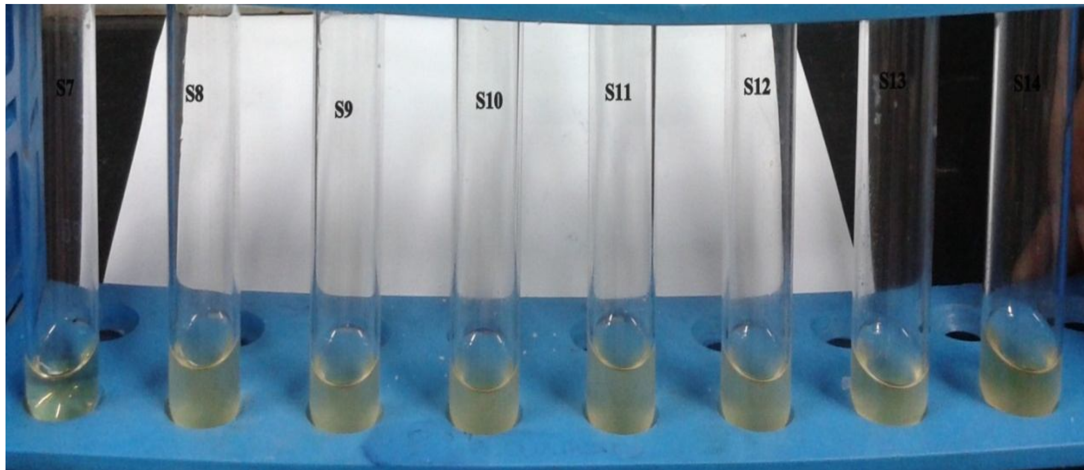


Fig.3 Voges Proskauer Test

Citrate Utilization Test – Fig.4 Shows All The Strains Showed Negative Result For Citrate Utilization Test As The Color of The Slants Remained Green After 48 Hours of Incubation.

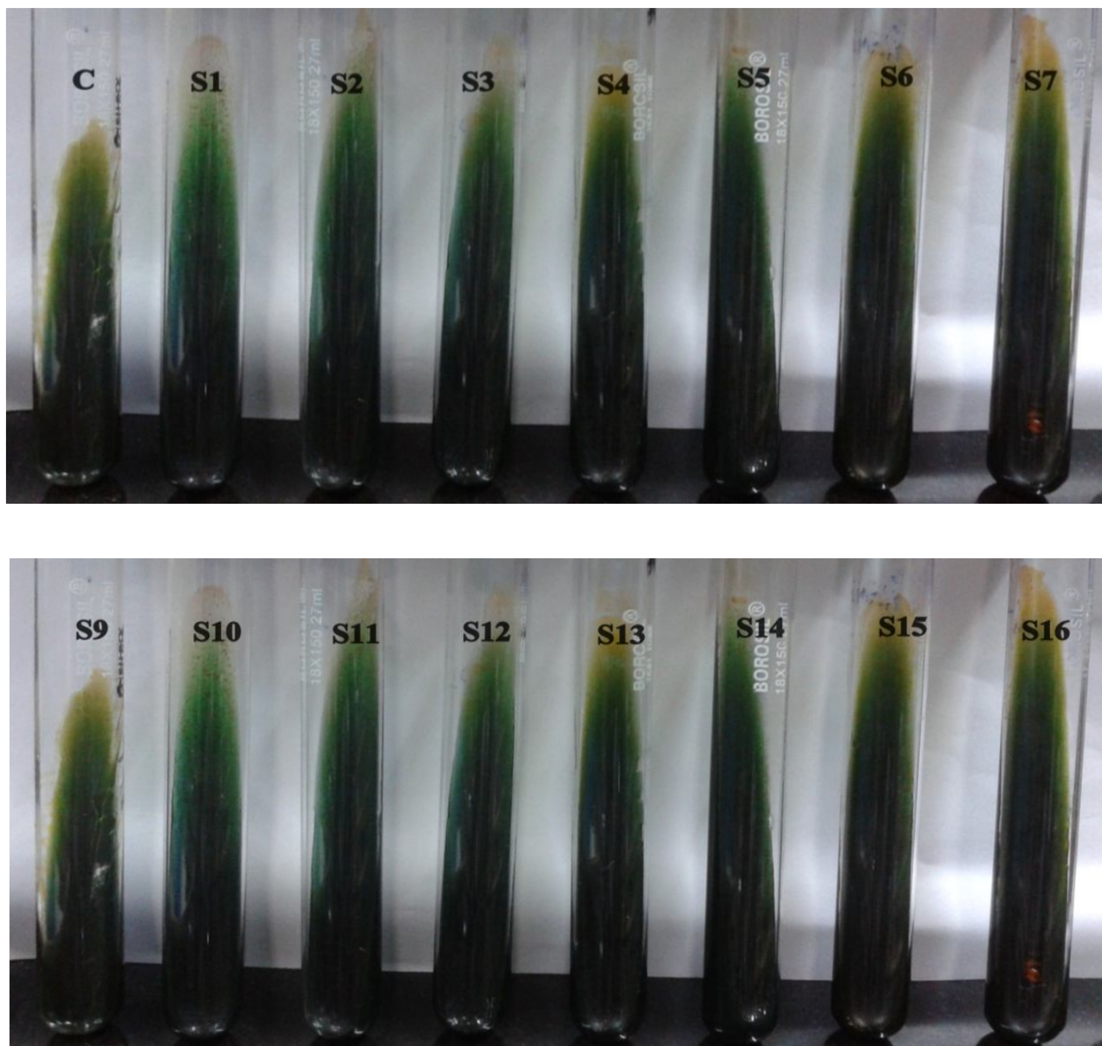


Fig.4 Citrate utilization test

Catalase Test – Fig.5 Shows All Samples Have Showed Positive Result For Catalase Test.

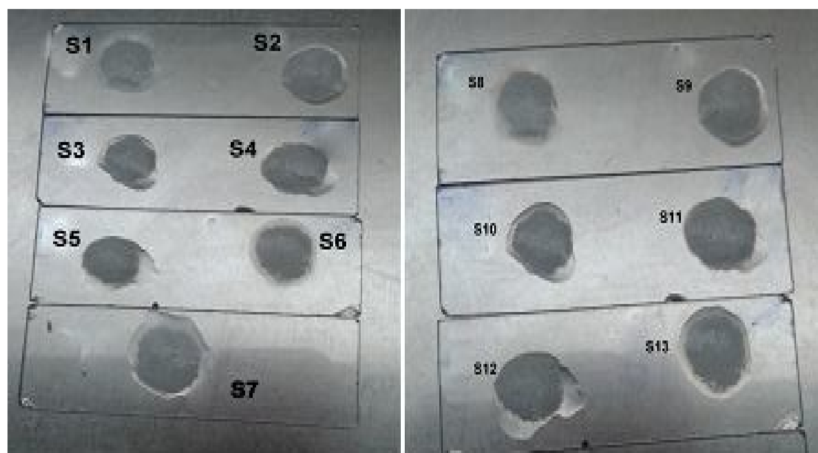


Fig.5 Catalase test

Urease Test – Fig.6 Shows All The Samples Revealed Negative Result For Urease Test.

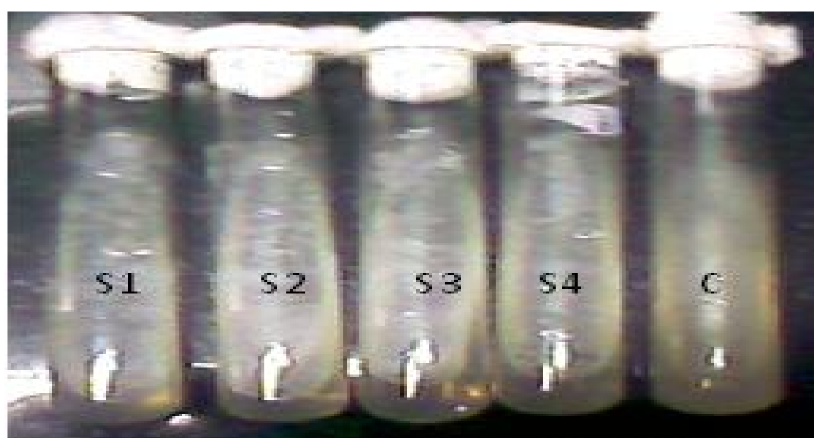


Fig.6 Urease test

Nitrate Reduction Test - Fig.7 Shows All Samples Showed Positive Result For Nitrate Reduction Test.

Fig.7 Nitrate reduction test

Starch Hydrolysis Test - Fig.8 Shows All The Samples Have Showed Positive Result For Starch Hydrolysis Test.

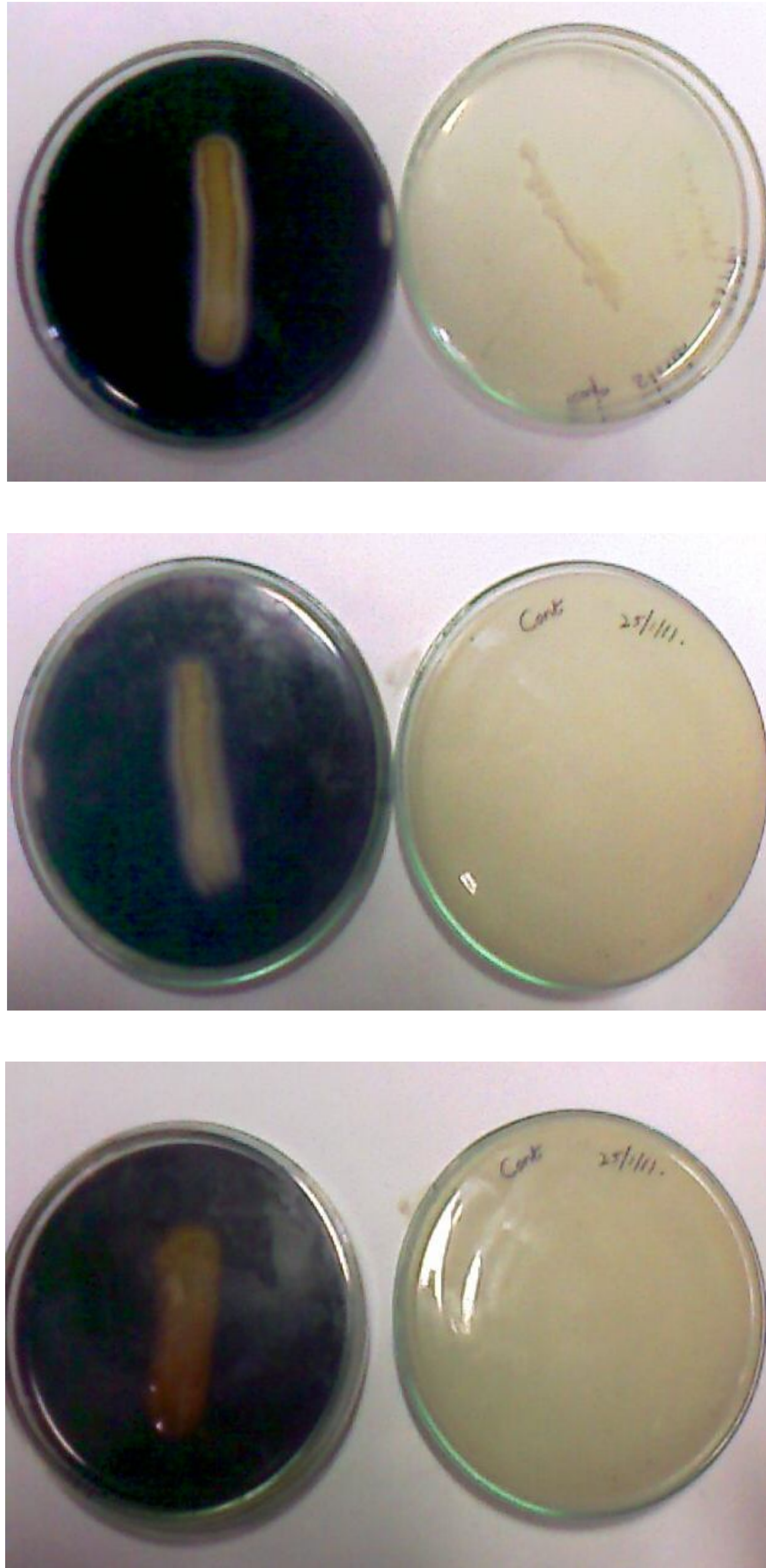


Fig.8 Starch hydrolysis test

Casein hydrolysis test – Fig.9 shows all samples have showed the negative result for casein hydrolysis test.

Fig.9 Casein hydrolysis test

Biochemical tests reactions (Table.1)

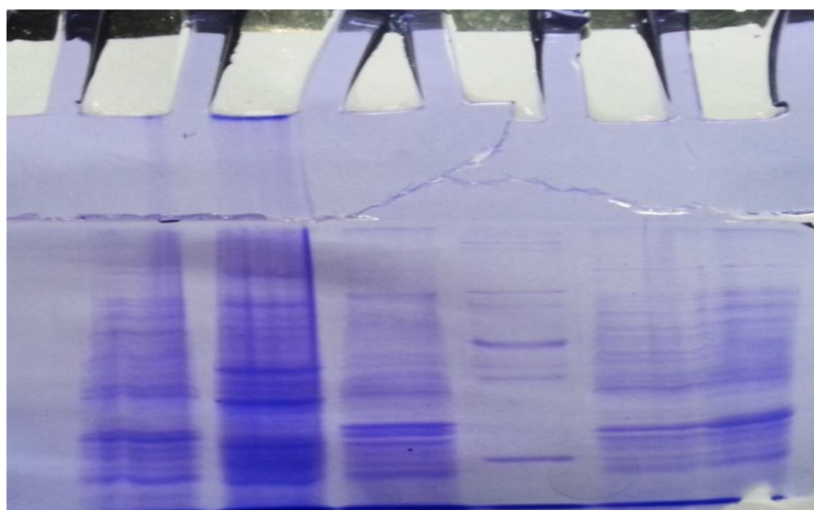
Table.1. Shows +/- reactions of Biochemical tests.

Biochemical test	Reaction
Lactose fermentation	+
Catalase	+
Simmon's citrate	-
Indole Production	+
Nitrate Reduction	+
Methyl Red	+
Voges- Proskauer	-
Urease	-
Acid from sugar	
Glucose	+
Mannitole	+
Lactose	+
Salicin	+
Sucrose	+

Recently, verocytotoxin producing *Escherichia coli* (VTEC), of serotype 0157:H7, have been cultured from retail fresh poultry products. In the past few years, VTEC have been associated with diarrhea, hemorrhagic colitis, haemolytic uremic syndrome and thrombotic thrombocytopenic purpura in humans. In recent outbreaks of these diseases, foods of animal origin such as hamburger, raw milk and sandwich meat are suspected sources of these organisms. Verocytotoxin-producing *E. coli* serotypes associated with human disease have been isolated from cattle; however, the importance of other food animals such as poultry, as reservoirs of VTEC for humans, is uncertain.

Protein Extraction: The proteins were extracted using ammonium sulphate and verotoxin was precipitated at 50% saturation of ammonium sulphate. The saturation above 50% did not precipitate any of the proteins.

SDS-PAGE: The proteins precipitated were determined using SDS-PAGE and it was found that 3 *E.coli* strains had bands of 32,000KDa. The presence of the band indicated that the protein was verotoxin and this three *E.coli* strains were vertoxin producers.
The E26 sampled showed bands with the respective molecular weight like 15, 21, 27, 38 and 41KDa. Bands with the molecular weight of 15, 20, 26, 32and 45 KDa were seen for E209. 16, 20, 21, 22, 30 KDa bands were seen in E29. E33 sample had bands with 14, 18, 20, 21, 32, 37, 42 and 53 KDa. E25 had similar bands as of E33 indicating that both the *E.coli* isolated is os the same strains (Fig.10).



E26 E209 E29 M E33 E25

Fig.10 SDS Gel

Lipopolysaccharide (LPS) from *E. coli* 0 157.H- strain 493- 1, isolated from a child with HUS was separated by the hot phenol water extraction method (procedure I) of Westphal and Jann and further purified using proteinase K (Sigma) followed by repeated phenol-water extractions and dialysis. Purified LPS was visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). There were no protein contaminants detected by Coomassie blue staining of the SDS-PAGE gel or after blotting on nitrocellulose. M. Bitzan *et al.* [22] showed the presence of free (faecal) verotoxin (FVT) was demonstrated in the stool filtrates of 16 patients associated with VTEC strains, and of 36 additional patients from whom VTEC was not isolated. HeLa and/or Vero cell toxicity of the stool filtrates ranged from 15 to 106 CD50 (geometric mean 4.7 x 102; median 2 x 102 CD50). The cytotoxic effect was neutralized by anti V'Tt/anti-Shiga toxin in 2 cases, by anti-VT2 in 44 out of 52 cases (85 %), and by a mixture of anti-VT1 and anti-VT2 in 6 cases (12%). None of the 9 E- patients excreted neutralizable FV'T.

Recently, Padhye *et al*. and Yutsudo *et al.* [23, 24] reported purification schemes for VTs that are immunologically unrelated to Shiga toxin. Padhye *et al*. present a scheme for purifying a unique Vero cytotoxin from culture filtrates by ultra filtration and anion exchange chromatography.

Yutsudo *et al*., describe a scheme for purifying VT2 from culture filtrates by ammonium sulphate fractionation, repeated chromate focusing chromatography, DEAE-cellulose chromatography and repeated high-performance liquid chromatography.

The strain (J-2) used by Yutsudo *et al.,* as a source of toxin is reported to produce only VT2. Other than the unique Vero cytotoxin that is not neutralizable with antibodies against Shiga toxin, Padhye *et al.,* do not comment on whether other cytotoxins are produced by their strain (EDL932). Strain EDL932, originally isolated at the Centers for Disease Control, was included in a study reported by Marques *et al.* [25] and it was founded to produce both SLT-I and SLT-II. Since only the neutralization data with anti-Shiga toxin has been reported, it is not clear that the toxin purified by Padhye *et al*. weather it is related to, or the same as, VT2 or SLT-II.

The production of a cytotoxin distinct from SLT-I and SLT-II is possible, since the culture and assay conditions used for detecting the SLTs may not have been optimal for other Vero cytotoxins. There were similarities and differences between the SLTII we purified and the

VTs reported by Yutsudo *et al*. and Padhye *et al*. Both VT2 purified by Yutsudo et al. and SLT-II consisted of A and B subunits of similar sizes. The both A and B subunits of SLT-II have molecular weights of 32,000 and 10,200 ± 800, respectively, whereas the A and B subunits of VT2 have molecular weights of 35,000 and 10,700, respectively.

The sizes of the subunits for SLT-II are also consistent with minicell and DNA sequence data reported for SLT-II. From the DNA sequence analysis of SLT-II, the predicted molecular weights of the mature A and B subunits were 33,135 and 7,817. The observed sizes of the B subunits for SLT-II and VT2 were larger than predicted for the mature B subunit from the nucleotide sequence data. The degree to which the B subunit is processed for SLT-II or VT2 is not known. Newland *et al*. [26] observed no processing of the SLT-II A and B subunits with polymyxin B treatment of labeled minicells. In contrast, Padhye *et al*., report that their toxin does not consist of subunits. The molecular weight of their toxin is 64,000 by SDS-PAGE and 54,000 by molecular-sieve chromatography. Although single bands were evident after Coomassie blue staining of SDS-polyacrylamide and isoelectric focusing gels, from their data it is not clear that the cytotoxicity migrated with these protein bands.

Few studies have addressed the frequency of non-0157 VTEC isolates from HUS patients [22, 27-29] or from patients with VTEC-associated diarrhea [30-34]. In our continuing study of the role of Vero toxin-producing *E. coli* in pediatric HUS in Germany, overall slightly < 10% of the HUS patients had evidence by bacterial isolation and/or LPS serology ofnon-O157 VTEC infection, with 026 being the most common serogroup (unpublished data). Serologic results presented in this study accord with the incidence of the implicated non-OI57 VTEC serovars. Our data indicate that patients with HUS associated with infection by non-OI57 VTEC strains can develop a robust, 0group- specific immune response similar to patients with *E. coli* 0157:H7 infection. We detected homologous antibodies in sera of 6 of 8 HUS patients with non-OI57 VTEC isolates. Ensuing, 7 of 17 VTEC culture- and 0157 LPS antibody-negative patients were found to possess elevated antibodies to non-O 157 LPS. In the absence of an immune response to 0157 LPS, detection of high antibody levels to non-O 157 LPS strongly suggests recent infection by corresponding *E. coli* serovars. Thus, a probable serologic diagnosis was possible in 41% of formerly undiagnosed patients using this panel of LPS ELISAs.

CONCLUSION

Based on our results we conclude that, the five *E.coli* samples were isolated from the clinical samples like urine, blood and stool of infected patients. From all the five samples proteins were extracted and precipitated. SDS-PAGE results shown that among five only three strains of *E.coli* strains had bands of 32,000KDa. The presence of the band indicated that the protein was verotoxin and this three *E.coli* strains were vertoxin producers.

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