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DETECTION OF SHIGA TOXIN GENES (*stx1 & stx2*) AND MOLECULAR CHARACTERIZATION OF SHIGA-TOXIGENIC *ESCHERICHIA COLI* ISOLATED FROM DIVERSE SOURCES IN GULBARGA REGION, INDIA

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ABSTRACT

We investigated the presence of Shiga toxin producing *E. coli* (STEC) in diarrhea patients (n=885) attending various health centers in Gulbarga as well as in fecal samples from diarrheic farm animals (n=158), ground beef (n=205), meat (n=157), sewage (n=182) and water (n=247) from ponds and bore wells. STEC was detected in 65 samples, in which detection of Stx1 & Stx2 gene was performed by polymerase chain reaction. Stx1 (n=56; 86.2%) was found to be present more frequently than Stx2 (n=7; 12.5%) and presence of both was noted in only two human stool samples (3.1%). Plasmid profiling and protein profiling by SDS-PAGE was performed on 30 and 18 of the isolated STEC strains respectively. A total of 9 different sized plasmids ranging from 1kb to 90 kb have been detected, forming about 12 different plasmid profiles in 30 SETC isolates. Plasmid profile was subjected for cluster analysis and the dendrogram developed indicated seven different clusters, the major cluster including as many as 12 isolates. Whole cell protein profiles were done for 18 STEC isolates. Seven distinguishing protein bands ranging in size from < 20 kDa to > 97.4 kDa were detected. The 66 kDa protein was found in only one isolate, while the protein of slightly less than < 97.4 kDa (n=10) was detected in maximum number of isolates. A large number of protein profiles were observed indicating a quite diversified nature of the STEC isolates. The dendrogram prepared using cluster analysis of the distinguishing proteins indicated the existence of only two closely related clusters of human and sewage isolates.

Keywords: Shiga toxin-producing *E.coli*, PCR, Plasmid profile, Protein profile, Molecular epidemiology.

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) is an important group of zoonotic human diarrheal pathogen associated with a wide spectrum of clinical conditions ranging from non-blood and bloody diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS).[1-4]

Though STEC serotype O157:H7 is considered epidemiologically significant worldwide, we cannot ignore non-O157, which has been linked to human infections at various geographical

locations.[5-7] STEC family is serologically diverse and more than 200 serotypes have been reported in which more than 100 are linked to human infections.[8]

Multiple virulence factors contribute to the pathogenesis of STEC. Its main pathogenic property is production of Shiga toxin (*Stx*), which inhibits the protein synthesis of the host cells leading to cell death. STEC has the ability to produce one or more *Stx's* (*Stx1*, *Stx2* or variants). *Stx1*, *Stx2* and their variants are heterogeneous and immunologically non cross reactive. Stx1 is virtually identical to shiga-toxin produced by *Shigella dysenteriae* type 1, while Stx2 shares only ~56% identity with Stx1.[9]

Some of the STEC strains harbour a ~90kb plasmid encoding several virulence determinants which includes enterohemolysin (*ehxA*), bi-functional catalase-peroxydase (*katP*), secreted serine protease (*espP*) and type II secreting system (*etpD*).[10,11]

Though cattle and sheep's are regarded as the principle reservoir of STEC, it is also prevalent in gastrointestinal tract of other domestic animals like pigs, dogs and cats.[12] Even a study from India showed presence of STEC in pigeon droppings.[13]

Transmission of STEC mainly occurs via consumption of contaminated food such as raw or undercooked meat products, which occurs as a result of contact of feces or intestinal content after slaughter and raw milk and even person to person contact is well documented during outbreaks.[14-16]

In the present study we investigated prevalence of STEC in diarrheal human stool samples, as well as in stool samples from cattle, in raw beef and mutton samples and in water and sewage sample. Isolated STEC strains were characterized to identify the predominant Shiga toxin genes (*Stx1* and *Stx2*), plasmid profiles and whole cell protein profile to understand how the Indian strains compare with STEC strains isolated elsewhere in the world.

MATERIALS AND METHODS

The research was carried out during several gastroenteritis epidemics in Gulbarga Region of Karnataka located in the southern part of India from 2004-2007.

Human Stool Samples

885 stool samples were collected from diarrheal patients attending different hospitals and health centers of Gulbarga city and surrounding rural places of Karnataka, India. Stool samples were collected in wide mouthed sterile bottles. Sterile cotton tipped swabs were used to take rectal swabs from patients from whom stool could not be obtained. Rectal swabs were placed in Cary-Blair medium and stool samples were transported to the laboratory within an hour for further processing.

Animal Fecal Samples

One hundred and fifty eight fecal samples were collected directly from the rectum of diarrheic animals from various livestock farms into sterile polythene bags and carried to the laboratory on ice.

Beef and Meat Samples

10 grams of ground beef (n=205) and meat samples (n=157) were collected in sterile flasks containing lactose broth and kept in ice box for transportation to the laboratory.

Water Samples

Two hundred and forty seven water samples were collected from wells, ponds and other water sources in sterile containers from different parts of Gulbarga region.

Sewage Samples

One hundred and eighty two sewage samples were collected from various regions of Gulbarga, in sterile screw capped containers.

Isolation and Screening of STEC

The samples were simultaneously streaked on to Sorbitol MacConkey's agar plates with cefixime-tellurite supplements (CT-SMAC agar) and were

incubated overnight at 37 ^{O}C for the isolation of STEC. Sorbitol negative colonies were tested for the production of ß-Glucuronidase, fermentation of cellobiose and growth in the presence of potassium cyanide by standard techniques to differentiate STEC strains from other strains of *E.coli* as they are unable to ferment sorbitol and MUG. Fermentation of different sugars and decaorboxylation of amino acids were tested. The sorbitol and MUG negative colonies were further tested for the presence of *stx* genes by the PCR.

Among the 1834 samples 65 were confirmed as STEC. Antibiotic susceptibility testing was performed on all the isolates using Kirby-Bauer's disc diffusion method as per National Committee for Clinical Laboratory Standards (NCCLS) guidelines as adapted by Naidu *et al.*[17]

PCR Detection of Virulence Factors

Crude DNA was obtained from the 65 STEC strains using boiling method. Sequence spanning of the Stx1 and Stx2 was amplified by polymerase chain reaction using *Taq* DNA polymerase (Qiagen, Germany). The PCR primers for Stx1 gene were FP-*Stx1* (5'-CAACACTGGATGATCTCAG-3'). RP-*Stx1* (5'-CCCCCTCAACTGCTAATA-3') and for Stx2 gene were FP-*Stx2* (5'-ATCAGTCGTCACTCACTGGT-3'), and RP-*Stx2* (5'- CTGCTGTCACAGTGACAAA-3') giving a product size of 349 bp and 100 bp respectively. Cycling condition for both were the following: 1 cycle at 94 ^{O}C for 1 min, 40 cycles at 94 ^{O}C for 45 sec, 55 ^{O}C for 1 min and 72 ^{O}C for 1 min with a final extension at 72 ^{O}C for 10 min.

Plasmid Isolation and Profiling

Each Single colony was inoculated individually in 3 ml of LB medium and grown overnight at 37 ^{O}C with shaking (~250 rpm). Plasmid DNA was isolated using the HiYield^{TM} Plasmid Mini Kit (Real Biotech. Corporation, Taiwan). A single digestion with *EcoRI* (New England Biolab, UK) was carried out according to manufacture's guide. After digestion, Plasmids were electrophoresed for 2 hours at 100 V on a 1.5% agarose gel using TAE buffer.

The Whole Cell Protein Profiles

The extraction of proteins from STEC cultures were performed as follows. Single colony was inoculated in 3 ml of LB broth and incubated over night at 37 ^{0}C. Samples were centrifuged for 5 min at 6000 rpm. The pellet was washed once with 1X PBS buffer (pH 7.4) and re-suspended in sonication buffer (1X PBS, pH 7.4, 0.5% Triton X-100 and protease inhibitor). After sonication (30:30 pulse) centrifugation was carried out at 13200 rpm for 30 min and the supernatant was collected. 24µl of supernatant was added with 7µl of 4X protein loading buffer and boiled for 5min at 90 ^{O}C. Total protein analysis was carried out by using SDS-PAGE by the method described by Nei M.[18]

Cluster Analysis

Different fragments on the gel were numbered sequentially and the presence or absence (presence 1, absence 0) of fragments in each sample was scored as described previously[19] according to the genetic distance method of Leotta GA.[20]

RESULTS

Polymerase Chain Analyses (PCR) of *Stx1* and *Stx2* Genes

Among the 65 isolated and phenotypically characterized STEC from 1834 samples, maximum isolation was observed from sewage (n=27/182), followed by animal feces (n=15/158), human stool (n=12/885), water (n=6/247), Beef (n=4/205) and meat (n=1/157). Majority of the isolated STEC harbored *Stx1* gene (n=56; 86.2%) compared to Stx2 gene (n=7; 10.8%), while only two of the human fecal isolates harbored both *Stx1* and *Stx2* genes (3.1%) (Table1).

Analysis of the Plasmid Profiles of STEC

A total of 30 out of the 65 STEC isolates were processed for the presence of plasmids (Table3). The 30 STEC isolates presented as many as 12 different plasmid profiles. One strain, 3S and 24S, isolated from sewage did not show the presence of any plasmid. The number of plasmids present in any one STEC isolate varied from just 1 to as many as six.

A mega plasmid of 90 kb was present in 70% (21) of the isolates, while the 21 kb plasmid was detected in 23.3% (7) of the isolates and the smaller plasmids 3 kb and 2 kb plasmids were detected in 16.6% (5) of the isolates each. The smallest (1kb) plasmid was observed in 5 isolates and the 50 kb plasmids were observed in 4 isolates.

Out of the eight human isolates, seven possessed the 90 kb mega plasmid, while the other one isolate contained a single 50 kb plasmid. The 18 kb, 4 kb and the 3 kb plasmids were not detected in the human isolates. Similarly, out of the 6 STEC isolates from cattle two possessed the 90 kb plasmid. All the isolates from beef and meat also contained the mega (90 kb) plasmid and the other plasmids, 21 kb, 4 kb, 3 kb and 1 kb, were randomly distributed among the STEC isolates from the food samples. Majority of the single mega plasmid (90 kb) containing isolates are either from the human stool, or from the sewage and water samples and rarely from the other sources. The largest plasmid (>90 kb) was also most frequently recovered from the water and sewage samples. The genetic relatedness of the 30 STEC isolates based on their plasmid profile has been analyzed by the Nei's genetic identity. The dendrogram thus generated indicates that the 30 STEC isolates from different sources form seven different clusters based on their plasmid profiles. Twelve isolates forming the major cluster possess only one mega plasmid. (Cluster 1). The isolate numbers 52, 49, 37, 20 (Cluster 3); 9, 38 and 25 (Cluster 4); 19 and 2 (Cluster 5); 58, 61, 26 and 53 (Cluster 6); 50, 58, and 48 (Cluster 7) formed the other close clusters. Cluster 2 possesses isolate 3S and 24S did not show any plasmid forming a separate cluster branching directly with Cluster 1.

Whole Cell Protein Profiles of STEC

A total of 18 STEC isolates were subjected for whole cell protein profile by SDS-PAGE analyses. The size range of these protein bands varied from the smallest band of <20 kd, to the largest band at >97.4 kd position. The protein which formed the band at <97.4kd (10) was observed in the maximum number of isolates. Interestingly five of these isolates are from the human stool samples from the diarrheic patients. The two proteins with molecular weights of >97.4 and <29 were found in 7 isolates each and the other protein with a molecular weight of <43 kd was recorded with 3 STEC isolates. The distribution of these 7 distinguishing protein molecules among the 18 STEC isolates was very diverse. Each pattern of this diversity is exhibited by only one isolate in most of the cases. While the three protein profiles patterns, with only one protein of >97.4 kd, two proteins of <97.4 kd and <29 kd and three proteins of >97.4 kd, <29 kd and <20 kd are exhibited by two isolates each. The largest protein with a molecular weight >97.4 kd is observed in STEC isolated from human (2), beef and cattle (2 each) samples only. Both the largest protein molecules of >97.4 kd and ≤97.4 kd together are detected in only three isolates, one from the cattle and two from humans. The smaller distinguishing proteins are more discretely distributed. Out of the six human STEC isolates, five differed from each other by possessing only one distinguishing band, while the remaining isolates possessed three distinguishing bands. The maximum numbers of four distinguishing protein bands were detected in only one STEC isolate from Cattle, while the remaining 17 isolates recorded the presence of one to three distinguishing bands. The genetic relatedness of the 30 STEC isolates based on their whole cell protein profile has been analyzed by the Hierarchical Cluster Analysis software of the Pop-Gen not SPSS (Statistical Programme for the Social Scientists)

and the dendrogram developed using the Average Linkage between the isolates is presented in fig. 2. The dendrogram is prepared based on the presence or absence of one or more of the 7 distinguishing protein bands. From the dendrogram it can be clearly observed that the 18 STEC isolates are much diverse in their protein profiles forming 3 different Clusters. Cluster 3 contains 3 closely related sub clusters A, B and C Interestingly the former cluster included 5 isolates from human sources, along with two from sewage forming sub-cluster C.

DISCUSSION

E. coli are the normal gut flora of the human beings, but certain subsets of this species have acquired virulence genes that enabled them to cause diarrhea and other extra-intestinal infections. Such is shiga toxigenic *E. coli* containing stx genes, which has direct enterotoxic properties resulting from selective targeting of Gb3 containing absorptive villus epithelial cells in the ileum.[2]

Members of stx family are compound toxins comprising a single catalytic 32 KDa A subunit and a multimeric B subunit (7.7 KDa Monomer) that is involved in the binding of the toxin to specific glycolipid receptors on the surface of the target cells.[2]

The profound sensitivity of vero cells to *Stx* and cytotoxicity for this cell line remains the gold standard for confirmation of putative *Stx* producing isolates.[15] However considering the clinical significance of O157, rapid, specific and sensitive detection methods are needed to identify toxin producing strains, and PCR is a sensitive and specific technique. For rapid detection of STEC from clinical samples, PCR has proven to be the widely used method for the detection of *Stx* genes.[21] The use of PCR technology permits the detection of *Stx* genes from samples that are microbiologically complex, including samples containing nonviable organisms.

In majority of the molecular typing of the epidemiological isolates of STEC the amplification of *Stx1* and *Stx2* genes with specific primers have been used. Direct PCR and multiplex PCR are currently more popular, wherein the presence of STEC in the stool samples can be identified within a short time.

Among the *Stx1* and *Stx2, Stx2* is considered to be the most important virulence factor associated with the human disease. It is about 400 fold more toxic to mice than *stx1* and also been shown to induce feto-placental re-absorption, intra-uterine haematoma, fibrin deposition and neutrophil infiltration. *Stx2* has approximately 55% amino acid identity to *stx1*.[9] In our study we observed the predominance of *Stx1* gene over *Stx2* gene. Only *Stx2* gene was found in human stools (n=2), Animal faeces (n=2) and Sewage water sample (n=3) and both *Stx1* and *Stx2* were observed only in 2 human stool isolates. A high preponderance of *Stx1* gene (n=56) was observed in all the STEC strains, which is similar to other studies from different parts of India and also similar to a study from Palestine.[6] On the contrary studies from other parts of the world, like Australia, New Zealand, Argentina and Brazil, have indicated the predominance of *Stx2* was found more frequently.[21-23] Also variability in the distribution of *Stx1* and *Stx2* has been noted in Bangladesh and Iran.[11,15]

A large number of plasmids have been detected in *E.coli* as well as a large number of other bacterial species and their role in antibiotic resistance and other variable characters has been well established. The occurrence of multiple antibiotic resistances has also been shown to be due to a greater genetic mobilization of the antibiotic resistance genes carried by the plasmids through transposons. Plasmid profile analysis has been used for typing of bacterial strains with high discriminatory potential and good reproducibility.[24] It has been proved as an useful method for differentiating isolates.[25] However, plasmid profiles do not reveal stable genetic differences of the strains. In fact, plasmid

profiles of the isolates are generally a useful tool for obtaining knowledge about resistance of the isolates to the antimicrobial substances and transfer of a plasmid among closely related isolates from different sources. Plasmid profile is one of the several useful methods for determining the relatedness or unrelatedness of bacterial strains that contain plasmid DNA. Out of the 30 isolates investigated plasmids were detected in 28 isolates. In majority of the isolates only one plasmid was present while in few two to four plasmids were detected.

Protein profiling by SDS-PAGE is also a very reliable and reproducible molecular technique with good discriminatory potential that has been used by many workers to type various microorganisms of epidemiological interest.[19] SDS-PAGE usually combined with dendrogram derived from the numerical analysis of the whole cell protein patterns of the strains has been used extensively to study the differences among bacterial genera, species and even strains.[25] SDS-PAGE typing has been proved to be more discriminating for typing the isolates.[19] In the present investigation the protein profiles of the 18 STEC individuals has been determined and this analyses indicates a greater diversity of the isolates as indicated by only two close sub-clusters and unrelatedness of majority of the isolates. However, the clusters included isolates mostly from the same source, indicating the usefulness of the protein profiles as one of the methods, which can be used in the characterization of the bacteria, especially STEC.

We observed presence of STEC more in water and sewage samples compared to beef and meet samples. Thus the spread of STEC infection among the humans could have been from contamination of food with the water and sewage signifying poor level of hygiene maintained. However we cannot overlook the presence of STEC in the food chain also, where the feeding habit of a majority of population is beef and meet. Various studies from India and globally have shown presence of STEC in meat and beef.[26,27] This probably could be attributed to the asymptomatic carriage of STEC in the feces of the farm animals, and the fecal contamination of the meat during slaughter.[28]

In our previous publication we have reported high degree of antibiotic resistance among these STEC strains with a high percentage resistance to ampicillin (73.8%), streptomycin (70.8%), cephalexin (63%) and almost 50% of all the STEC isolates showed resistance to 9 or more tested antibiotics.[17] The presence of STEC in diverse source having a high level of antibiotic resistance is of utmost public health concern in the region of Gulbarga.

Based on the plasmid and protein profile it can be hypothesized that the gastroenteritis epidemics in southern part of India mainly in Gulbarga region was due to environmental contamination of water and meat related products with the infected strains. With an expanding knowledge of virulence factors possessed by STEC isolated from humans and other sources analyses such as ours, although tedious, can help delineate the characteristics that are necessary for the pathogenicity of diarrheogenic *E. coli*.

ACKNOWLEDGMENTS

We are thankful to all who have provided the samples.

Table 1: Frequency distribution of *Stx1* and *Stx2* genes in the Shiga toxigenic *E. coli* (STEC) strains isolated from diverse sources

Sample	STEC	Stx1	Stx2	Stx1 & Stx2
Human diarrheal stool (n=885)	12 (1.4%)	8	2	2
Beef (n=205)	4 (2%)	4	-	-
Meat (n=157)	1 (0.6%)	1	-	-
Water from Ponds and Bore wells (n=247)	6 (2.4%)	6	-	-
Sewage (n=182)	27 (14.8%)	24	3	-
Farm animal faeces (n=158)	15 (9.5%)	13	2	-
Total (n=1834)	65 (3.5%)	56	7	2

Table 2: Size based presence or absence of plasmids in the STEC

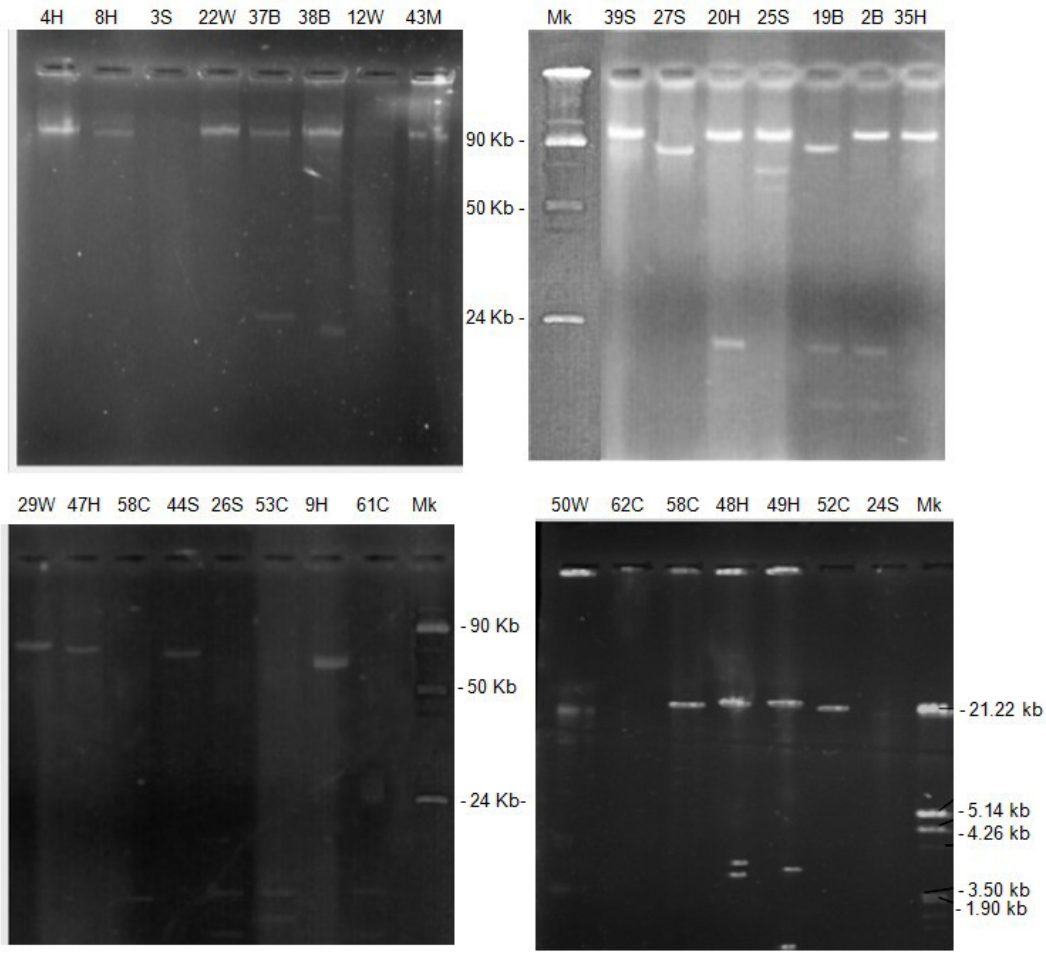
Strain No	No of plasmids	90kb	>50kb	50kb	21kb	18kb	4kb	3kb	2kb	1kb
4H	1	+	-	-	-	-	-	-	-	-
8H	1	+	-	-	-	-	-	-	-	-
3S	-	-	-	-	-	-	-	-	-	-
22W	1	+	-	-	-	-	-	-	-	-
37B	2	+	-	-	+	-	-	-	-	-
38B	3	+	+	-	-	-	-	+	-	-
12W	1	+	-	-	-	-	-	-	-	-
43M	1	+	-	-	-	-	-	-	-	-
39S	1	+	-	-	-	-	-	-	-	-
27S	1	-	+	-	-	-	-	-	-	-
20H	2	+	-	-	+	-	-	-	-	-
25S	3	+	+	+	-	-	-	-	-	-
19B	3	+	-	-	-	-	+	-	+	-
2B	3	+	-	-	-	-	+	-	-	-
35H	1	+	-	-	-	-	-	-	-	-
29W	1	+	-	-	-	-	-	-	-	-
47H	1	+	-	-	-	-	-	-	-	-
58C	1	-	-	-	-	-	-	+	-	-
44S	1	+	-	-	-	-	-	-	-	-
26S	2	-	-	-	-	-	-	+	-	+
53C	2	-	-	-	-	-	-	+	-	+
9H	1	-	+	-	-	-	-	-	-	-
61C	2	-	-	-	-	-	-	+	-	+
50W	4	+	-	-	+	-	-	-	+	+
62C	1	+	-	-	-	-	-	-	-	-
58C	4	+	-	-	+	-	-	-	+	+
48H	4	+	-	-	+	-	-	-	+	+
49H	2	+	-	-	+	-	-	-	-	-
52C	1	-	-	-	+	-	-	-	-	-
24S	-	-	-	-	-	-	-	-	-	-

N.B.- (+) indicates as presence and (–) indicates as absence

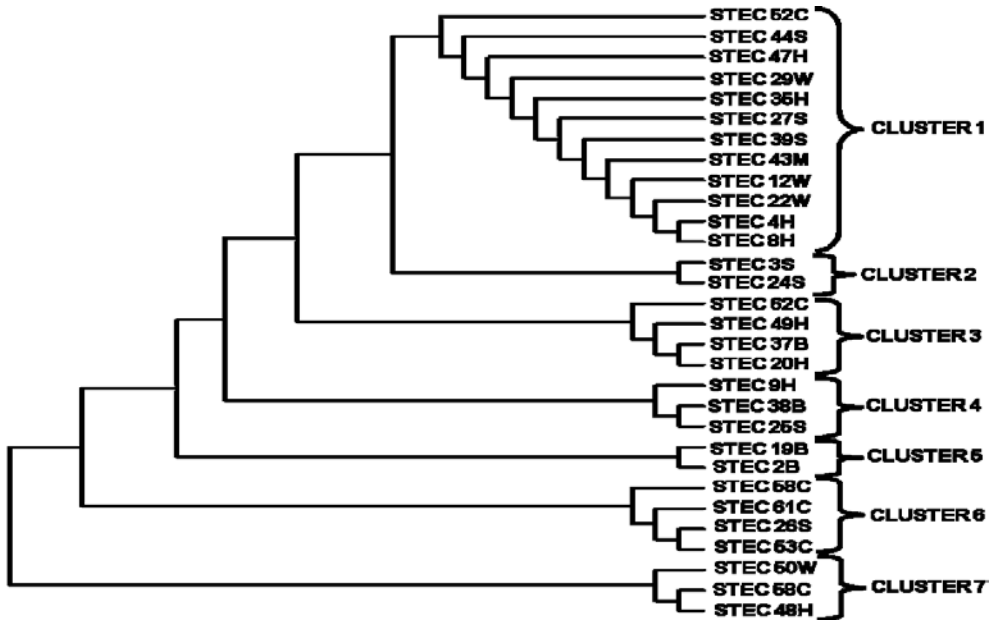
Table 3: Presence of Distinct protein band in the STEC strain

Strain No	Distinct Band	Molecular Weight of Protein in kDa						
		>97.4	≤ 97.4	>66	≤66	≤ 43	≤29	≤20
19B	3	+	-	-	+	-	-	+
12W	2	-	-	-	-	+	-	+
2B	1	+	-	-	-	-	-	-
50W	3	-	+	-	-	-	+	+
47H	3	+	+	-	-	-	-	+
49H	1	+	-	-	-	-	-	-
62C	4	+	+	+	-	-	+	-
37B	2	-	-	-	-	-	+	+
25S	2	-	+	-	-	-	+	-
22W	2	-	-	-	-	-	+	+
4H	1	-	+	-	-	-	-	-
8H	3	+	+	-	-	-	-	+
38B	1	-	+	-	-	-	-	-
58C	3	+	-	-	-	-	+	+
48H	1	-	+	-	-	-	-	-
43M	2	-	-	-	-	+	+	-
24S	2	-	+	-	-	-	+	-
20H	1	-	+	-	-	-	-	-

N.B; (+) indicates as presence and (–) indicates as absence



A.



B

Figure 1(A,B): The dendrogram based on plasmid profiles of Shiga Toxin Producing *E. coli* isolates

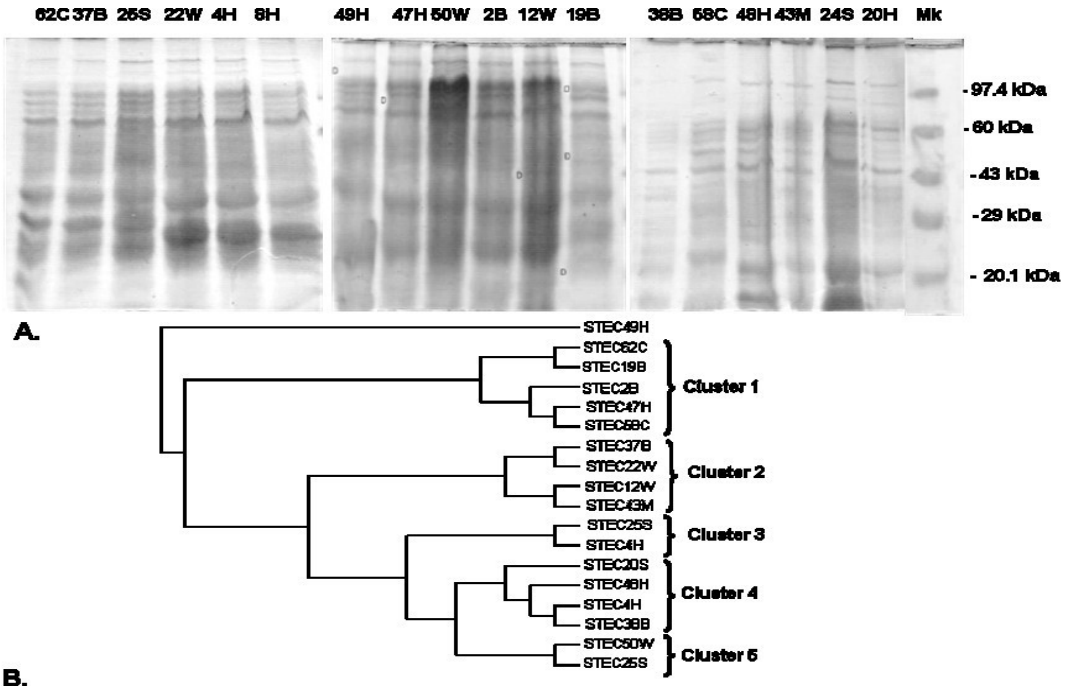


Figure 2(A,B): The dendrogram based on whole cell protein profiles of Shiga Toxin Producing *E.coli* isolates.

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