IDENTIFICATION AND CHARACTERIZATION OF ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) VARIANT AND SCREENING OF THEIR ANTIGENIC PROTEINS.

142553

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CERTIFICATE

This is to certify that the work titled "**Identification and Characterization of Enteropathogenic *Escherichia coli* (EPEC) variant and screening of their antigenic proteins."** submitted by "**Deeksha Gupta"** in partial fulfillment for the award of degree of M.Tech Biotechnology from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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LIST of ABBREVIATIONS

WHO	World Health Organization
EPEC	Enteropathogenic *Escherichia coli*
aEPEC	Atypical Enteropathogenic *Escherichia coli*
tEPEC	Typical Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
NSP4	Non-structural protein 4
EHEC	Enterohemorrhagic *Escherichia coli*
IVAC	Vaccine Access Center
UNICEF	United Nations Children's Fund
GAPPD	Global Action Plan for Pneumonia and Diarrhoea
ORS	Oral rehydration salts
BFP	Bundle forming pillus
LPS	Lipopolysacchride
$PAMP_S$	Pathogen associated molecular patterns
PRR	Pattern Recognition Receptors
NF-kappa B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PMN	Polymorpho nuclear leucocytes
T3SS	Type 3 secretion system
Nle	Non-LEE-encoded effector
Tir	Translocated intimin receptor
EAEC	Enteroaggregative *Escherichia coli*
EICE	Enteroinvasive *Escherichia coli*
ICDDR,B	International Centre for Diarrhoeal Disease Reasearch, Bangladesh
LEE	Locus of enterocyte effacement
LAL	Localized adherence-like
CheA-Z	Chemotaxis- related proteins
flhA	Flagellar biosynthesis genes
MCP_s	Methyl-accepting chemotaxis proteins

Aer	Aerotaxis receptor protein
cdt	Cytolethal distending toxin
HUS	Hemolytic uremic syndrome
lysp	Lysine Permease
mdh	Mallate Dehydrogenase
°C	Degree Centigrade
IGMC	Indira Gandhi Medical College
rpm	Revolutions per minute
BSA	Bovine Serum Albumin

ABSTRACT

Diarrhoea is the second most common cause of deaths in young children. *Escherichia coli* and its different pathotypes are generally associated as one of the major etiologic factors. Enteropathogenic *E.coli* (EPEC) pathotype have been explored extensively as major contributor among all pathotypes of diarrhoeagenic *Escherichia coli*. However, novel and unusual strains of EPEC have also been reported by several groups. One of the unusual patterns producing EPEC is *Escherichia albertii* which shows the new emergence, associated with gastrointestinal disorders in various parts of world. In the present study an attempt was made to answer the questions that arose during the analysis of Next Generation Sequencing (MiSeq). 16sRNA genes analysis through MiSeq from diarrhoea stool samples of paediatric patients revealed the presence of *E.albertii* in our region. Therefore, EPEC strains of diarrhoeagenic *Escherichia coli* were further taken forward for the biochemical and molecular characterization. 45 EPEC strains (*eae* gene +ve) were targeted by multiplex PCR for *lysp* (lysine-specific permease) and *mdh* (malate dehydrogenase) genes. Among them, total 11 strains were positive and marked the presence of *lysp* and *mdh* along with *eae* gene confirming these as EPEC-variant strains. Their biochemical and molecular profiles were similar as with the reported *Escherichia albertii* profile. The *lysp* and *mdh* amplified genes also confirmed by Sanger sequencing and results overlapped with the genome of *Escherichia coli*. SDS-PAGE technique was done to observe comparative membrane proteins profiling of pathogenic and non-pathogenic strains so that the novel membrane proteins may be further processed as antigenic proteins. SDS-PAGE profile revealed the differences in the banding patterns of pathogenic (EPEC and EPEC-variant) and non-pathogenic (*E.coli*- DH5α) *Escherichia coli*. Remarkable biochemical, molecular and proteomic assets were observed in these atypical EPEC strains which correspond to mysterious clades in phylogeny of *Escherichia* genus, which further needs to be explored.

CHAPTER 1

Introduction

Diarrhoea is a disturbance in the normal mechanisms controlling secretion and absorption of water which causes excess loss of water in faeces. World Health Organization (WHO) defines diarrhoea as passage of loose or watery stools for more than three times per day. Enteric infections cause diarrhoea which is the major factor of morbidity and mortality, globally. Diarrhoea, behind pneumonia is considered to be the second most common cause of death among children under five, globally. It is estimated that diarrhoea accounted for 9% of the 5.9 million deaths among children under-five in 2015 (Geneva:WHO Report, 2015). India, accounts for the greatest number of child deaths due to diarrhoea. In one of the studies it was found that the major etiological factor for the death of children under-five include Enteropathogenic *Escherichia coli* (EPEC), Enterotoxigenic *Escherichia coli* (ETEC) , calicivirus and rotavirus in year 2011 (Lanata *et al*. , 2013; Srivastava et *al*., 2015).In one of the recent studies, the major etiological agent of nosocomial diarrhoea was diarrhoeagenic *Escherichia coli*, found in 49% of the cases and the leading pathotype was Enterotoxigenic *Escherichia coli* (ETEC) i.e. 17% and the least affecting pathotype found was EPEC, i.e 7%. *Clostridium difficle* was not the major cause of the nosocomial diarrhoea. It was only detected in 9% of the cases (Chandra *et al*., 2012; Carlton *et al.,* 2015). Diarrhoeagenic *Escherichia coli* includes 5 different types of pathotypes in which Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *Escherichia coli* (EHEC) are misidentified with the new species in the *Escherichia* genus, i.e. *Escherichia albertii*. *Escherichia albertii*, earlier tentatively recognized as *Hafnia alvei* as they share similar biochemical properties like inability to ferment lactose or D-sorbitol, unable to produce indole, and shows similar antimicrobial susceptibility profiles (Nimri.,2013).Conventional biochemical characterization, susceptibility to the cephalothin antibiotics and *Hafnia*-specific phage, presence of *phoE* gene and partial 16s rDNA sequencing have supported the fact that this atypical species belong to the *Escherichia* genus (Huys *et al.,* 2003). *E.albertii* is a potential diarrhoeagenic pathogen in humans and also seen to cause infection in birds and animals. There are no reports of *E.albertii* from India. In 2014, next generation sequencing (MiSeq) was performed for whole DNA isolated from diarrhoea stool samples of paediatric patients. The positive results of sequencing marked the presence of *E.albertii* in this region. Phylogenetic analysis revealed that genus *Escherichia* comprised of species *albertii* instead of *coli* in diarrhoea disease patients. Worldwide, improvements in preventing the diarrhoea is heading towards the success and the latest three years reports published by Vaccine Access

Center (IVAC) at the Johns Hopkins Bloomberg School of Public Health shows that there is decrease in the number of death (annual child mortality rate) of under five year children due to diarrhoea. The published statistics shows that there is reduction from 6.9 million to 5.9 million deaths of below five age group children in 2015 compared to 2013. World Health Organization (WHO) and United Nations Children's Fund (UNICEF) have set the goals to end preventable deaths by introducing a Global Action Plan for Prevention and Control of Pneumonia and Diarrhoea by 2025. The Integrated Global Action Plan for Pneumonia and Diarrhoea (GAPPD) states certain interventions for prevention of diarrhoea which includes excluding breastfeeding for six months and then continued breastfeeding with some other supplements, vaccines against rotavirus, proper diagnosis of the diarrhoea in patients, oral rehydration salts (ORS) with low osmolarity formulae with zinc supplements, solving the hygiene issues in the society and the air pollution problems in rural areas. The major goal of this plan is to reduce the prevalence of diarrhoea cases in children (under five) by 75% as compared to 2010 levels. IVAC has evaluated progress in the 15 highest burden countries of under-five children deaths due to diarrhoea and pneumonia. The evaluation of diarrhoea component was included in the year 2013. These 15 countries includes India, Nigeria, Pakistan, The Democratic Republic of the Congo, Ethiopia, Angola, China, Afghanistan, Indonesia, Kenya, Sudan, Bangladesh, Niger, Chad, and Uganda. Around 75% of the deaths occur in these 15 countries, though these countries covers only the half of the population of the under-five children. In 2013, India and Nigeria are the top two countries which have the highest mortality rate due to diarrhoea and lowest GAPPD intervention score i.e. 22%. In 2014, the Nigeria and Uganda showed gains in their GAPPD- diarrhoea scores. Overall, diarrhoea treatment rates in the 15 highest-burden countries continue to be well below the GAPPD target (90%), with zinc coverage being drastically lower than ORS coverage. In 2015, prediction of 5.6 million children death (under five) worldwide where diarrhoea is responsible for 9% of the deaths is done. Together these diseases are responsible for 1.5 million deaths in a single year. In each passing minute, six children are lost due to diarrhoea. The burden was same as of in the 2014 but between 2014 and 2015, Uganda and Kenya dropped from the list of the 15 countries with the greatest number of under-five deaths from diarrhoea (Geneva:WHO Report, 2015).. Vaccination programme for the diarrhoeal diseases are important for saving the life of children and adults. Vaccine for rotavirus is currently available and is recommended for global use by the WHO. Vaccine for EPEC is not yet developed.

CHAPTER 2

2. **Review of Literature**

2.1 Enteropathogenic *E.coli* (EPEC)

Enteropathogenic *E.coli* is the first pathotype identified among all the diarrhoeagenic *E.coli* (Neter *et al*., 1955). Initially, all *E.coli* was named as EPEC but now EPEC pathotype is distinguished from others based on their pathogenic characteristics. This group of pathotype belongs to the attaching and effacing (A/E) pathogens as they attach to the enterocytes present in microvillus of the intestinal lumen and efface and causes lesions on the surface of intestinal epithelial cells. EPEC is classified into two types, atypical strains and typical strains. This classification is done on the basis of presence or absence of *E.coli* adherence factor plasmid (pEAF) (Croxen *et al*., 2013). MLST results shows that strains of EPEC are divided into 4 clonal lineages, EPEC1 to EPEC4 and it seem to evolve through acquisition of LEE and pEAF (Lacher *et al*., 2007). Vaccine for this pathotype is not yet developed. The possible candidates for vaccine development against EPEC are BfpA and EspB. BfpA is the structural protein subunit A of bundle forming pillus (BFP) and EspB is the type 3 secreted pore forming protein (Flores *et al*., 2002). Due to the similar pathogenesis, the possible vaccine candidates for *E.albertii* could be BfpA and EspB.

2.2 EPEC and *Escherichia albertii*

Escherichia albertii, a species in genus *Escherichia*, is a facultative anaerobic Gram negative rod which is an emerging enteric pathogen associated with diarrhoea in humans. It is considered to be a potential food-borne pathogen and is also responsible for infection in birds and finches (Nimri, 2013; Fiedoruk *et al*., 2014; Oaks *et al*., 2010). Diarrhoeagenic *Escherichia coli (E.coli)* are classified into five different pathotypes like Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enterohemorrhagic *E.coli* (EHEC), Enteroaggregative *E.coli* (EAEC), and Enteroinvasive E.coli (EICE). Other bacteria responsible for causing diarrhoea are *Shigella* spp., *Vibrio cholerae*, *Salmonella* spp, *Campylobacter jejuni, Clostridium difficile, Yersinia* species. Rotavirus, norovirus, astrovirus, enteric adenovirus are also responsible for causing enteric infections. Parasites like *Giardia lamblia* and *Entamoeba histolytica* are potential cause of parasitic diarrhoea (Hodges *et al*., 2010; Thapar *et al*., 2004). Diarrhoea is basically altered movement of ions and water which is due to colonization of infectious micro-organism on the mucosal surface

of intestine. Genus *Escherichia* is already composed of five different species, *E.blattae*, *E.hermannii*, *E.vulneris* , *E.fergusonii* and the type strain *E.coli* (Farmer et al, 1999 ; Abott *et al*., 2003). *E.albertii* is the sixth species, recently described when its first outbreak in six Bangladeshi children happened (Hyus *et al.*, 2003). *E.albertii*, a novel species is misidentified as EPEC or EHEC due to the presence of similar genes which are responsible for pathogenesis (Janda *et al*., 1999). Recent cases of diarrhoea have seen the presence of *E.albertii* in stool samples but unfortunately it is misidentified as EPEC or EHEC and misleads the presence of actual infectious microorganism, causing diarrhoea. Recently, determination of the complete genome sequencing of three *E.albertii* strains is being done (NIAH_Bird_3, CB9786 and EC06-170) and the draft genome sequencing of 26 *E.albertii* strains is also published (Ooka *et al*., 2015). This gives the confirmation that *E.albertii* is different from *E.coli* and is also responsible of causing diarrhoea.

2.3 History

In year 1991, *Hafnia.alvei* was isolated from 9-month-old female's stool sample brought to the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and was suspected as a possible cause of the watery diarrhoea. Light and electron microscopy showed the "attachment-effacement' mechanism of pathogenesis by *H.alvei*. It is already a well-known virulence characteristic shown by the EPEC, causing diarrhoea. The locus responsible for pathogenesis is locus of enterocytes effacement (LEE) - encoded type III secretion system. This locus consists of intimin, an *eae* gene-encoded outer membrane protein, and type III secretion system effectors. Earlier, this piece of information set a hypothesis that this mechanism is conserved among diverse species within the family of *Enterobacteriaceae* (Albert *et al*., 1991). Further research with the true *H.alvei* and other seven strains isolated from diarrhoea samples at the ICDDR, B showed differences on the basis of biochemical characteristics, antimicrobial susceptibility test and the mechanism of pathogenesis, and was concluded that they either are unusual biotype of *E.coli* or the novel species in the *Escherichia* genus (Albert *et al*., 1992). Additionally, partial 16S rRNA sequence analysis showed relatively low level of similarity between the *eae*-positive and *eae*-negative *H.alvei*. Positive results of PCR for intimin encoding *eae* gene is the major reason that earlier and still today, this novel species is misidentified as EPEC or EHEC (Janda *et al.*, 1999).

In year 2003, Huys and his colleagues, manifested and finally concluded the taxonomic position of the five strains recovered from diarrhoeal stool samples at the ICDDR, B by

DNA-DNA hybridization, phenotypic characterization and 16S rDNA sequencing. DNA-DNA hybridization results showed that above isolates constitutes a homogenous taxon and is closely related to the genus *Escherichia* but still below 70% related to, *E.coli* and *Shigella flexneri*. Also, phenotypic results indicated that this novel group of *Escherichia* strains are different from the closely related enterobacterial species. This concluded that ICDDR, B strains represents a novel species in the genus *Escherichia*, which was proposed to be *Escherichia albertii* (Hyus *et al.*, 2003).

2.3.1 Outbreaks

Sporadic infections as well as food-borne outbreaks caused by *E.albertii* have been reported, although rarely. First outbreak of *E. Albertii* was reported in Fukuoka City of Japan in 2003. The suspected cause of this outbreak was boxed lunches. Food was consumed by 31 people and among them 20 people showed symptoms like abdominal pain, fever and developed diarrhoea. Earlier the isolated strains of this outbreak were identified as EPEC, though phenotypically they showed atypical *E.coli* i.e. no lactose and sucrose production. Serotyping (OUT:H-) and molecular results (*eae* positive) concluded that the strains isolated in outbreak were EPEC OUT:H-. Recently the study has been conducted to confirm whether previously identified strains are *E.coli* or *E.albertii.* All *eae* positive strains were examined for the confirmation. Biochemical characterization showed the characteristics correspond to the features of *E.albertii*. Molecular characterization was done by targeting *lysp, mdh* and *clpx* genes for PCR. All the three genes were amplified in the outbreak isolated strains and only *clpx* was amplified from EPEC (Fuk-EC606). This molecular profile confirmed the presence of *E.albertii*. This current study revealed that earlier the strains were misidentified as EPEC but actually there was an outbreak of *E.albertii* (Asoshima *et al*., 2014).

Second outbreak of gastroenteritis also occurred in Fukuoka City of Japan in 2005. The major cause of this outbreak was contaminated water consumption by the 393 students and 16 teaching staff at the Oita Prefecture town where they went for camping. Symptoms like watery diarrhoea, abdominal pain, vomiting and fever were seen in 174 students and 2 staff members. Food was not the reason for the infection in this outbreak. Water resource was spring water and water saved in tank. Both stool and water sample were taken for the testing. 20 patients stool samples were tested in Fukuoka City Institute for Hygiene and the environment whereas 5 water samples were examined at the Oita Prefectural Institute of Health and Environment. Only 11 patients were positive for *eae* and negative for other

virulence gene (VT-, LT-,ST-,*invE-*,*ipaH-*,*bfpa-*,*aggR-*). Serotyping results showed presence of OUT:H- serotype and one of the 11 patients showed presence of O168:H serotype. The biochemical properties of these isolates are: Motility-,TSI agar: R/Y, Beta-galactosidase+ and Beta glucouronidase-. The *eae* positive *E.coli* strains isolated from water resources were also sent for serotyping and three different kind of serotypes were found (OUT:HNT,O119:HNT,O168:H-). The biochemical properties of O168: H serotype strain isolated from storage tank water and bathroom water shares the biochemical properties with the same as of patient isolate. Onset of symptoms varied greatly and it was concluded that it is caused by EPEC *E.coli* and infection source was spring water of campground (Baba *et al*., 2006).

In May 2011, the third outbreak occurred in Kumamoto, Japan where people attended two parties in restaurant. Total number of people who attended first party is 86 and among them only 43 showed symptoms whereas total 8 people attended second party and among them only 5 showed symptoms. The main symptoms of patients were diarrhoea (83%), abdominal pain (69%), fever (44%) and nausea (29%). Total 54 faecal specimens were taken for the examination. Results showed the presence of atypical *E.coli* in 24 specimens which were lactose negative and OUT: H- strains. All these strains were *eae+*. *E.coli* O183:H18 strain was isolated from 3 specimens and was s*tx2d+*, *ast+* and *eae-*. The atypical *E.coli* strain was suspected as *E.albertii*. To confirm the presence of *E.albertii* multilocus sequencing analysis (MLST) was done and they examined intimin subtyping by sequencing *eae* gene, *cdt* typing, and the chromosome integration site of the locus of enterocytes effacement encoding the *eae* gene and a set of type III secretion system genes. Results showed that *E.albertii*-like OUT: HNM strains belong to *E.albertii* lineage, as all 6 strains showed identical sequences. Presence of intimin sigma in *E.albertii* was identified, which is the rarely identified in EPEC and EHEC. The locus of enterocytes and effacement integration was found into the pheU tRNA gene and *cdt* type II/III/V are present in *E.albertii*. These above results concluded the presence of *E.albertii* at the larger scale than *E.coli* O183:H18. Symptoms caused by *E.albertii* infection were higher in comparison to the *E.coli* O183:H18 infection. This made the *E.albertii*, the major cause of outbreak (Oaka *et al*., 2013).

In November 2011, the fourth outbreak was occurred in Akita Prefecture in 22 patients, Japan, where 1 of the patients was detected with EPEC infection and 2 of the patients with norovirus infection. Norovirus infected patient was also infected with one another strain designated EC1506. This new strain was characterized at biochemical level, molecular level,

and 16S rRNA sequencing. Differentiation between EC1506 and other diarrhoeagenic *E.coli* was done by PCR. Results showed that EC1506 is only positive for *eae* but negative for other virulent genes of diarrhoeagenic *E.coli* (*stx,aggR,astA,elt,est,invE*). At this stage EC1506 was suspected as *E. Albertii*. Biochemical characterization showed different results, like indole negative but PCR results identified EC1506, as *E.albertii*. Differentiation between EPEC and EC15062 was clearly done on the basis of these respective virulence genes: *uidA+,lysp-,mdh-,stx-,eae+,cdtB-* (profile shown by EPEC), *uidA-,lysp+,mdh+,stx-eae+,cdtB+* (profile shown by EC15062). *Cdt* typing was also done and EC1506 strain showed presence of two *cdt* type i.e III and V. Normally, in *E.coli* CDTs are classified into 5 types. Significant association between *cdt* and diarrhoea is still unclear but it is considered that *cdt* can be a putative virulence marker in *E.albertii.* Conclusion cannot be made that *E.albertii* is identified as etiological agent in this outbreak as co-infection with norovirus is seen. Moreover, *E.albertii* infection was only seen in 1 patient. It is the first study of Japan where isolation of *E.albertii* from diarrhoeal patients is reported (Konno *et al*., 2012).

2.4 Pathogenesis

E.coli is a mucosal pathogen and acquires certain strategy to infect the mucosal surfaces of the host. Diarrhoeagenic E.coli colonizes the mucosal surface in a very conserved manner. Diarrhoeagenic E.coli strain has specific fimbriae which adhere to the surface of small bowel mucosa of the host (Nataro *et al.*, 1999). After colonization, the pathogenesis of the diarrhoeagenic *E.coli* varies depending upon its pathotype. Three different types of strategies of the diarrhoeagenic *E.coli* are seen which include production of enterotoxin by ETEC and EAEC, invasion by EIEC and intimate adherence with membrane signalling by EPEC and EHEC for pathogenesis. Here, pathogenesis of EPEC or EHEC is explained in detail because the variant of EPEC, *E.albertii* shows the similar pathogenesis. The hallmark of the EPEC pathogenesis is attaching-and-effacing (A/E) histopathology observed on the mucosal surface. The A/E lesions were observed on the mucosal surface and it was first seen by the Staley *et al*, 1969 and Polotsky *et al*., 1977. (Chen *et al*., 2004). EPEC is a prototype organism for strains causing A/E histopathology and other members of A/E pathogen family are *E.albertii*, LEE-positive STEC, and other animal pathogens such as rabbit-EPEC, porcine-EPEC, dog-EPEC and *Citrobacter rodentium*-EPEC (Goosney *et al*., 2000). A three-stage model of EPEC pathogenesis was first described in the early 1990s by Donnenberg and Kaper. The pathogenesis model include localized adherence to host cells, signal transduction, and intimate attachment (Donnenberg *et al.*, 1992). Localized adherence of both typical

EPEC and atypical EPEC are different. They get attached to the small intestine, enterocytes cells. In tEPEC, the initial attachment is by type IV BFP (bundle forming pilli) which tethers the bacteria on one another and forms the localized adherence (LA). This can be seen after 3hours of infection in Hep-2/ HELA cells. EPEC1 and EPEC 2 possess large EAF plasmids. The self-transmissible EAF plasmid pMAR2 is found among strains of the EPEC1 lineage and contains an intact transfer region, unlike pB171, which is more common among EPEC2 strains (Brinkley *et al*., 2006). These plasmids are virulent and cause infection in human volunteers. In contrast, aEPEC strains do not harbour pEAF and thus do not produce BFP, resulting in the formation of loose clusters of bacteria on tissue culture cells. This pattern, known as "localized adherence-like" (LAL), is slower to establish and can be seen after 6 hours of infection. Signal transduction and intimin attachment is done. Intimin is a protein encoded by the *eae* gene and it gets attached to the bacterial translocated intimin receptor, Tir (Gruenheid *et al.*,2001). There are different subtypes of intimin based on the change in C-terminus of the intimin protein. tEPEC has α and β subtypes, whereas aEPEC strains have ε, ζ and γ subtypes (Tardelli *et al*., 2010). As soon the intimin gets attached to the intestinal cells it induces varities of signal transduction pathways within the infected host and leads to the subversion of many cellular processes which benefits the pathogen. These attaching and effacing pathogens have a locus of enterocytes effacement (LEE) which encodes type three secretion systems (T3SS). The function of T3SS is to secrete protein components like EspA, EspB and EspD and drives effectors directly into the host (Muller *et al*., 2009). The genome of EPEC encode for seven LEE-encoded effector genes in which Tir is one of the extensively studied receptor. The function of Tir is effacement of microvillus and it also recruits cytoskeletal proteins for pedestal formation (Dean *et al*., 2009). The other six effectors include Map, EspF, EspG, EspZ, EspH, and Esp B which play roles in A/E pathogen infection. The function of Map is to stimulate formation of membrane filopodia and epithelial barrier disruption along with mitochondrial dysfunction (Ma *et al*., 2006). EspF and EspG, both shows multifunctional properties which affect aquaporin localization, leading to diarrhoea and EspF also disrupts tight junctions. (Guttman *et al*., 2007; Guttman *et al*., 2006) while EspG alters host cytoskeletal components through its interaction with tubulin (Hardwidge *et al.*, 2005) and EspH influence filopodium formation and participates in actin signalling during pedestal formation (Wong *et al*., 2012). Both EspH and EspB inhibit phagocytosis of EPEC by macrophages (Dong *et al*., 2010).

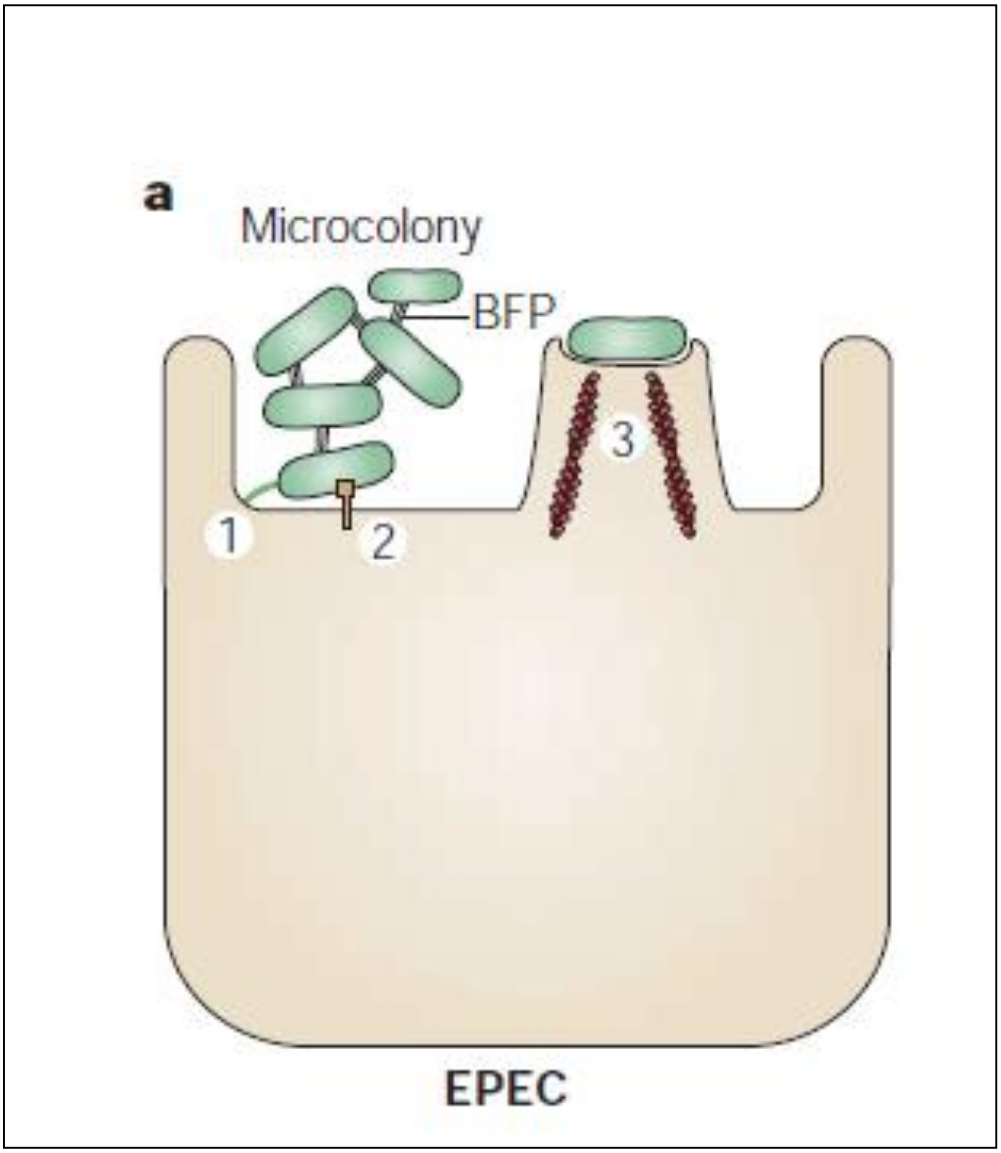

Figure 1. Mode of pathogenesis of Enteropathogenic *Escherichia coli*. 1. Initial adherence, 2.Translocation of proteins by T3SS and 3. Pedestal formation. (Adapted from Kaper *et al*., 2004).

2.5 Epidemiology

Previous outbreaks of *E.albertii* shows that it persists in infected hosts, birds, humans, and other mammals like poll finches (Gordon *et al*., 2003; Gordon *et al*., 2011; Oh *et al*.,, 2011,). Contamination in drinking water and food results in intake of the pathogen. This occurs due to the poor hygiene conditions. Outbreak in poll finches was due to the consumption of contaminated birds by finches (Foster *et al*,. 1998; Pennycott *et al*., 1998; Oaks *et al*., 2010). *E.albertii* can multiply in the food stuff, if not refrigerated. Humans are exposed to *E.albertii*

by the consumption of contaminated meat of chickens and pigs (Olesn *et al*., 2007). Number of infected people (Morbidity) by *E.albertii* was not known and develops non-lethal enteritis. Deaths have not been recorded in humans due to *E.albertii* (Albert *et al*., 1999). Birds were exposed but never showed any clinical signs (Gordon *et al*., 2011). Poll finches were found dead due to infection (Oaks *et al.*, 2010).

2.6 Clinical Manifestation

Incubation period of *E.albertii* in humans is 19 hours. After 19 hours the onset of clinical signs are seen. The clinical signs of enteric bacterial infection include abdominal pain, bloating, vomiting. diarrhoea, dehydration and fever (Albert *et al.*, 1999; Huys *et al*.,2003 ; Ooka *et al*., 2012). Generally, birds die immediately after the infection with few signs (Ooka *et al*., 2010).

2.7 Methods of Identification of *E.albertii*

E.albertii is distinct from the other five species in *Escherichia* genus and this distinction is done on the basis of biochemical and molecular identification methods. Originally, the biochemical properties of *E.albertii* were determined primarily by the API 50 CH system. Recently, the biochemical characterization for *E.albertii* identification was done based on conventional format, and other selected commercial systems (API 20E, Vitek, MicrScan). Biochemical commercial system would suggest final identification with an unacceptable probability or no identification at all. This triggers a more in-depth analysis with additional tests (Abbott *et al*., 2003). Potential variability is seen, because of its poorly defined genetic and biochemical properties. For clear identification, molecular studies including 16S ribosomal DNA sequencing, DNA-DNA hybridization and polymerase chain reaction are performed which shows that *eae* positive strains belong to a new species known as *E.albertii*. First complete genome sequence of *E.albertii* strain KF1 was done and results showed that its genome consists of 4,701,875-bp chromosome, G+C content=49.7% and four plasmids (Fiedoruk *et al*., 2014).The DNA-DNA hybridization values obtained, showed that the isolate constitutes a homogenous taxon in the genus *Escherichia* but still well below 70% related to *E.coli* (Huys *et al*., 2003). Phylogenetic analysis was done, which determined that there is a presence of *E.albertii*, in the Japan outbreak (Ooka *et al.*, 2013). Presence of housekeeping genes, *lysp* (lysine-specific permease) and *mdh* (malate dehydrognase) are specific, and shows amplification in the *E.albertii* species only. Presence of putative *cdtB* virulence factor

in *E.albertii*, is also reported. It is seen that *E.albertii* is positive only for *cdt* type III and *cdt* type V. *E.albertii* identification still remains unclear because it remain often unidentified or misidentified by the routine biochemical identification methods. Isolation and proper identification of isolates as *E.albertii* will be highly important to further understand the importance and significance of *E.albertii* as diarrhoeagenic pathogens (Nimri, 2013).

2.8 Biochemical Characteristics of *E.albertii*

The phenotypic and biochemical properties of *E.albertii* are quite similar to *E.coli*, so it is quite difficult to differentiate between both species. There are some key biochemical properties which differentiate but they are not the gold standard method due to variability in results. The biochemical characteristics distinguishing *E.albertii* from *E.coli* include a negative indole reaction and an inability to ferment lactose, D-sorbitol and D-xylose. Variability in the above tests is reported where isolates have shown indole positive reaction and ability to ferment sorbitol. The most consistent biochemical properties of *E.albertii* reported in studies include the non-motile nature and unable to ferment xylose and rhammose sugars (Murakami *et al*., 2013; Ooka *et al*., 2010; Ooka *et al*., 2012; Nimri, 2013). Recently, the reason behind the non-motile nature of *E.albertii*, despite the presence of flagellar biosynthesis genes (*flhA* and *fliD*) is the absence of chemotaxis- related proteins (CheA-Z) genes, four methyl-accepting chemotaxis proteins (MCP_s) and an aerotaxis receptor protein (Aer) in the *E.albertii* strains CB9786 and NIAH_Bird_3 (Ooka *et al*., 2015). Lactose fermenting strain of *E.albertii* is also reported (Ooka *et al*., 2012). Identification of *E.albertii* strains on basis of biochemical characteristics is difficult.

2.9 Genotypic Characteristics of *E.albertii*

E.albertii isolates are positive for *eae* gene, the major reason for its misidentified as EPEC and EHEC. The *eae* gene sequencing shows the presence of 5 new subtypes of intimin (N1 to N5) which shows <95% nucleotide sequence identity to 30 known intimin subtypes in *E.coli*. In addition, *cdt* (Cytolethal distending toxin) gene, present in *E.albertii* is of *cdt* type II/III/V. Presence of *pheU* tRNA gene is seen in *E.albertii* where LEE integration occurs (Ooka *et al*., 2012). A second type III secretion system is been found in the *E.coli/Shigella* lineage (Hayashi *et al*., 2001; Ideses *et al*., 2005). This region was found at the tRNA –*gly*U locus and this region is found deeply degraded in most of the strains. It is also known as ETT2 and is found intact in three completely sequenced *E.albertii* strains. This opens the

possibility of this system to play a role in the pathogenesis of the organism (Ooka *et al*., 2015). Recent studies shows presence of shiga-toxin genes in *E.albertii*. PCR analysis showed that 2 *E.albertii* strains possessed the *stx2f* gene. No gastroenteritis outbreak has been reported regarding this role of microorganism (Ooka *et al*., 2013). Virulence intensity of this gene is mild. Origin of the sample was from a symptomatic human and a cat (Ooka *et al.*, 2012). Another study revealed the presence of *stx2a* gene in *E.albertii* which is highly virulent and is able to cause life-threatening haemolytic uremic syndrome (HUS) in infected patients. The infected patient showed the symptoms of bloody diarrhoea (Brandal *et al.*, 2015).

2.10 Immune Response in EPEC

In the Peyer's patch, M cells sample the antigens from intestinal lumen and within the intraepithelial spaces of intestine present them to antigen presenting cells (APS) (Neutra *et al.*, 1996). EPEC is not taken up by the M-cells and they gets adhere to macrophages, where they do not get phagocytosied and this might influence the generation of innate and specific immune response. Exposure of EPEC fails to generate adaptive response due to inability of the M cells to take up the EPEC. Innate immune system gets activated as soon the entry of pathogen in the epithelial cells occurs. The innate immune system involves recognition of certain bacterial products like flagellin, lipopolysacchride (LPS) via pathogen associated molecular patterns ($PAMP_s$) expressed by the host cells. Other bacterial products include bundling, EspA, and EspC. Host cell also express specialized germ line encoded pattern recognition receptors (PRR_s) to recognize CpG DNA of the pathogen (Akira *et al*., 2006). Flagellin is alone responsible for inducing chemokine IL-8 secretion which is mediated by the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB) pathway (Schuller *et al*., 2009). IL-8 provides chemotaxis and recruits neutrophils, macrophages and other inflammatory associated immune cells such as polymorphonuclear leucocytes (PMN) (Helene *et al.* , 2007; Savkovic *et al*., 1996). Other bacterial products are responsible for creating inflammatory host response (Edward *et al*., 2011). The activation of such pro-inflammatory cytokines activates the innate immune system. Epithelial cells of intestine also express and upregulates specific anti-microbial products in response to the infection and it also triggers the pro-inflammatory cytokines (TNF and IL-1) and chemoattractant (IL-8). The infection is created when the pathogen breaches the innate immune system. In the case of EPEC, Type 3 secretion system (T3SS) of the pathogen transports various virulence factors into the host system and tends to manipulate the host cell

processes and antimicrobial responses for its survival and replication into the host. Around 10 years ago, it was observed that EPEC strains can inhibit NF-kappa B and mitogen-activated protein kinase (MAPK) activation and it also inhibits the production of pro-inflammatory molecules like IL-6 and IL-8 during the early stage of infection. These observations were made in the cultured epithelial cells, when infected with the EPEC strain (Hauf *et al*., 2003). These strategies are acquired by the organism to overcome the early immune system so that it can stay for longer period of time before the overall immune system clears the pathogenic bacteria. There are certain EPEC effector proteins secreted by T3SS which inhibits the inflammatory signalling and dampens the innate immune system and leads to the infection, causing diarrhoea. These effector molecules include NleE (Non-LEE-encoded effector E), NleC (Non-LEE-encoded effector C), NleD (Non-LEE-encoded effector D), NleH (Non-LEE-encoded effector H) and Tir (Pallett *et al.*, 2014). Due to similar pathogenesis shown by the *E.albertii*, it might be said that the immune response shown by this novel organism is similar.

CHAPTER 3

3. Materials and Methods

3.1 Materials

Bacterial Strains, Markers and Primers

The bacterial strains used are listed in Table 2, Markers in Table 3, and Primers in Table 4, along with source and reference.

3.1.1 Table 2: *Escherichia coli* Strains

Strain	**Source**
Enteropathogenic *Escherichia coli* (EPEC)	Already procured diarrhoeal stool samples.

3.1.2 Table 3: Molecular weight of Markers

DNA Marker	**Fragment Sizes (bp)**
100bp gene ruler	1000,900,800,700,600,500,400,300,200,100

3.1.3 Table 4: Primers (Adapted from Nguyen *et al*., 2005 and Konno *et al*., 2012)

Target gene	**Protein**	**Amplicon Size (bp)**	**Sequence (5'-3')**
eae F	Intimin	376 bp	5'CAGGATCGCCTTTTTTATACG3'
eae R			5' CTCTGCAGATTAACCTCTGC3'
lysp F	Lysine-specific Permease	252 bp	5'TCCAGATCCAACCGGGAGTATCAGGA 3'
lysp R			5'GGGCGCTGCTTTCATATATTCTT 3'
mdh F	Malate Dehydrognase	115 bp	5'CTTGCTGAACCAGATTCTTCACAATACCG 3'
mdh R			5'CTGGAAGGCGCAGATGTGGTACTGATT 3'
cdtB F	Cytolethal distending toxin	466 bp	5'GAAAGTAAATGGAATATAAATGTCCG 3'
cdtB R			5'AAATCACCAAGAATCATCCAGTTA 3'

3.2 Methods

3.2.1 Samples and strains used in the present study

Studies were carried out on the diarrhoeagenic *E.coli*, Enteropathogenic *E.coli* (EPEC) which was isolated in an ongoing study in our research lab (and ethical permission was already taken for the study).

3.2.2 Culturing and Isolation of Microorganism

3.2.2.2 Screening of microorganisms on different media

Nutrient Agar (NA): Microorganisms were maintained on the nutrient agar plate which is a non-selective media and is also used to check the purity of the culture. The culture was streaked on Nutrient Agar and was incubated at 37°C for 18-24 hours.

MacConkey Agar: To selectively isolate diarrhoeagenic *Escherichia coli* (DEC), the culture was streaked on MacConkey agar and was incubated at 37°C for 18-24 hours.

3.2.3 Morphological Characterization

Gram staining of the isolated colonies was done for the morphological identification. Use a sterile toothpick to pick up the colony from pure culture and make a thin smear on a glass slide. Heat fix the smear. Put crystal violet stain for 1 minute over the smear. Crystal violet penetrates the cell wall and cell membrane of both gram positive and gram negative organisms. It stains the cells purple. Then wash off the stain and add gram's iodine to the smear and keep it for 2 minutes. Gram's iodine acts a mordant. It increases the affinity of the primary stain and turns the smear color dark purple. Wash the slide using 100% ethanol. Put safranin stain over the smear for 1 minute. Rinse off the stain using tap water. Let it air dry and observe under the light microscope.

3.2.4 Biochemical Characterization

The biochemical characteristics of the isolates were determined by the IMVic test. IMVic test is done to identify the members of *Enterobacteriaceae* family. It consists of indole test, Methyl Red (MR) test, Voges-Proskauer (VP) test, and Citrate test.

3.2.4.1 Indole Test

Tryptophanase is an enzyme produced by certain bacteria which can cleave indole ring of tryptophan amino-acid. Peptone water contains tryptophan which is converted into indole, pyruvic acid and ammonia in the presence of tryptophanase and water. Presence of indole is detected by Kovac's reagent which consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and con. HCl. Cherry or red color ring on top of media is formed when indole reacts with the aldehyde group of the reagent which is an indication of positive results. Negative result is indicated by the no change (yellow color ring) on top of the media. Autoclave 1% of peptone water in two different test-tube at 15 psi for 15 minutes and inoculate with 50µl of test culture in one test-tube. Negative control was the one in which no inoculation was done. Incubate for 48 hours (2days) at 37°C and then add 500µl (or few drops) of Kovac's reagent in both test-tubes.

3.2.4.2 Methyl Red (MR) Test

This test is done to check the ability of a microorganism to produce stable mixed acid end product from glucose fermentation. Glucose phosphate broth is used for the test which contains glucose and a phosphate buffer. Production of high acid end product overcomes the buffering action of the system. Methyl red (MR) is used as a pH indicator which shows red color when the test is positive, at pH 4.4 or less and yellow color for the negative test. Autoclave glucose phosphate media in two different test-tubes at 15 psi for 15 minutes and inoculate with 50µl of test culture in one test-tube. Negative control was the one in which no inoculation was done. Incubate for 48 hours (2days) at 37°C and add 5 drops of MR solution. Do not shake the tubes.

3.2.4.3 Voges-Proskauer (VP) Test

VP test is done to detect the presence of an intermediate acetyl-methyl carbinol (acetoin) in the butylene glycol production. Acetoin is oxidized in presence of air and even KOH which produces diacetyl and this diacetyl reacts with guanidine components of peptone, in the presence of alpha- naphthol to produce red color. Alpha-naphthol acts as a catalyst and a color intensifier. Appearance of red color indicates positive result whereas no color appearance indicates negative results. Autoclave glucose phosphate media in two different test-tubes at 15 psi for 15 minutes. Inoculate with 50µl of test culture in one test-tube. Negative control was the one in which no inoculation was done. Incubate for 48 hours

(2days) at 37°C. For this test, 40% KOH solution in distilled water was prepared along with 5% solution of alpha-naphthol in ethanol. Add 600µl of alpha- naphthol solution to both the test-tubes after incubation. Then, add 200µl of 40% KOH to the same test-tubes. Shake them well and wait for 15 minutes for color appearance.

3.2.4.4 Citrate Utilization Test

This test is done to check the ability of a microorganism to utilize citrate as a sole carbon source for energy. This media contains a pH indicator called bromthymol blue which changes its color from green to blue in an alkaline condition. Inorganic ammonium salts are also present which is utilized as a sole nitrogen source. Presence of citrate in a microorganism breaks down the oxaloacetate into pyruvate and carbon dioxide. Utilization of citrate results in alkaline environment. Positive result indicates changes of media color from green to blue and negative result indicate no change in media color and it remains green. Autoclave Koser's citrate medium in two different test-tubes at 15psi for 15 minutes. Make a slant, immediately after autoclaving and streak a loopful of culture in one test-tube. Do not streak in another test-tube (Negative control). Incubate for 48 hours (2days) at 37°C. Color change will be seen, if results are positive.

3.2.4.5 Triple Sugar Iron (TSI) Agar

This test is done to check the fermentation of three different sugars, glucose, lactose and sucrose. In TSI media sodium thiosulphate and ferric or ferrous ions make H_2S indicator system. Phenol red is the pH indicator. The results of this test are summarized like, if alkaline slant / acid butt (yellow butt, red slant) then only glucose is fermented. Acid slant / acid butt(yellow butt, yellow slant), then all the three sugars, glucose, lactose and sucrose fermented and if alkaline slant/alkaline butt (red butt and red slant), then no sugar is fermented. Bubbles or crack means gas production. Presence of black precipitate means H_2S production. Autoclave TSI medium in two different test-tubes at 10psi for 10 minutes Make a slant, immediately after autoclaving and streak a loopful of culture in one test-tube. Do not streak in another test-tube (Negative control). Incubate for 48 hours (2days) at 37°C. Note down the color change.

3.2.5 Molecular Characterization

Polymerase chain reaction (PCR) is used for genotyping and this method is developed by Kary Mullis in the 1980s. DNA polymerase used in PCR has an ability to amplify the target sequence by adding nucleotides to the existing 3'OH group in the primer, where the first nucleotide adds. The purpose of using PCR is to get billions of copies of the target region at the end of the reaction. The components required for PCR are: DNA template (target sequence), forward and reverse primers, nuclease free water and Master Mix (contains DNTPs and DNA polymerase).

3.2.5.1 Genomic DNA Isolation from Enteropathogenic *E.coli*

DNA isolation of Enteropathogenic *E.coli* was done by the phenol/chloroform extraction method. Phenol and chloroform are the two liquids which separates mixtures of molecules based on the differential solubilites. Both the liquids are immiscible in water. Phenol being the dense (density- 1.07g/cm^3) sits at bottom and it denatures the proteins and solublizes it in organic phase. Chloroform aids the separation and allows sharper separation of the organic and aqueous phase as it is denser than phenol (density- 1.47 g/cm^3). Isoamylalchol is added to prevent foaming. DNA gets dissolved in aqueous phase due to like dissolves like phenomena. RNA and lipids gets dissolved in organic phase and proteins in interphase (Sambrook). DNA isolation was done by following certain steps. Inoculate the culture in nutrient broth and incubate overnight at 37°C and harvest the cells by centrifugation at 7000 rpm for 10 minutes at 4°C. Dissolve the pellet in 200µl-400µl of SDS-lysis buffer. Incubate at 60°C for 1 hour. Add 200µl-400µl of phenol: choloroform: isoamylalchol in the ratio 25:24:1. Centrifuge at 13,000 rpm for 10 minutes at 4°C. Collect the supernatant. Add 200µl-400µl of chloroform: isoamylalchol in the ratio 24:1 in the supernatant. Centrifuge at 13,000 rpm for 10mins at 4°C. Add equal volume of isopropanol and 1/20th volume of NaCl (.5M). Keep at 4°C for 1 hour. Centrifuge at 13,000 rpm for 10mins. Wash the pellet with 70% ethanol for 5mins at 13,000 rpm, twice. Dry the pellet. Resuspend the pellet in 100µl of nuclease free water. DNA quantification was done at 260nm. Store DNA at -20°C.

3.2.5.2 Polymerase chain reaction (PCR) for *eae*, *lysp* and *mdh* genes

Biochemically confirmed EPEC and other strains which were different from the EPEC pattern was subjected to PCR for the amplification of *eae* gene. Briefly, PCR conditions for *eae* gene were denaturation at 96°C for 4 min, followed by 30 amplification cycles (94°C

for 20 secs, annealing at 58° C for 30 secs, 72°C for 40 secs), and a final extension cycle (72°C for 7 mins). All *eae* harbouring strains were again subjected to multiplex PCR, where two sets of lineage specific primers of genes, *lysp* (amplicon size- 252bp) and *mdh* (amplicon size-115bp), are used to identify the variant of EPEC. Briefly, multiplex PCR conditions for *lysp* and *mdh* genes were denaturation at 95°C for 5 min, followed by 30 amplification cycles (95°C for 30 secs, annealing at 40° C for 30 secs, 72°C for 40 secs), and a final extension cycle (72°C for 2 mins). All isolates which are suspected as variant of EPEC by multiplex PCR is further targeted for c*dtB* gene (amplicon size- 466bp), which is a putative virulence factor responsible for producing cytolethal distending toxin in *E.alberti*. *CdtB* gene was not standardized. After PCR, 1.8% agarose gel for *lysp, mdh* and *eae* and 2% agarose gel for *cdtB* was prepared. The PCR product was mixed with 0.5µl dye and run on the electrophoresis gel. The gel was observed in Gel Doc for checking the amplification.

3.2.6 Sequencing

PCR products specific for *lysp* gene amplification and *mdh* gene amplification were sent for Sanger sequencing.

3.2.7 Proteome Analysis

3.2.7.1. Protein Isolation from Enteropathogenic *E.coli* (EPEC), EPEC-variant and E.coli DH5-α strains

Total membrane or envelope protein of the EPEC variant, EPEC and non-pathogenic *E.coli* strain- DH5α was isolated for further comparisons of the protein profiles. The protocol was modified from the original one (Haigh *et al*., 2013). Inoculate the three different cultures in 200ml LB media in different flask and incubate overnight at 37°C on shaker at 150rpm. Harvest the bacterial culture by centrifugation at 6,700Xg for 10mins at 4°C. Wash the pellet twice in 20ml (for 25 ml culture) of 10mM Tris Cl buffer (pH- 7.5). Again, wash the pellet in 10mM Tris base (pH-7.5) to separate membrane proteins. Resuspend the pellet in 1ml Tris base and incubate at -80°C overnight to weaken the cell wall. Thaw the bacterial suspension on ice. Sonicate the samples for cell lysis in cycles of 15 secs sonication followed by 45 secs of cooling. Then, centrifuge at 3,000 rpm for 15 minutes at 4°C and collect the supernatant. Again, centrifuge at 10,000rpm for 15minutes at 4°C to separate total membrane proteins from cytoplasmic proteins. Pellet will contain total membrane protein. Total membrane protein comprises of both inner and outer membrane protein. Wash the pellet twice, from

500µl of 10mM Tris HCl (pH- 7.4) and then finally re-suspend in 50µl of the same buffer, according to the membrane pellet size. Store the pellet at -80°C.

3.2.7.2 Protein Estimation by Bradford Assay

Protein concentration is quantified by Bradford assay. Principle behind this assay is that the Coomassie Brillant Blue G-250 dye binds to the protein molecules under acidic condition which results in a color change from brown to blue. Presence of basic amino-acid in the protein contributes to formation of the protein-dye complex. Seven different dilutions of the BSA protein standard were prepared, 0µg/ml, 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, 125µg/ml, and 150µg/ml. Dillutent used here was Tris HCl (pH 7.5). Blank did not contain the BSA protein. Pipette 10µl of standard dilution and 10µl of isolated protein samples on different wells of micro-titter plate. Then, add 200µl of Bradford reagent to each well. Incubate at room temperature (25°C) for five minutes. Measure the absorbance at 595nm. It was done in triplicates and then average was calculated (H.Fanglian, 2011).

Table 5: Concentration of BSA protein for standard curve.

Concentration (µg/ml)	BSA (µl)	Dillutent (Tris HCl) (µl)
0	0	1000
25	25	975
50	50	950
75	75	925
100	100	900
125	125	875
150	150	850

3.2.7.3 Protein Profiling by SDS-PAGE technique

Total membrane protein profile is analyzed by SDS-PAGE technique. Firstly, prepare 10% resolving gel. All the constituents were added in the same order (Appendix 7.2.6.4). Add TEMED when the mixture is ready to pour between the plates. After adding TEMED pipette the mixture between the plate. Add a layer of water on the surface of the gel to make the surface smooth. Wait for solidification. Prepare 5 % stacking gel. All the constituents are added in the same order (Appendix 7.2.6.4). Add TEMED when the mixture is ready to pour

between the plates. Remove the layer of water from the resolving gel. Pour the stacking solution on it. Immediately apply combs on it and wait for solidification. After solidification remove the combs and gel is ready to load the samples. Samples were prepared by adding sample buffer (Appendix 7.2.6.2) and were incubated in water bath for 10 minutes at 95°C. Cast the gel, and load 30 µl of samples and apply voltage. Initially the voltage is kept low i.e. 55Vso that the sample in stacking gel easily and slowly align itself to give a same start. After 15 minutes the voltage was changed to 65V. After gel is fully run, disassemble and stain the gel. Staining is done overnight with agitation as agitation helps in full circulation of dye and makes its penetration easy. Destaining is done to remove excessive dye by destaining solution, keeping it on agitation (Appendix 7.2.6.5, Appendix 7.2.6.6).

*All procedures and protocols were approved by institutional ethics committee for ongoing ICMR project.

CHAPTER 4

Results

4.1 Sample and strains used in study

Strains used in the study were already collected from two different regions (Shimla and Solan). The presence of lactose fermenting colonies on MacConkey agar plates showed the presence of *E.coli* (Figure 2).

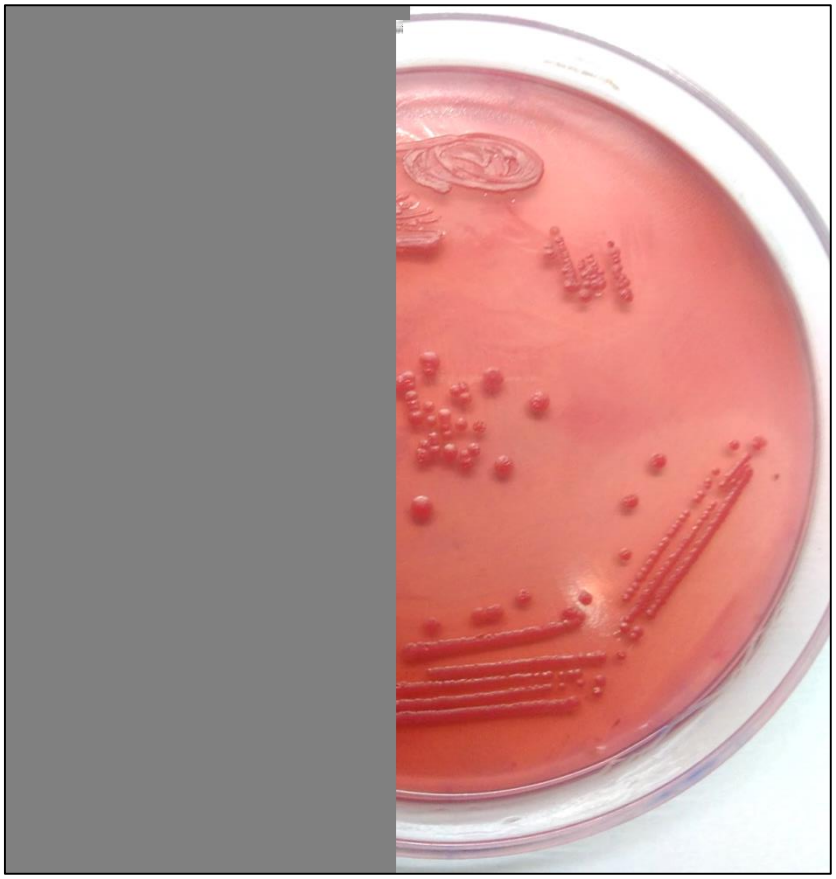

Figure2: Culture patterns of *E.coli* (Lactose fermenting colonies on MacConkey Agar).

4.2 Morphological Characterization

Gram negative pink color rods were seen under microscope. This morphology confirms that the culture is pure and gram negative bacilli.

4.3 Biochemical Characterization

Among all 45 Enteropathogenic *E.coli* strains (EPEC), only 8 strains showed unusual biochemical profile (Figure.3a) which varies from the *E.coli* pattern (n=37) (Figure.3b). The unusual biochemical profile of these strains were quite similar with the reported biochemical properties of *E.albertii* i.e. negative for indole but many variations in results are seen in these 8 strains. A variation in results of biochemical profiling makes the molecular characterization of these strains important. The PCR of all 45 strains was done to see the different genes as well as to re-confirm the Enteropathogenic *E.coli* (EPEC) 35 strains.

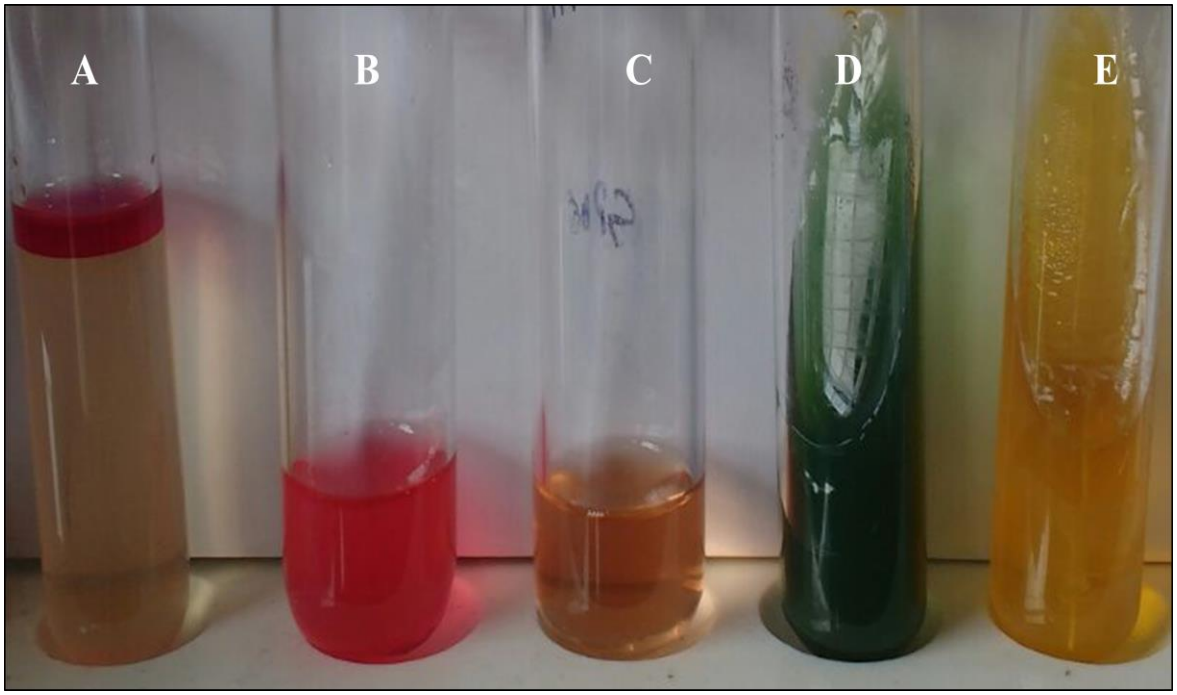

Figure 3a: EPEC strains characterized through biochemical test: IMVic (A, Indole, B, MR, C, VP, D, Citrate) and E, Triple Sugar Iron Agar. IMVic pattern: +, +, - , - , A/A.

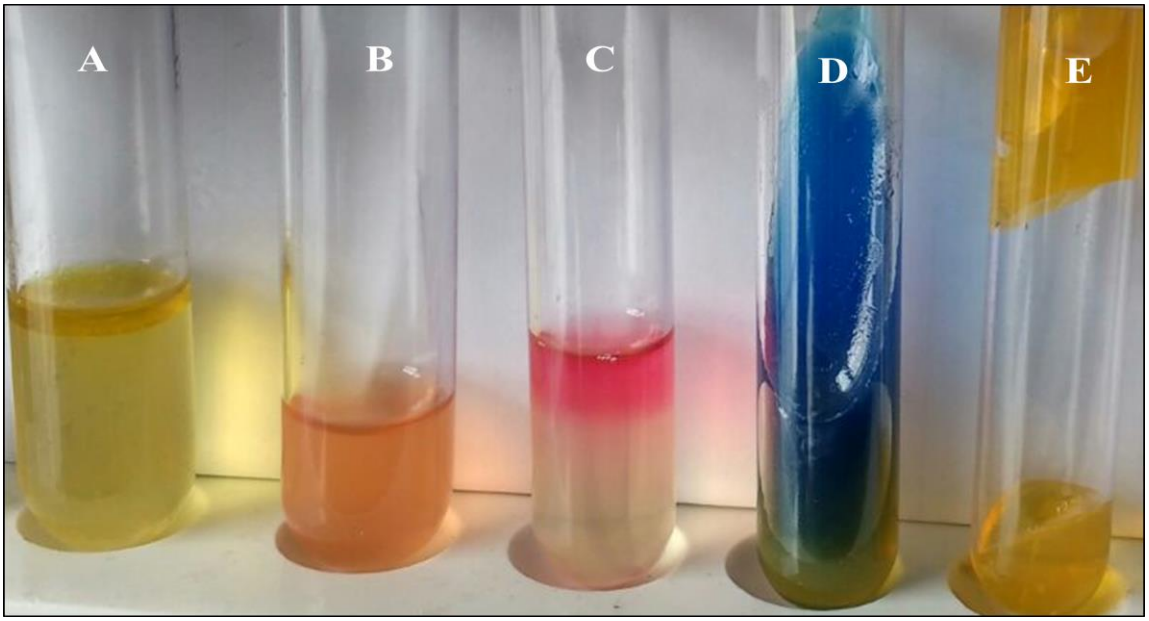

Figure 3b: Unusual strain (EPEC-variant strain) characterized through biochemical test: IMVic (A:Indole, B: MR, C: VP, D: Citrate) and E: Triple Sugar Iron Agar. IMVic pattern: -, -, +, +, A/A.

4.4 Molecular Characterization

4.4.1 Genomic DNA isolation

Genomic DNA was isolated from 45 strains**.** Concentration of DNA of all the samples range between 414ng/µl to 515ng/µl. The ratio of 260/280 of all the samples range between 1.8-1.9. Representative picture is shown in figure 4.

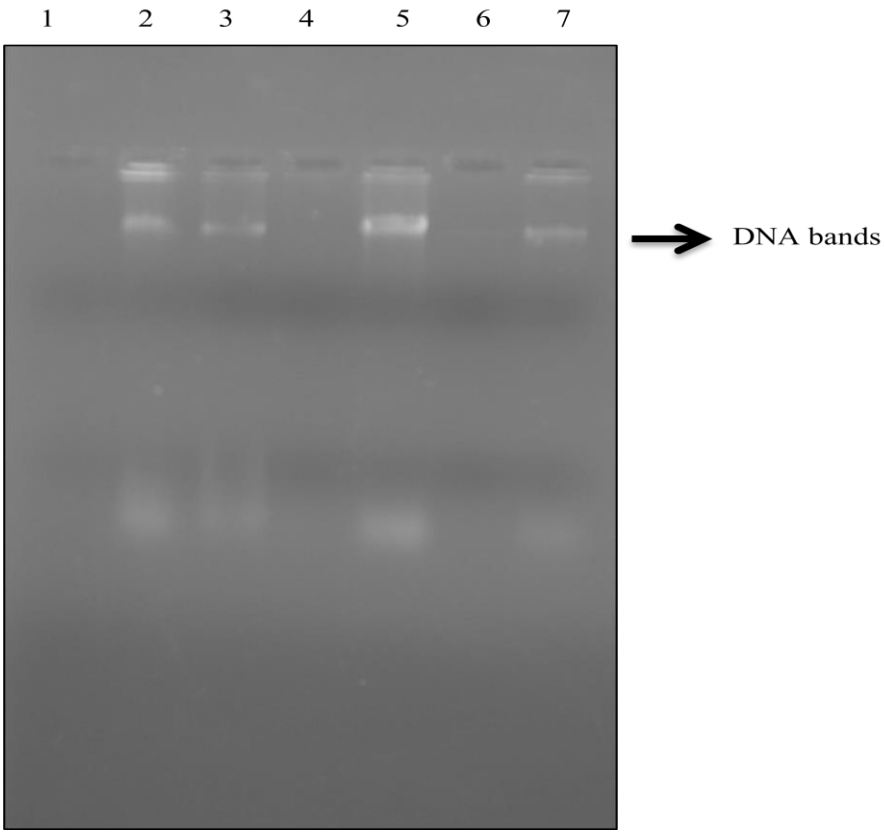

Figure 4: Gel electrophoresis image of 0.8% agarose gel depicting genomic DNA isolated from *E.coli* samples. Lanes: 1, empty, 2, 3, Sample ID: 79 and 336 respectively, 4, Sample ID: 419, 5, 218, 6, 308, 7, Empty.

4.4.2 Amplification of *eae, lysp* and *mdh* genes in EPEC strains

PCR characterization of 45 lactose fermenting isolates was performed for *eae* gene in which all the 45 strains showed the amplification of the gene. The amplification of *eae* gene confirms that the all the isolated *E.coli* strains are EPEC. A representative gel picture is shown in figure 5.

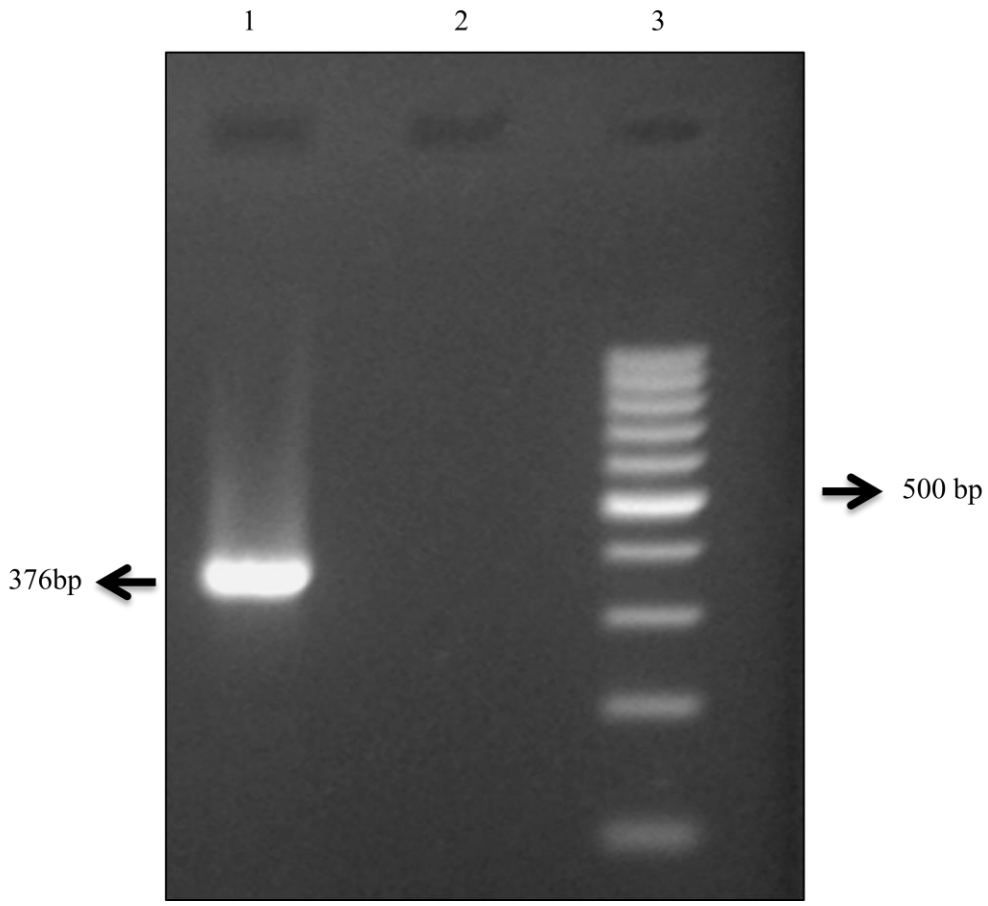

Figure 5. Gel electrophoresis image of 1.8% agarose gel depicting the detection of *eae* gene in diarrhoeal sample isolates of EPEC. The amplicon size of *eae* is 367bp. Lanes: 1, *eae* gene, 2, Negative control, 3, 100bp ladder.

In all the 45 positive strains for *eae*, only 11 of the strains showed positive amplification for *lysp* gene and *mdh* gene, which shows that they are variant strains of EPEC and somehow this profile matches with the *E.albertii*, because these genes are reported as housekeeping genes in *E.albertii*. Representative picture is shown in figure 6. Sequencing results matches with the genome of *E.coli*, so they are confirmed as variant of EPEC but not *E.albertii*. An attempt was made to standardize the third gene, *cdtB*, however, it did not show amplification at the required base pairs.

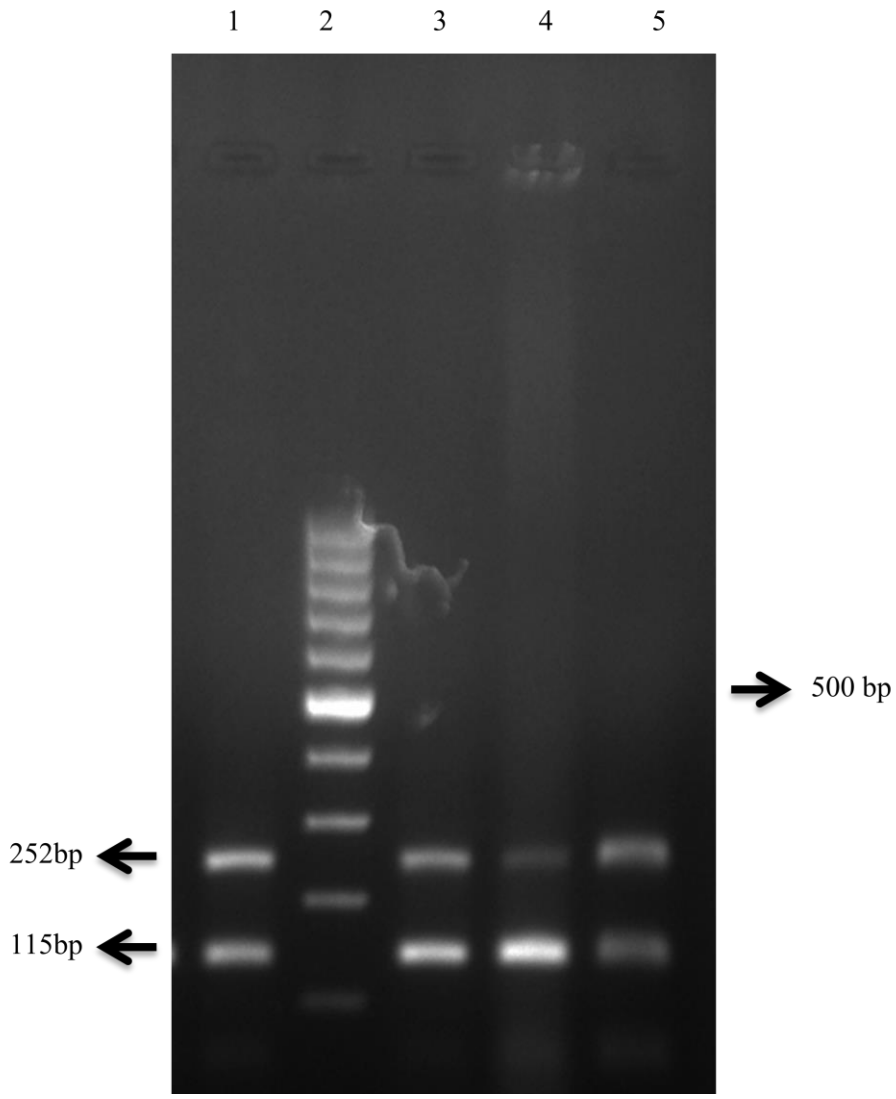

Figure 6. Gel electrophoresis image of 1.8% agarose gel depicting the detection of *lysp* gene and *mdh* gene (multiplexing) in diarrhoeal sample isolates of EPEC. The amplicon size of *lysp* and *mdh* is 252bp and 115 bp respectively. Lanes: 1, 79, 2, 100bp ladder, 3, 449, 4, 73, 5,44.

4.5 Protein Analysis

4.5.1 Protein Isolation and Protein estimation

Three different (DH5-α, EPEC-variant and EPEC) 200 ml overnight grown cultures were used for isolation of proteins (methodology in detail is given in materials and methods section). Precipitated membrane protein was quantified using Bradford assay. Concentration is plotted on x-axis and Optical Density (OD) on y-axis (Figure.7). Concentration of unknown protein sample was quantified using the standard curve. The value of R^2 is 0.948, which is an acceptable range.

Table 6: Optical Density (OD) at different concentrations of BSA protein.

Concentration (µg/ml)	BSA (µl)	Dillutent (Tris HCl) (µl)	Optical density(OD) (nm)
0	0	1000	0
25	25	975	0.0416
50	50	950	0.1001
75	75	925	0.138
100	100	900	0.204
125	125	875	0.220
150	150	850	0.0225

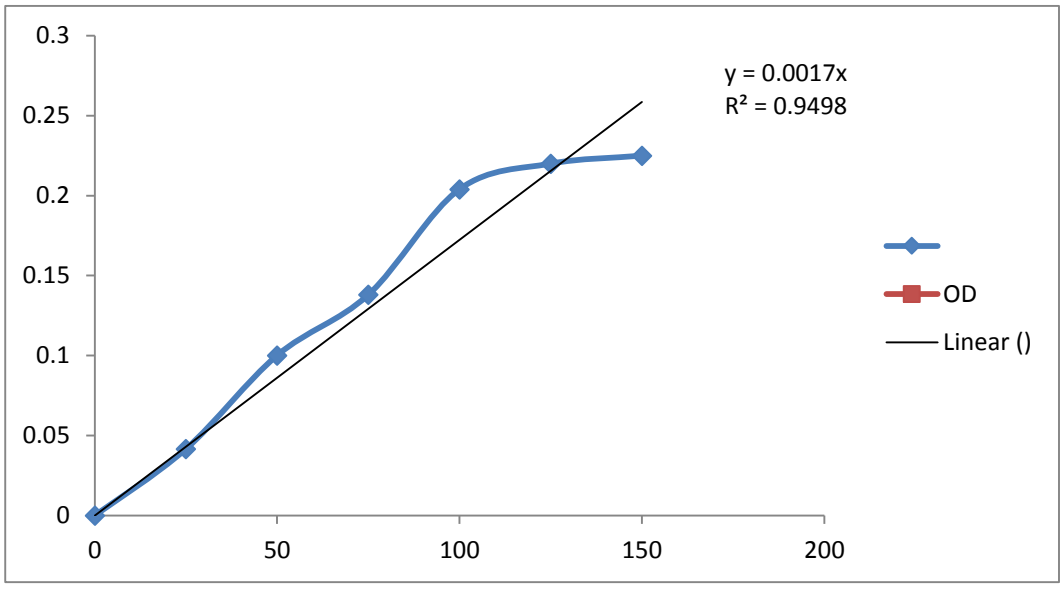

Figure 7: Standard curve for estimation of BSA protein concentration.

O.D of the unknown samples was used to know the concentration from the standard curve. Software Graph Pad was used to calculate the concentration of unknown protein samples.

Table 7: Optical Density (OD) and concentrations of unknown samples.

Strains	O.D (nm) of the unknown samples	Concentration of the unknown sample(μg/ml)
DH5α	0.176	101.706
EPEC-variant	0.378	226.211
EPEC	0.177	102.322

4.5.2 Protein profiling using SDS-PAGE technique

SDS-PAGE profile of total cell membrane proteins of three different strains (*E.coli* DH5-α, EPEC and EPEC variant) showed a gross similar profile as seen in (Figure.8). Majority of the proteins are present between 14 kDa to 97 kDa. However, there are also several differences in protein expression of all the three strains. Protein expression profile of pathogenic (EPEC and EPEC-variant) strains differed from the non-pathogenic (*E.coli* DH5-α) strains (marked regions in Figure.8). In pathogenic (EPEC and EPEC-variant) strains the proteins 30-35 kDa are highly expressed in comparison to the non- pathogenic (*E.coli* DH5-α) strain (range between 30-32 kDa). Further, between the pathogenic strains of Enteropathogenic *E.coli* (EPEC) difference in the protein expression was also observed. The EPEC-variant strains showed higher protein expression in comparison to the Enteropathogenic *E.coli* (EPEC) strains (Figure.8). These specific membrane proteins observed in the pathogenic strains may be further investigated and characterized to screen the surface antigens for immunogenic properties.

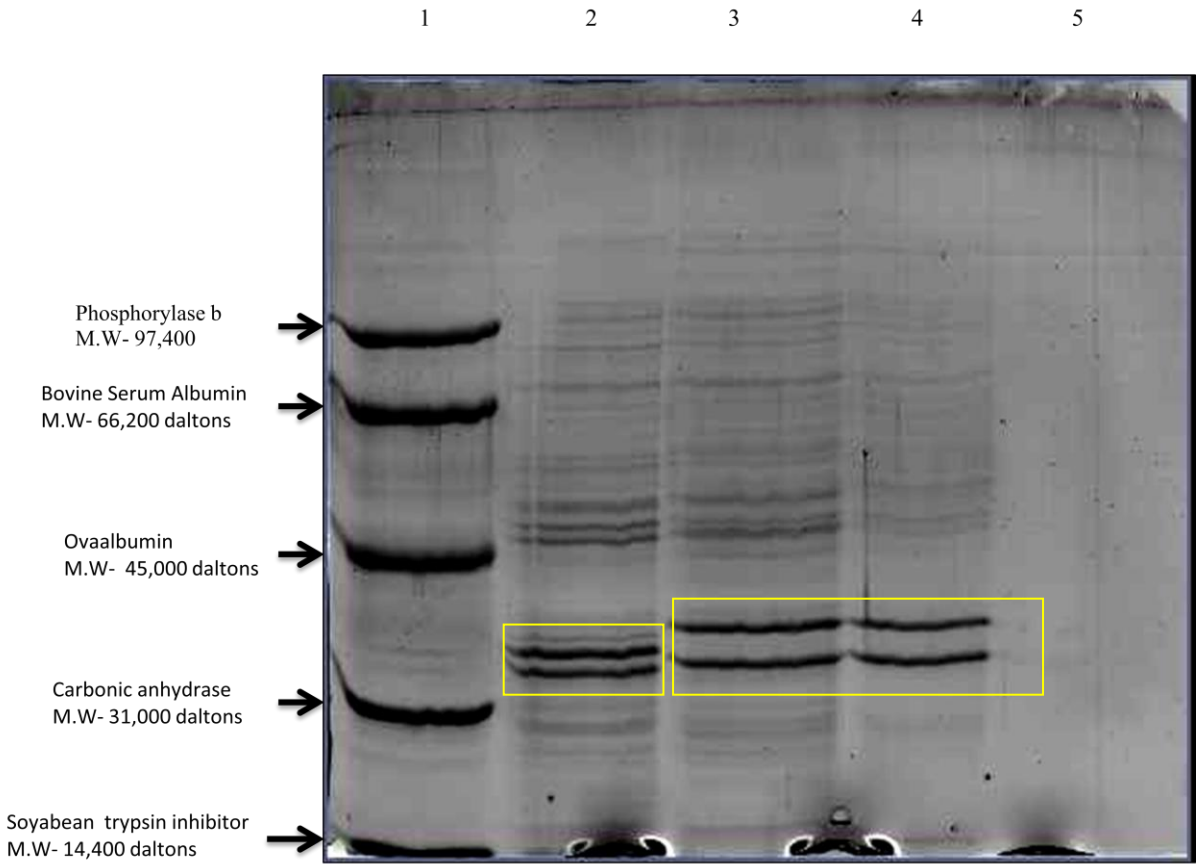

Figure 8: SDS-PAGE profiling of the membrane proteins of *E.coli*. Lanes: 1, Bio Rad SDS-PAGE Low Range Marker, 2, *E.coli* DH5α, 3, EPEC-variant, 4, EPEC, and, Empty.

CHAPTER 5

Discussion and Conclusion

Diarrhoea is hitting globally, and causing millions of deaths of infants (under-five) and young children, annually. The report published in U.S Department of Health and Human Services, Center for Disease Control and Prevention states that 1 in 9 child deaths are due to diarrhoea and 2,195 children die daily of diarrhoea i.e. 801 thousand in a year. This concludes that children are dying more because of diarrhoea than AIDS, malaria and measles together. Current studies on Enteropathogenic *E.coli* reveal that this category possesses some novel strains, but there are great chances of error in identification system which can mislead further investigation. Several reports on this novel strain have concluded that *E.albertii* is one of the misidentified strains with the Enteropathogenic *E.coli* category and this novel species in *Escherichia* genus is known to cause gastroenteritis. In this study, 45 Enteropathogenic *E.coli* strains were biochemically characterized and results showed that Enteropathogenic *E.coli* -variant strains deviates its biochemical properties from the Enteropathogenic *E.coli* strains in many aspects like Enteropathogenic *E.coli* -variant strains are indole negative. Variations in the biochemical results are seen as some of the Enteropathogenic *E.coli* -variant strains are indole positive and MR negative. This concludes that biochemically it is difficult to distinguish between the EPEC strains and the EPEC-variant strains. Reported studies on *E. albertii* (an EPEC variant) also conclude that it has poorly defined biochemical properties (Nimri *et al*., 2013). Further, Enteropathogenic *E.coli* strains are characterized for the presence of two genes, *lysp* and *mdh*, and the positive results in 11 strains of Enteropathogenic *E.coli* revealed that there is the presence of Enteropathogenic *E.coli*-variant. Molecular profile of Enteropathogenic *E.coli* -variant strains matched with the *E. albertii*. Although, research on this novel species of *Escherichia* is going in many countries where the properties of the misidentified strains of Enteropathogenic *E.coli* are redefined and attempts are made to determine its accurate phylogenetic position. Amplification followed by Sanger sequencing of *lysp* and *mdh* gene in Enteropathogenic *E.coli* -variant strains adds up the possibilities of *E. albertii.* An attempt was made to study the comparative protein expression profiles of pathogenic (EPEC and EPEC-variant) and non-pathogenic (*E.coli* DH5α) strains by SDS-PAGE technique. Difference in protein expression profiles was observed between the pathogenic (EPEC and EPEC-variant) and non-pathogenic strains (*E.coli* DH5α). In pathogenic strains, Enteropathogenic *E.coli*- variant strain showed slight higher expression in comparison to the Enteropathogenic *E.coli* strain. This could be further investigated and characterized to screen the surface antigens for immunogenic properties. Our

results suggests the presence of Enteropathogenic *E.coli* and its variant strains in the Himachal region which needs to be further researched to conclude that these strains are novel species, *E.albertii* or actually the other variants of Enteropathogenic *E.coli*.

CHAPTER 6

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CHAPTER 7

7.0 Appendix

7.1 Bacteriological Media

All the media were prepared in Milli RO grade water and autoclaved at 15 pounds per square inch for 15mins unless otherwise indicated.

7.1.1 Nutrient Broth Medium (HiMedia Laboratories)

Ingredients	**gms/Litre**
Peptone	10.000
Beef extract	10.000
Sodium chloride	5.000

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.2 Nutrient Agar (HiMedia Laboratories)

Ingredients	**gms/Litre**
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.3 MacConkey Agar (HiMedia Laboratories)

Ingredients	**gms/Litre**
Peptones (meat and casein)	3.000
Pancreatic digest of gelatine	17.000

Lactose monohydrate	10.000
Bile salts	1.500
Sodium chloride	5.000
Crystal violet	0.001
Neutral red	0.030
Agar	13.500

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.4 Peptone water

Ingredients	**gms/Litre**
Proteose peptone	10.000
Sodium chloride	5.000
Disodium phosphate, anhydrous	3.500
Monopotassium phosphate	1.500

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.2 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.5 MR-VP Medium (Glucose Phosphate Broth)

Ingredients	**gms/Litre**
Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000

The components were dissolved in 950ml Milli RO water and the pH adjusted to 6.9 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.6 Simmons Citrate Agar

Ingrediets	**gms/Litre**
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000

The components were dissolved in 950ml Milli RO water and the pH adjusted to 6.8 with 5N NaOH and the volume adjusted to 1000ml with Milli RO water.

7.1.7 Triple Sugar-Iron Agar Medium

Ingredients	**gms/Litre**
Beef extract	3.000
Peptone	20.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Dextrose monohydrate	1.000
Ferrous sulphate	0.200
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.000

7.2 Reagents and Buffers

7.2.1 DNA Isolation Reagents

SDS lysis buffer (10ml)

Tris Cl 1mM pH 7.4	200µl
1% SDS (Stock- 10%)	1ml
0.5mM EDTA (Stock-.5M)	100µl

Phenol: Chloroform: Isoamylalchol (25:24:1) (50ml)

Solution contains 25 parts phenol, 24 parts chloroform and 1 part isoamylalchol.

Phenol	25ml
Chloroform	24ml
Isoamylalchol	1ml

The solution is stored in dart bottles at room temperature.

Isopropanol

NaCl (.5M) (1/20th of isopropanol)

7.2.2 Buffers for Electrophoresis

TAE Buffer (50X)

Tris base	242 g
Glacial Acetic Acid	57.1 ml
.5M EDTA (pH8.0)	100 ml
Final volume	1000 ml

7.2.3 Dye for Gel Loading

1X dye for agarose gel electrophoresis

Bromophenol Blue	1µl

7.2.4 Protein Isolation Buffers

Tris HCl buffer

Tris-HCl buffer of desired strength (10mM) was prepared by dissolving appropriate amount of Tris in distilled water and adjusting pH with concentrated HCl. The required pH was 7.5. Molecular weight of Tris HCl is 157.6 g/mol.

Tris base buffer

Tris base buffer of desired strength (10mM) was prepared by dissolving appropriate amount of Tris base in distilled water and adjusting pH with concentrated HCl. The required pH was 7.5. Molecular weight of Tris base is 121.14 g/mol.

7.2.5 Protein estimation Reagents

Bradford reagent (1litre)

Coomassie Brillant Blue G-250	50mg
Methanol	50ml
85% w/v Phosphoric acid	100ml
Distilled waterr	850ml

The solution is stored in dark bottles at 4°C.

7.2.6 SDS-PAGE Reagents

7.2.6.1 Stock solutions

30% Acrylamide, 25ml

Components

Acrylamide	7.3 g
Bis-acrylamide	0.2 g

For 1.5M Tris (pH 8.8): Dissolve 18.6g tris base in 100ml distilled water.

For 1M Tris (pH 6.8): Dissolve 12.11g in 100 ml distilled water.

10 % Ammonium per sulphate (APS): 20 mg in 200 µl distilled water.

7.2.6.2 Sample Buffers, Laemmli, 2 X SDS-PAGES, 30 ml

Components

0.5 M Tris-HCl (pH 6.8)	3.75 ml
50% Glycerol	15.0 ml
1.0% Bromophenol blue	0.3 ml
10% SDS	6.0 ml
Distilled water	30 ml

Add Beta-mercaptoethanol (50 µl to 950 µl sample buffer) before use.

7.2.6.3 Running Buffers, 10x SDS-PAGE, 1liter

250 mM Tris base (pH 8.3)	30.30 g
1.92 M glycine	144.10 g
1% SDS	10.00 g

7.2.6.4 Stacking and Resolving Gels

10% Resolving gel, 10ml

Components

Distilled water	3.9 ml
30% Acrylamide	3.3 ml
1.5 Tris (pH 8.8)	2.5 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	4 µl

5% Stacking gel, 5ml

Components

Distilled water	3.4 ml
30% Acrylamide	830 µl
1.5 Tris (pH 8.8)	630 µl
10% SDS	50 µl
10% APS	50 µl
TEMED	5 µl

7.2.6.5 Staining Solution, 1liter

Components

Coomassie Brilliant Blue R-250	2.5 g
Methanol	450 ml
Glacial acetic acid	100 ml
Distilled water to 1 liter	

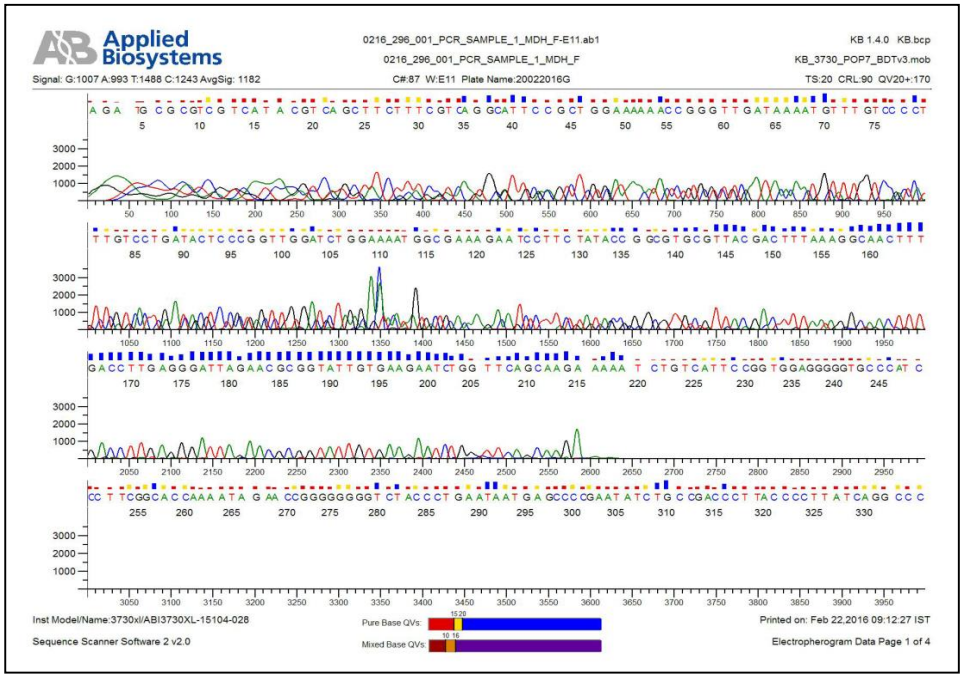
7.2.6.6 Destaining Solution, 4 liters

Components

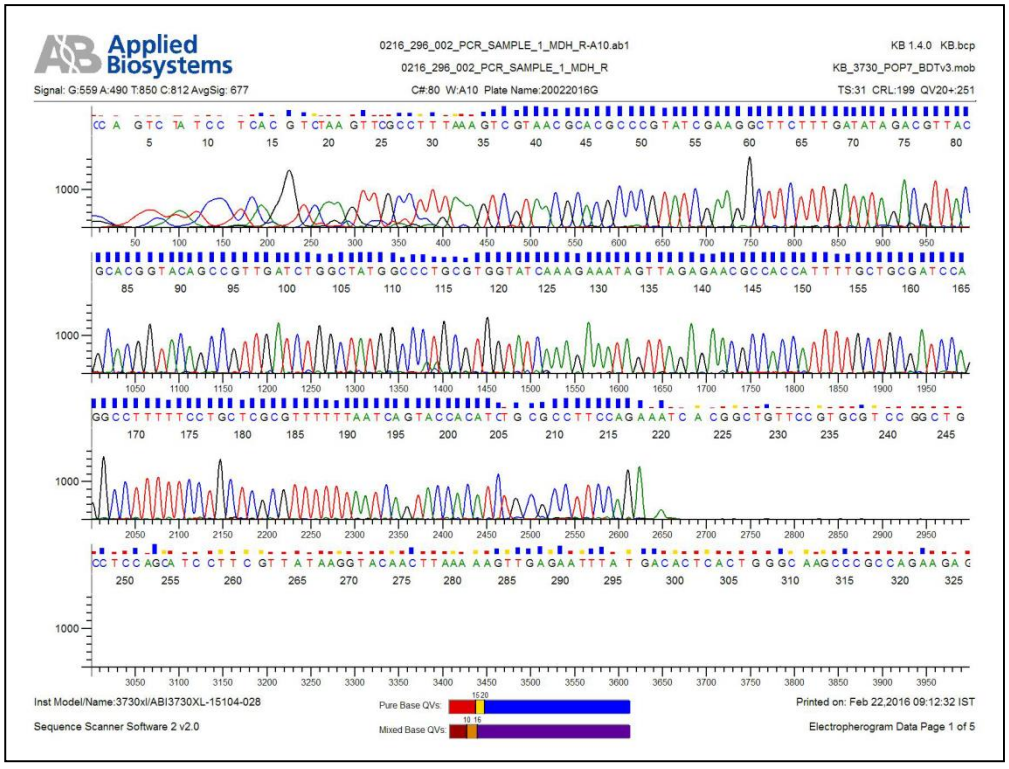
Methanol	300 ml
Acetic acid	400 ml
Distilled water to 4 liters.	

7.3 Sequencing chromatograms for *lysp* gene and *mdh* gene amplified in Enteropathogenic *E.coli* variant.

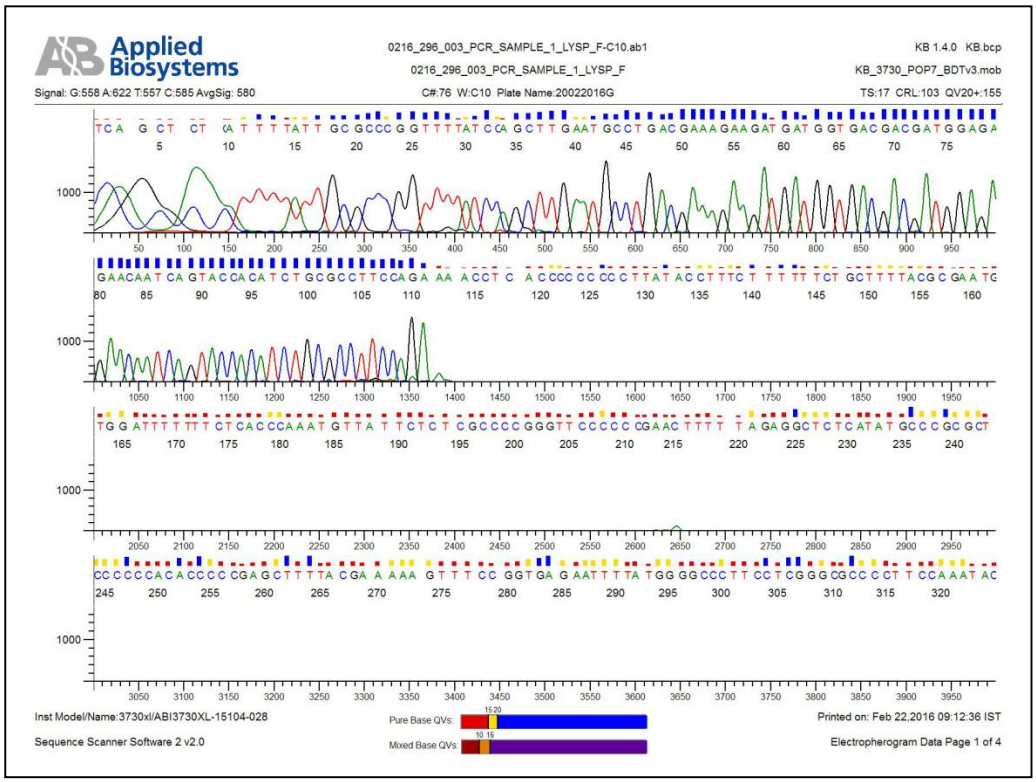
A. *mdh* F



B. *mdh*R



C: *lysp* F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Mixed Base QVs:
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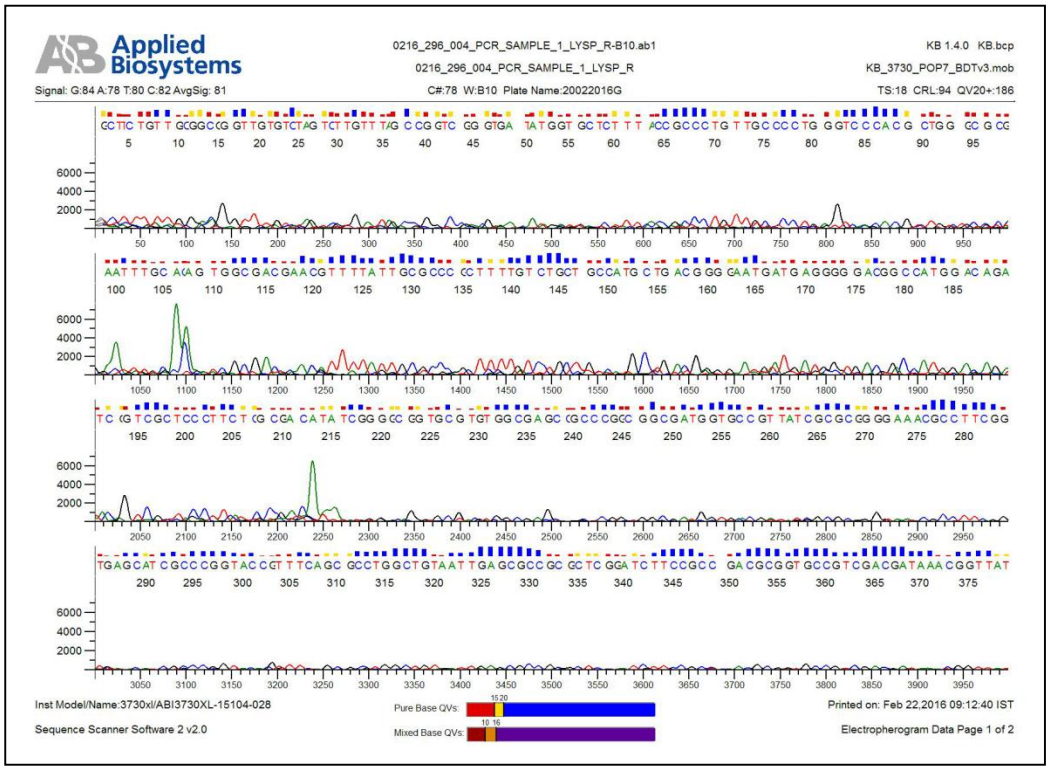
Sequence Scanner Software 2 v2.0
Electropherogram Data Page 1 of 4

D: *lyspR*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Sequence Scanner Software 2 v2.0
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