Ph.D. NUTAN JUIT, WAKNAGHAT 2019

IDENTIFICATION AND CHARACTERIZATION OF MAJOR CAUSATIVE BACTERIAL AGENTS OF DIARRHEA FROM REGIONS OF HIMACHAL PRADESH

Thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY & BIOINFORMATICS

BY

NUTAN

Department of Biotechnology & Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA.

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Dedicated to my beloved Parents

Mr. Roop Singh Thakur

Mrs. Amrita Thakur

& Brother

Sushil Thakur

DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Identification and characterization of major causative bacterial agents of diarrhea from regions of Himachal Pradesh"** submitted at the **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Jitendraa Vashistt**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Identification and characterization of major causative bacterial agents of diarrhea from regions of Himachal Pradesh"** submitted by **Ms. Nutan** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Supervisor: **Date:**

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Nutan

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LIST OF ABBREVIATIONS

%	Percent
'O'	Lipopolysaccharide O antigen
≤	Less than equal to
^{0}C	Degree Celsius
AA	Aggregative adherence
AAF	Aggregative adherence fimbriae
AK	Amikacin
AQP	Aquaporin
AST	Antibiotic susceptibility test
bfpA	Bundle forming pilus encoding gene
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CFA	Colonization factor antigen
CFT	Ceftazidime
CFTR	Cystic fibrosis transmembrane receptor
CFX	Cefixime
CIP	Ciprofloxacin
COT	Co-trimoxazole
CPM	Cefepime
CT	Cholera toxin
DALYs	Disability Adjusted Life Years
DEC	Diarrheagenic *E. coli*
DNA	Deoxyribonucleic acid
DRA	Downregulated in adenoma
E. coli	*Escherichia coli*
eae	Intimin encoding gene

EAEC	Enteroaggregative *E. coli*
EAF	*Escherichia coli* (EPEC) adherence factor
EAG	Empowered Action Group
ECP	*E. coli* pilus
EDTA	Ethylene diamine tetracetate
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
eltB	Heat labile toxin encoding gene
EPEC	Enteropathogenic *E. coli*
ERD2	ER lumen protein-retaining receptor protein
estA	Heat stable toxin encoding gene
ETEC	Enterotoxigenic *E. coli*
ETL	Epidemiological Transition Level
Gb3s	Globotriaosylceramides
GBD	Global Burden of Disease
GI	Gastrointestinal tract
HBC	Hyperimmune bovine colostrum
HCP	Hemorrhagic coli pilus
HIV	Human immunodeficiency virus
HUS	Hemolytic uremic syndrome
I	Intermediate
IAEC	Institute Animal Ethics Committee
ial	Invasion encoding gene
ICMR	Indian Council of Medical Research
IHME	Institute for health Metrics and Evaluation
IPM	Imipenem

IRTKS	Insulin receptor tyrosine kinase substrate
IVOC	In vitro organ culture
KDa	Kilo Dalton
LB	Luria-Bertani
LE	Levofloxacin
LEE	Locus of enterocyte effacement
LT	Heat labile toxin
MDa	Mega Dalton
MDG	Million Death Study
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
ml	Milliliter
MLC	Myosin light chain
mM	Millimolar
MSD	Moderate to severe diarrhea
NA	Nalidixic acid
Nck	Non-catalytic region of tyrosine kinase adaptor protein
NEB	New England Biolabs
NET	Netilimicin
NHE	Na^+/H^+ exchanger
NOR	Norfloxacin
NTS	Non-typhoidal *Salmonella*
N-WASP	Neural Wiskott–Aldrich syndrome protein
PAI	Pathogenicity associated island
PBS	Phosphate buffer solution
pCVD	Plasmid for aggregative phenotype
PHFI	Public Health Foundation of India

PMN	Polymorphonuclear
psi	Pounds per square inches
R	Resistant
rRNA	Ribosomal ribonucleic acid
S	Svedberg unit
S	Sensitive
SD	Standard Deviation
SGLT	Na^+/glucose co-transporter
SNP	Single nucleotide polymorphism
Spp.	Species
ST	Heat stable toxin
Stx	Shiga toxin
T3aSS	Type III, subtype a, secretion system
TAE	Tris acetate ethylene diaminetetraacetate
TCBS	Thiosulphate citrate bile salt agar
tcpA	Toxin coregulated pilin
Tccp	Tir cytoskeleton-coupling protein
TJ	Tight junction
TLR	Toll-like receptors
TNF	Tumor necrosis factor-α
TOB	Tobramycin
UNICEF	United Nations Children's Fund
vt1	Verocytotoxin encoding gene I
vt2	Verocytotoxin encoding gene II
W/V	Weight/Volume
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar
µg	Microgram

µl	Micro liter
µM	Micromole

ABSTRACT

ABSTRACT

Diarrhea is responsible for considerable global morbidity and mortality rates among all age groups. Diarrhea illness leads to an estimated 1.31 million deaths/annum among all age groups and mortality rate is higher (0.49 million deaths/annum) among children population under the age of five years. Disease burden is mainly concentrated in South East Asia particularly in India, where diarrhea incidences are quite higher as compared to other nations. Significant variations in diarrhea incidences have been reported from different parts of the country. Comprehensive surveillance studies exploring diarrhea incidences, their correlations with bacterial pathogen specific clinical parameters and antibiotic resistance are curbed in Himachal Pradesh. Therefore, present study was focused to investigate incidences of bacterial pathogens from an unexplored northern hilly state, Himachal Pradesh.

A total of five hundred seventy two stool specimens were collected from diarrhea in-patients admitted to regional and tertiary care hospitals. All collected samples were screened for various bacterial pathogens through standard microbiological and biochemical approaches. Following biochemical analysis, predominant bacterial pathogens were further characterized through molecular methods and their associations with antibiotic resistance were also correlated.

Microbiological and biochemical characterization revealed diarrheagenic *E. coli* (DEC) as the predominant bacterial pathogen followed by *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. among all diarrheal age groups. Owing to higher incidences of DEC and *Vibrio* spp., these bacteria were further characterized using molecular approach. Incidences of various DEC pathotypes were observed up to a level of 21% and DEC infection rates were quite higher in children less than five years of age as compared to other age groups. Enteropathogenic *E. coli* (EPEC), a molecular pathotype of DEC was found as a predominant pathotype with highest infections of 13.7%. Two other molecular pathotypes enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) accounted for 5.7% and 1.3%, respectively in all diarrhea incidences. Serogrouping of various identified DEC molecular pathotypes showed frequency of somatic antigens O26,

O2 and O3 as the major serogroups among EPEC, ETEC and EAEC pathotypes, respectively. DEC pathotypes exhibited high levels of resistance against all tested antibiotics, while low resistance profile for co-trimoxazole & norfloxacin reinforcing their continuance as potent antibiotics against DEC pathotypes.

Vibrio spp. was other bacteria characterized with higher prevalence in diarrheal patients. Molecular characterization of biochemically confirmed isolates showed 4.5% of *Vibrio* spp. incidences among all cases. Highest incidences of *Vibrio* spp. were observed among adolescent and adult ages than other age groups. Antibiotic susceptibility assays showed higher levels of resistance against cephalosporins and nalidixic acid while, a low level of resistance were exhibited for carbapenems, aminoglycosides and quinolones classes of antibiotics.

Our study revealed DEC pathotypes as predominant etiological agent of diarrhea significantly contributing to diarrhea related morbidity among hospitalized patients. Defining impact of antibiotic use among significant risk groups observed in current study and judicious use of antibiotics could aid in management of diarrheal disease in view of alarming resistance levels exhibited by predominant pathogens.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Diarrhea is a global health concern which leads to higher morbidity and mortality [1]. Diarrhea is one of the predominant infectious causes of hospitalization and outpatient visits in developing as well as in developed countries. Sub-Saharan & South East Asian regions account for highest burden of the disease, which also accounts for more than 72% of the total global burden [2]. Unfortunately, India bears highest toll of the disease [2] which demands acceleration in interventions for diarrhea prevention and cure. Diarrheal pathogens are transmitted through fecal oral route and are responsible for worldwide inexorable outbreaks from time to time [3, 4].

Infectious diarrhea is multifactorial in nature and is associated with vast array of etiological agents [5]. Consequently, for a successful treatment regime, identification of diarrheal agents is of utmost importance. Various etiological agents associated with diarrhea include bacteria, virus and parasitic pathogens. Predominant diarrheal pathogens from these classes include; Diarrheagenic *E. coli* (DEC), *Vibrio* spp., *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., rotavirus, norovirus, astrovirus, *Cryptosporidium parvum* and *Giardia lamblia* [6]. Among all these, rotavirus, DEC, *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. have been associated with moderate to severe forms of diarrhea among all age groups [7, 8]. Diarrheal pathogens possess amalgam of certain virulence factors which is responsible for specific disease pathophysiology and is quite useful in diagnosis and choice of treatment protocol. For diagnosis, specific molecular markers have been developed by various research groups and are successfully utilized in identification during surveillance and epidemics.

Bacterial diarrhea contributes to significant proportion of morbidity and mortality in developing and developed countries [9]. Among above mentioned bacterial agents, diarrheagenic *E. coli* is an extremely versatile microorganism and also signifies as one of the important leading etiological agent of moderate to severe diarrhea worldwide [10-12]. In low to middle income countries, >40% of diarrheal episodes are caused by diarrheagenic *E. coli* [13]. DEC is further catalogued into various pathotypes based upon occurrence of unique virulence determinants contributing to specific pathophysiology [14] viz. enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enterohemorrhagic *E. coli* and enteroinvasive *E. coli* [14-16]. Various DEC pathotypes display unique virulence arsenal which transforms the predominant repertoire available for diagnostic and therapeutic

approaches. These pathotypes also play a considerable role in diarrhea morbidity in the Indian population [17-19]. Published studies from parts of India acclaimed 5-65% of DEC pathotypes infections associated with diarrheal burden [18-20].

Diagnosis of DEC is ascertained on the basis of biochemical, serological and molecular techniques. *E. coli* possesses various extracellular structures like adhesin and lipopolysaccharide (LPS) which help in elaborating pathogenicity in host. The *E. coli* LPS constitutes major 'O' antigen and has been utilized historically as an epidemiological marker for strain characterization [14, 15]. DEC pathotypes possess a large number of various 'O' somatic antigen, therefore their continuous monitoring is helpful in subtyping of strains and enhancing phylogenetic studies. Associations and prevalence of different DEC pathotypes with 'O' antigens observed to vary across different regions of world [21, 22].

Vibrio is an important diarrheal pathogen responsible for several global epidemics and pandemics [23, 24]. *Vibrio,* member of family *Vibrionaceae* is a comma shaped, gram negative, aquatic bacterium which colonizes small intestine of human [25]. *Vibrio* spp. causes profuse watery diarrhea and disease become severely fatal, if left untreated [23, 26]. Symptoms of *Vibrio* mediated gastroenteritis include rice watery stools, severe dehydration, fever and vomit. *Vibrio* species expresses several virulence factors like toxins, colonization factors (lipopolysaccharide, flagellar components, outer membrane proteins, hemagglutinin, toxin coregulated pilin (*tcpA*)), protease (hemolysins, cytolysins, thermolysins; metalloproteases) and iron acquisition systems [27].

Shigellosis, an acute infection of intestine is caused by *Shigella* spp. which is endemic in temperate and tropical climates. Shigellosis is characterized by mild to severe forms of watery, bloody or mucoid diarrhea and symptoms may be aggravated among immunocompromised persons and young children [28]. Among four species of *Shigella* (*S. dysentriae, S. flexneri, S. boydii* and *S. sonnei*), *S. flexneri* typically predominates in developing nations whereas, *S. sonnei* prevails in developed nations. Success of this pathogen is based upon the fact that as few as ten bacterial cells can cause infection in a healthy individual [28]. Therefore, bacterial identification in diarrheal illness is necessary to screen *Shigella* spp. as causative agent.

Non-typhoidal *Salmonella* (NTS) is another important cause of acute gastroenteritis which is characterized by rapid diarrhea onset, fever and abdominal cramps. *Salmonella* is known to

cause several food borne outbreaks and younger children with nutritional deficiency and elder people with immunocompromised status are at higher risk of acquiring infections [29]. However, there has been significant reduction in NTS infections from past decade [30].

Since, diarrheal disease is generally self-limiting, anti-diarrheal agents are not usually recommended for treatment of diarrhea. However, traveler's diarrhea, persistent diarrhea and acute invasive diarrhea display high severity of infection and extended recovery periods, which reinforce the use of various antimicrobials [31]. In addition, alarming resistance rates have narrowed down the choice of treatment protocols. WHO's Global Antimicrobial Surveillance System (GLASS) 2016-2017, revealed *E. coli* and *Salmonella* spp. to be the most common resistant organisms and also confirmed situation of serious antimicrobial resistance worldwide [32].

Diarrhea associated high rates of hospitalization and outpatient visits reinforce information on infectious etiology, its determinants, preventive and control interventions for better policy making strategies in health services. Comprehensive studies investigating the pathogen-specific attributable incidence are sparse in Himachal Pradesh and are mainly reported during outbreaks and to lesser extent in surveillance studies. However, baseline studies analyzing epidemiological significance of bacterial pathogens are not reported till date from Himachal Pradesh. Present study focuses on characterization and prevalence of pathogen-specific diarrhea incidences and determination of microbial resistance levels in Himachal Pradesh, a northern hilly state of India. Molecular methods were utilized to better define incidences of major bacterial pathogens, their etiology and clinical outcomes. Correlations of different pathogens with different age groups and clinical symptoms were also analyzed. Analysis of resistance patterns exhibited by bacterial pathogen would help in prioritizing treatment regimens against prevalent pathogens.

Present dissertation is classified into following chapters:

- Chapter 1: A short introduction to present study
- Chapter 2: Review of literature related to the study
- Chapter 3: Objectives of the study
- Chapter 4: Materials & Methods used in this study
- Chapter 5: Results observed in study
- Chapter 6: In detail discussion of our results
- Chapter 7: Conclusion of present study
- Chapter 8: List of references cited in this dissertation, followed by an appendix.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Diarrhea

Diarrhea is an acute gastrointestinal symptom characterized by passage of three or more loose stools/day. Diarrhea constitute an important public health issue imposing great economic burden and also leading to malnutrition and growth faltering conditions [2]. In general, diarrhea illness is transmitted by fecal-oral route; via consumption of contaminated food and drinks [33]. Diarrhea is more prevalent in regions with suboptimal hygienic conditions and lower access to clean drinking water. Factors contributing for contamination of food and water include; human or animal defecation in/near water bodies and use of contaminated water for preparation of food and irrigation [1]. Among several factors like pneumonia, congenital abnormalities, preterm birth complications etc. which contribute to global child mortality, diarrheal disease constitutes an important infectious cause of global children deaths (8%) (Figure 2.1) [34].

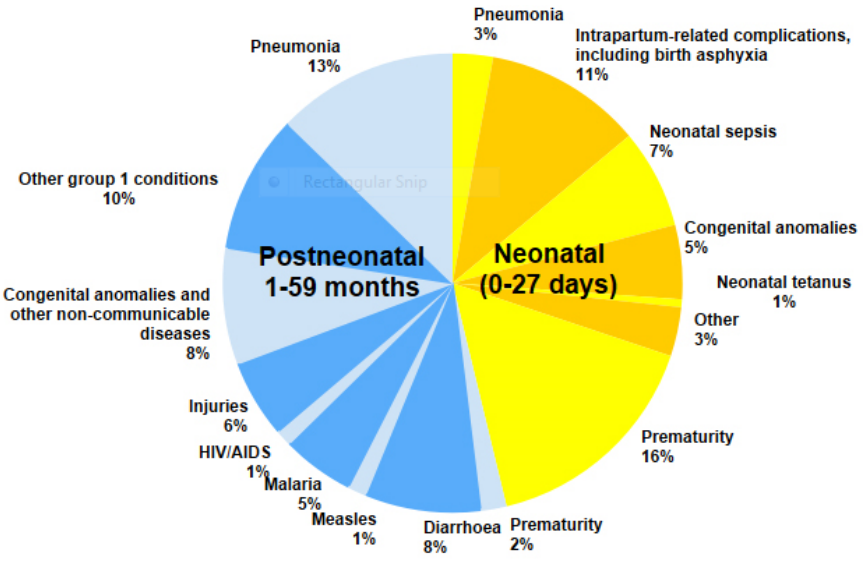


Figure 2.1: Global mortality rate estimates for various causes among children under the age of five years. (Source: WHO, Global health observatory data repository 2018) [34].

Globally, overall incidences of diarrhea are higher in South East Asian region, particularly India is one of the leading nation in diarrhea associated morbidity and mortality cases [35]. In adult population, only few succumb to severe complications of diarrhea, but elderly population is at higher risk of death, emergency hospitalization and longer hospital stay as compared to younger peoples [36]. Geographically, there are huge variations in seasonal

drivers associated with the diarrhea. Usually, incidences of diarrhea peak during humid and optimal temperature conditions [37, 38].

2.2 Global scenario of diarrhea

Comprehensive analysis in Global Burden of Disease (GBD) 2016, estimated a total of 2390 million episodes of diarrhea among all ages, of which approximately 957 million episodes of diarrhea have been reported among children <5 years of age [8]. GBD study detected trivial variations in diarrhea mortality rates on gender basis [8].

In developed nations, diarrheal diseases constitute common cause of outpatient visits and hospital admissions. However, sporadic diarrhea outbreaks have also been reported from developed regions at different time intervals [39, 40]. The primary vehicles of transmission involved during outbreaks mainly included farm animals, seeds, household cross contamination and ice [41, 42].

Diarrhea is a global health scourge, however South Asian and sub-Saharan African regions account to highest proportions of diarrhea morbidity and mortality [35]. In low to middle income countries, diarrhea is a widespread illness and average number of diarrheal episodes is observed to be much higher as compared to developed nations. Children from low-income countries, suffer from severe complications of diarrhea as each episode of diarrhea deprives the child of the nutrition necessary for growth. As a result, diarrhea is a major cause of malnutrition, and malnourished children are more likely to fall ill from diarrhea [8].

Although, diarrhea is still a major public health issue, however continuous credible efforts of organizations, research groups and policy makers have led to an overall substantial reduction of 57% in diarrhea mortality rates from past decade (2000-2015) (Figure 2.2) [35]. Though, global diarrhea mortality rates have dropped to a significant proportion but globally, there is no satisfactory decline in diarrhea morbidity rates. For example, East Asia, Latin America and Eastern & Western sub-Saharan Africa regions with highest mortality rates have shown >65% reduction however, there has been a very narrow window change for diarrhea incidences globally [8].

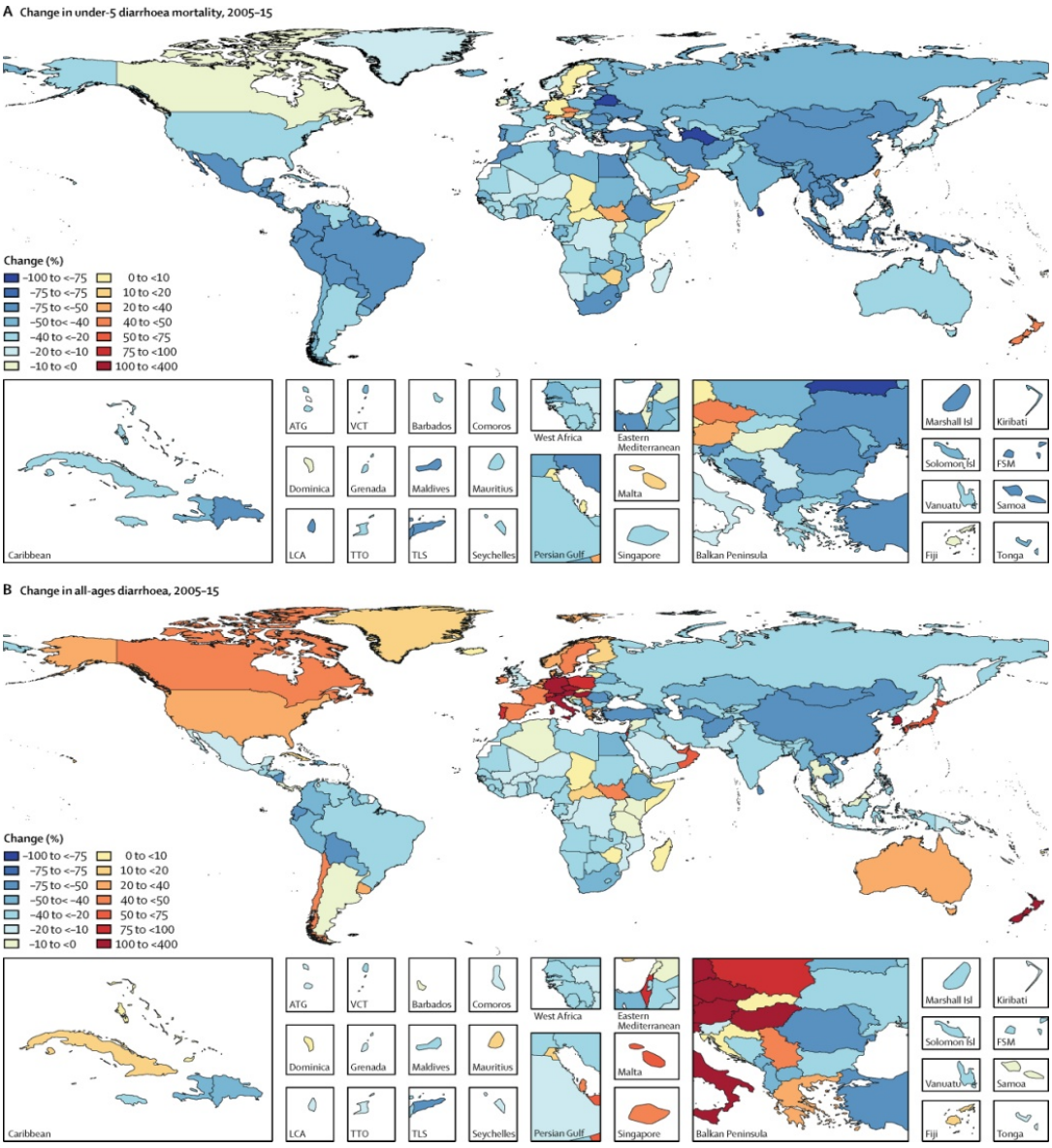


Figure 2.2: Global diarrhea mortality rate changes per 100,000 during 2005 and 2015 among under five and all age groups. ATG= Antigua and Barbuda. VCT= Saint Vincet and the Grenadines. LCA= Saint Lucia. TTO= Trinidad and Tobago. TLS= Timor-Leste. FSM= Federated states of Micronesia.(Source: Creative common License No CC BY 4.0) [8].

2.3 National level scenario of diarrhea

India harbors world's one fifth population and possesses considerable region wise variations in its population health status and causes of health loss. Diarrheal illness accounts for a significant proportion of morbidity and mortality in the country and also imposes additional economic burden [43].

Young age, low socioeconomic status, poor maternal literacy and hygiene practices, low birth weight, inadequate breastfeeding, and malnutrition are several listed factors known to contribute escalated diarrhea incidences among Indian population [17]. According to a recent statistical analysis, infectious diseases like diarrhea, lower respiratory tract and tuberculosis have attributed to high of mortality rates (15.5%) among all age groups from past fifteen years (1990-2016) [44].

India state-level disease burden initiative estimated state wise burden of diarrheal disease and disability adjusted life years (DALYs) attributable to diarrhea (Figure 2.3 and Table 2.1). According to the report, Orissa and Chhattisgarh were observed to have highest number of diarrheal deaths. Furthermore, DALYs attributable to diarrhea were observed to be highest in Orissa, Chhattisgarh and Bihar. Himachal Pradesh, the region under study was estimated to have moderate levels of diarrhea mortality and DALYs rates. However, potential causative agents are not explored from the region till date.

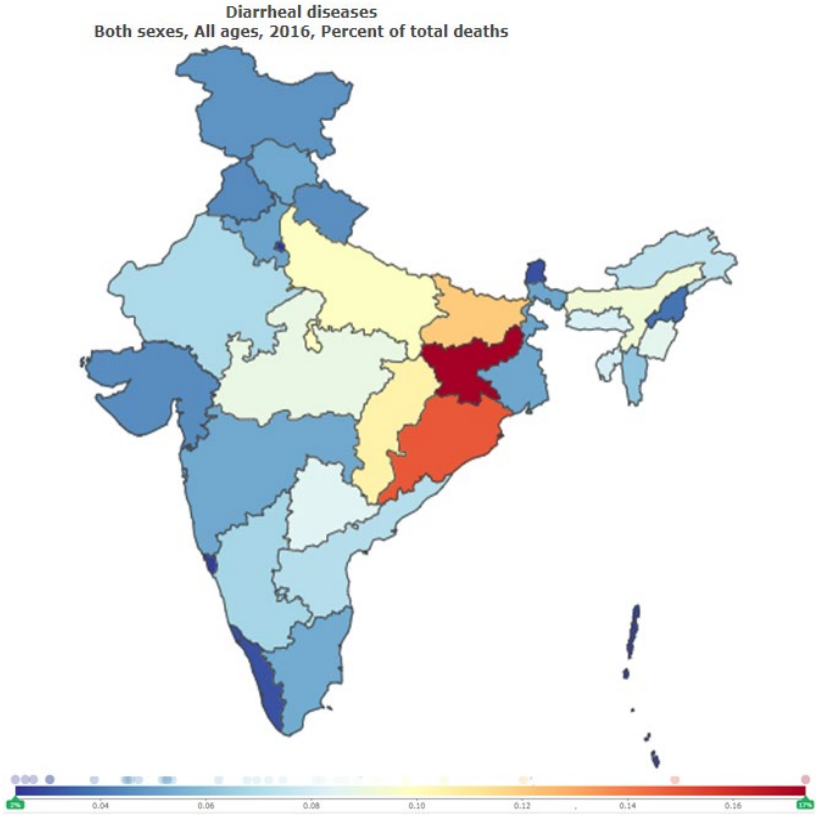


Figure 2.3: Percent of deaths attributable to diarrheal disease in year 2016 estimated by Global Burden of Disease Study. (Source: https://vizhub.healthdata.org/gbd-compare/india) [44].

Overall, diarrhea was also one of the leading causes of DALYs contributing significantly to a total proportion of 5% [44]. DALYs due to diarrhea is more prevalent in the empowered action group states (EAG) and lowest among highest epidemiological transition level (ETL) groups. These observations highlight broad trend and the importance of exploring diarrhea disease burden and etiology from different regions of India.

Table 2.1: DALYs rate due to diarrhea in different states of India. (Adopted from creative common License no CC BY 4.0). EAG; Empowered Action Group, ETL; Epidemiological Transition Level

States of India	**DALYs rate**	**States of India**	**DALYs rate**
Empowered Action Group (EAG) States	**2351**	Other states	
Lowest Epidemiological Transition Level (ETL) group	**2354**	**Lower-middle ETL group**	**959**
Bihar	2827	Gujarat	959
Chhattisgarh	2193	**Higher-middle ETL group**	**1048**
Jharkhand	3420	Haryana	1109
Madhya Pradesh	1835	Delhi	485
Odisha	2978	Telangana	1391
Rajasthan	1490	Andhra Pradesh	1292
Uttar Pradesh	2380	Jammu & Kashmir	821
Lowest Middle ETL group	**1059**	Karnataka	1228
Uttarakhand	1059	West Bengal	993
North East states	**1944**	Maharashtra	927
Lowest ETL groups	**2231**	Union territories other than Delhi	443
Meghalaya	1344	**Highest ETL group**	**781**
Assam	2309	Himachal Pradesh	749
Lower middle ETL groups	**1158**	Punjab	881
Arunachal Pradesh	1204	Goa	402
Mizoram	919	Tamil Nadu	920
Nagaland	526	Kerala	438
Tripura	1464		
Sikkim	606		
Manipur	1364		

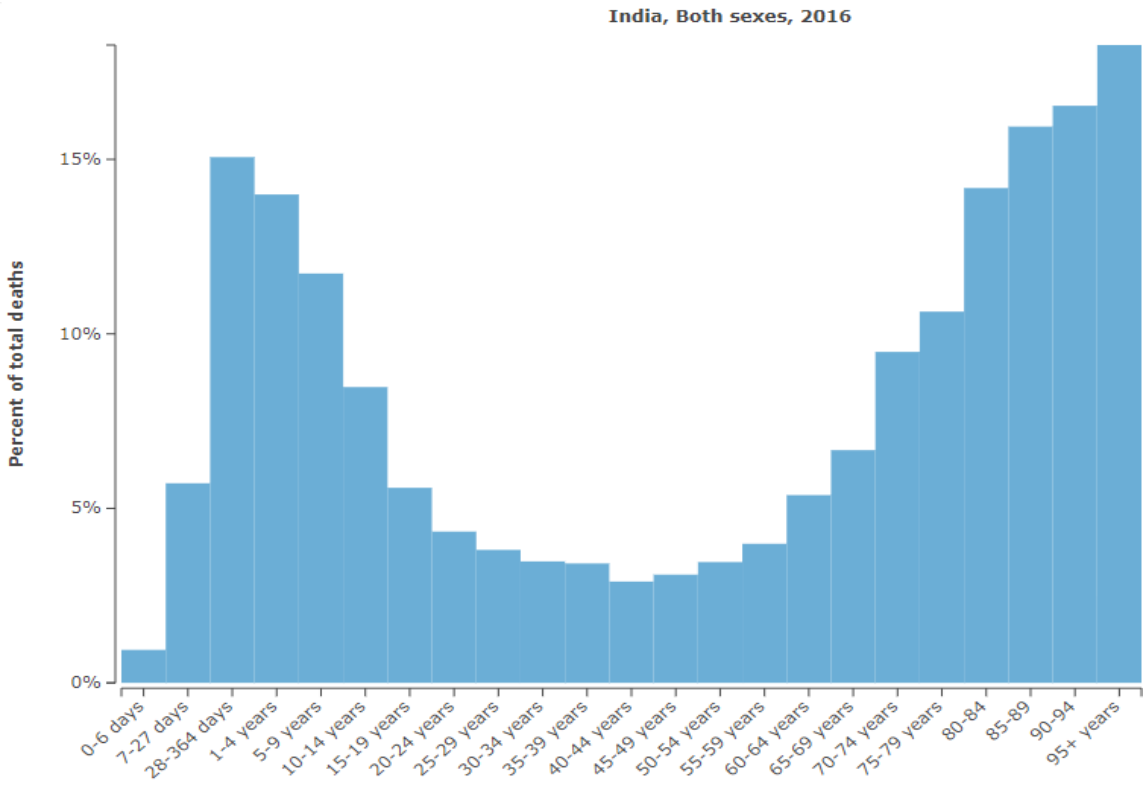


Figure 2.4: Age wise categorization of diarrhea mortality rates in India during year 2016. (Source: https://vizhub.healthdata.org/gbd-compare/india). [44].

GBD for India also explored highest risk age groups vulnerable for diarrhea disease. From Figure 2.4, mortality rates were quite higher among children and elderly (>65 years) groups. Approximately 3-5% mortality rates were observed for 20-60 years age group. Table 2.2 summarizes studies of bacterial enteropathogens from different parts of the India published during years 2010-2018.

Table2.2: Studies addressing burden of diarrhea from different parts of India from 2010-2018.

Diarrheagenic *E. coli*							
Year	Region	Study	Patients	Identification method	Etiological agents	Co-infection	Ref
2010	Vellore	Surveillance 2003-2006	394 children with diarrhea/ 198 controls	PCR, stool specimens	52% DEC [EAEC 14.7%, EPEC 10%, ETEC 4.1%, EHEC 2%, EIEC 1%, DAEC 0.5%] in patients and 63% in control [EAEC 23.7%, EPEC 8%, ETEC 2.5%, EHEC 0%, EIEC 0%, DAEC 0%]	87 cases	[45]
2010	Kotputli	Outbreak	1175 [652 adults,	69 Rectal	*V. cholerae*, *E. coli, Enterobacter*	Not reported	[46]

			515 children]	swabs, PCR	*cloacae, Proteus vulgaris*		
2012	Mangalore	2002-2004 Hospital based study 115 *E.coli*	115 [95 adults, 20 children]	PCR, stool samples	DEC (17.4%), atypical EPEC (10.4%), EAEC (3.4%) and STEC (3.4%).	Not reported	[17]
2013	Kolkata	2008–2011 Hospital based surveillance	3826 All age groups	PCR, stool samples	DEC 11.8% [EAEC (5.7%), ETEC (4.2%) and EPEC (1.8%)]	69.5%	[18]
2014	Hyderabad	Hospital, based surveillance	502 children [6months to 5 years] outpatient clinic or admitted	PCR, stool samples	DEC (45.6%)(EPEC 35%, ETEC 11%, STEC 30%, *E. coli* O157:H7 24% and non DEC 55.8% >70% of *E. coli* resistant to norfloxacin, amoxycillin, co-trimoxazole, ampicillin, ceftriaxone, cefotaxime and metronidazole	Not reported	[20]
2016	Tamil Nadu	2012-2014	90 patients and 95 case controls of 0-60 months of age	PCR, stool samples	65.5% DEC [only ETEC was detected]	Not reported	[19]
2017	Odisha	Outpatient hospital based surveillance	130 children <5 years	PCR, stool samples	*E. coli* (30.07%), rotavirus (26.15%), *Shigella* (23.84%), adenovirus (4.61%), *Cryptosporidium* (3.07%), and *Giardia* (0.77%)	(33.84%)	[47]
2017	Bihar	2 years consecutive study Outpatient and hospitalized patients	633 0-60 months	PCR, stool samples	DEC (30.2%), EAEC (69.1%) ETEC (10.5%), EPEC (8.4%), EHEC (2.6%), EIEC (1.6%). 37.6% of DEC isolates were ESBL producers.	7.8%	[48]
2017	Andaman Islands	2013-2016 Hospital based surveillance	1394 <5 years old children	PCR, stool samples	DEC (6.82%), EAEC (70.1%), EPEC (19.6%) [63.2% were atypical EPEC], ETEC (10.3%)	Not reported	[49]
2017	New Delhi	2013-2014 case control	80 stool samples from	PCR, stool samples	21.5% DEC	Not reported	[50]

		study	children with diarrhea, 40 from healthy controls				
***Vibrio* spp.**							
Year	Region	Study	Patients	Identification method	Etiological agents	Co-infection	Ref
2010	Odisha	Epidemic study	68 rectal swabs and 28 water samples	Mismatch amplification of mutation (MAMA)-PCR assay.	*V. cholerae* O1 Ogawa biotype El, Sensitive to tetracycline, gentamicin, azithromycin, and chloramphenicol Resistant to ampicillin, ciprofloxacin, norfloxacin, co-trimoxazole, nalidixic acid, neomycin, and furazolidone,	Not reported	[51]
2012	Gujarat	Outbreak	117 case of gastroenteritis	Conventional microbiology	14.5% *Vibrio cholerae*01 serotype ogawa	Not reported	[52]
2014	Kolkata	Hospital based surveillance	3,607 acute diarrheal patients	Serogrouping, PCR	4.9% *V. parahaemolyticus*	Not reported	[53]
2017	New Delhi	Surveillance Study	11,570 diarrheal /dysentric fecal samples	Biochemical reactions, serological & AST assay	2.4% *V. cholerae,* resistant to nalidixic acid and ampicillin	Not reported	[54]
2018	New Delhi	Case report	1 5-month-old	PCR, DNA sequencing	HCT (ctxB7) in a nonO1/nonO139 isolate	Not reported	[55]
Salmonella							
Year	Region	Study	Patients	Identification method	Etiological agents	Co-infection	Ref
2010	Pondicherry Bangalore Mangalore	Hospital Surveillance study	21 isolates of non typhoidal *Salmonella*	Serotyping, PCR and sequencing	47.6% *S. Agona*, 23.8% *S. Typhimurium* , 14.3% *S. Enteritidis*, 4.7% *S. Senftenberg*, 4.7% *S. Lexington* and 4.7% *S. Kirkee*	Not reported	[56]
2014	Chandigarh	2002–2010 Hospital based surveillance	7813	Biochemical tests, serotyping and PCR, stool samples	Among total 77 *Salmonella* strains, 83.11% non-typhoidal *Salmonella* and 15.6% Typhi and 1.3% Paratyphi	Not reported	[57]
2016	Karnataka	2011–2014 Hospital based	3187	Biochemical assays, stool samples	Total 320 (10.04%) were enteric pathogens of which 64 (20%) NTS, 46.5% DEC, 11.8% *Aeromonas*	Not determined	[58]

		surveillance			species, 10.6% *Vibrio cholerae* O1, 6.2% *Shigella* species, 3.1% *Salmonella* species other than NTS and 0.6 % unusual pathogens		
Shigella							
Year	Region	Study	Patients	Identification method	Etiological agents	Co-infection	Ref
2010	Kolkata	2001-2007 Hospital surveillance	4478	Microbiology assays and serotyping, stool samples	Total 516 (11.5%) Shigella isolates that includes 60.4% *S. flexneri,* 23.8% *S. sonnei*, 9.3% *S. dysenteriae* and 6.4% *S. boydii*	Not reported	[59]
2011	Port Blair, Andaman and Nicobar	2008-2009 Hospital surveillance study	311	Microbiology assays and serotyping, stool samples	Total 44 (14%) were *Shigella* isolates that includes 66% *S. flexneri*, 23% *S. sonnei* and 11% *S. dysenteriae*	Not reported	[60]
2014	Port Blair and Nicobar	2006-2011 pediatric samples	943	Microbiology assays and PCR	Total 88 (9.33%) *Shigella* isolates (62.5 % *S. flexneri*, 26.1 % *S. sonnei*, 9.1% *S. dysenteriae*, and 2.3 % *S. boydii*	Not reported	[61]
2016	New Delhi	2009- 2012 Hospital surveillance study	6339	Microbiology assays and PCR	121 (1.9%) *Shigella* that includes 66.1%, *S. flexneri*, 19.8%, *S. boydii*, 12.4%, *S. sonnei* and 1.7% , *S. dysenteriae*	Not reported	[62]
2016	New Delhi	2011-2012 Hospital surveillance study	325 under age of 12 years	Microbiology assays and PCR	Total 56 (17.2%) were *Shigella* spp., 66.1%*S. flexneri*, 24.1% *S. boydii* and 12.5% *S. sonnei.*	Not reported	[63]
2016	Kolkata	Hospital surveillance study	37	Microbiology assays, Real-time PCR and ELISPOT assay	56.7% *Shigella*; 57.1% *S. flexneri* 3a, 23.8% *S. flexneri* 2a, 9.5% *S. sonnei*, 4.7% *S. flexneri* 4 and 4.7% untypeable *S. flexeri*. 8.1% *V. fluvialis*, 5.4% *Campylobacter jejuni*, 2.7% *Pseudomonas* spp., 2.7% *V. cholerae*, 2.7% *V. parahaemolyticus*	Not reported	[64]

2.4 Infectious agents of diarrhea

Diarrhea is a symptom of infections caused by bacterial, viral and parasitic organisms, most of which are transmitted by fecal-oral route [33]. The multiple etiologies associated with diarrheal illness are bacteria, viruses and protozoans [7] (Figure 2.5). Bacterial diarrheal pathogens involve diarrheagenic *Escherichia coli*, *Vibrio* spp., *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., and *Clostridium* spp. etc. According to Global Enteric Multicenter Study (GEMS), *Shigella* and ETEC are among the leading pathogens associated with moderate-to-severe diarrhea (MSD) in low middle income countries [65].

Among viruses, rotavirus, norovirus, astrovirus, adenovirus and calcivirus are the leading causes of diarrhea [66-68]. Rotavirus is responsible for 25% of MSD illnesses [7] and 30% of the diarrheal deaths, among children [69]. Burden of rotavirus mediated morbidity and mortality will decrease significantly following introduction of rotavirus vaccines into National immunization programs.

Parasitic agents of diarrhea include *Giardia lamblia, Entamoeba histolytica,* and *Cryptosporidium* spp. [70]. According to World Health Organization (WHO), enteric protozoa accounted for a significant morbidity and DALYs between years 2010-2015 [71]. Epidemiologically, *Giardia, Cryptosporidium* and *Entamoeba* species infections are more frequent causes of persistent diarrhea among children [72, 73].

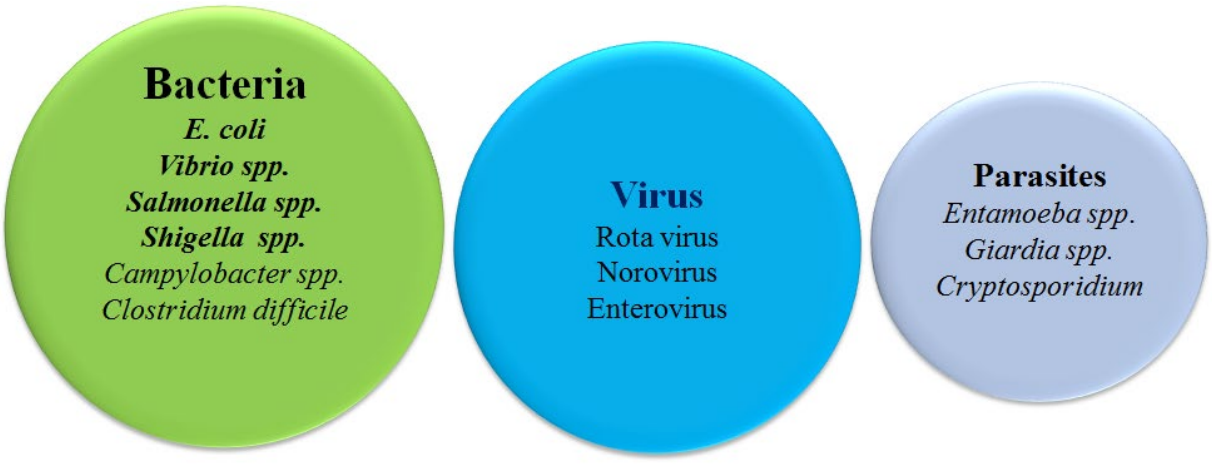


Figure 2.5: Various etiological agents of diarrhea.

In many cases, identification of etiological agent is a cumbersome process and globally approximately 40% of the cases identified none of the pathogens [74, 75]. Furthermore, co-

infections of gut pathogens are quite frequent which increases severity and duration of disease [76, 77]. In such cases, in vivo studies exploring virulence potential of strains should be validated. Bacterial co-infections involving DEC, *Vibrio* spp., and *Shigella* spp. have been reported so far [77, 78].

2.4.1 Bacterial agents of diarrhea

Bacterial pathogens contribute to 20-40% of diarrheal episodes and are associated with significant mortality in developing nations and also with substantial morbidity in developed nations [79]. Diarrheagenic *E. coli* and rotavirus are known to cause significant morbidity and mortality among children less than five years of age [7] and illness ranges from mild to severe diarrhea [79].

Bacterial pathogens can either cause inflammatory or non-inflammatory diarrhea. Inflammatory diarrhea is usually caused by bacterial colonization of distal ileum and colon. Colonized bacteria either secrete toxins or invade epithelial cells, resulting in inflammation [80, 81]. Bacteria capable of causing inflammatory diarrhea include cytotoxin-producing, invasive and noninvasive organisms. Invasive pathogens like *Shigella* spp., *Campylobacter* spp., *Salmonella* spp., *Yersinia* spp., and enteroinvasive *E. coli* (EIEC) infections result in acute inflammatory reaction with disruption of mucosal epithelium. Invasive organisms generally result in presence of mucus, red blood cells and polymorphonuclear (PMN) leukocytes in the stool. Non-invasive organisms like enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC) and *C. difficile* associated clinical symptoms include watery diarrhea, with or without blood and abdominal cramps [80-82].

Non-inflammatory diarrhea is the infection caused by etiological agents colonizing in small intestine. These pathogens usually interrupt the normal absorption/secretion physiology of intestine, without resulting in acute inflammation or epithelial cell damage. Bacterial diarrheal agents usually resulting in the non-inflammatory diarrhea include ETEC, *V. cholera* and *Clostridium perfringens* [80]. Many of these organisms (Like *V. cholerae*, ETEC and rotavirus) secrete enterotoxins that stimulate intestinal secretion and hence, the production of watery diarrhea without any blood or pus. Patients with non-inflammatory diarrhea generally have few systemic signs or symptoms such as abdominal cramping, nausea or vomiting, and fever is typically absent.

2.4.1.1 Mechanism of diarrhea by bacterial agents

Diarrhea is categorized into acute watery, acute bloody and persistent forms. Acute watery and bloody diarrhea can last from hours to days (<14days). However, complications associated with acute illness include moderate to severe dehydration and intestinal epithelium damage. Persistent diarrhea occurs more than 14 days and can cause severe dehydration and malnutrition in children [1]. Pre-existing malnutrition can also expose children for diarrhea and severe life threatening complications. Dehydration is caused by excessive fluid loss from the body and is attributed to most diarrhea related deaths.

Basic mechanisms of diarrhea interfere with the normal absorptive and secretory functions of the intestinal epithelial monolayer (Figure 2.6**)** [83]. **a.** ETEC and *Vibrio cholerae* secrete toxins which lead to increased chloride ion secretion via cystic fibrosis transmembrane receptor or reduced chloride ion uptake via inhibition of downregulated in adenoma (DRA) channels. **b**. Decreased sodium ion absorption is caused due to inhibition of Na^+/H^+ exchanger 3 (NHE3). **c**. Down regulation of the Na^+/glucose co-transporter also leads to decreased sodium uptake via enterocytes (SGLT1).

d. Direct inhibition of water channels or aquaporins (AQPs). **e.** Disruption of epithelial barrier function by promoting myosin light chain (MLC) phosphorylation (possibly by stimulating tumor necrosis factor-α (TNFα) production), leading to contraction of the peri-junctional actomyosin ring and opening of the tight junctions.

f,g. Direct alteration of tight junction (TJ) protein localization leading to disruption of barrier and fence functions and pro-inflammatory signaling through engagement of Toll-like receptors (TLRs) by bacterial ligands, eventually leading to neutrophil transmigration into the lumen, and release of 5 -AMP and its conversion to adenosine, which subsequently causes cyclic AMP (cAMP)-dependent Cl secretion. MLCK, MLC kinase; NF-κB; nuclear factor-κB; PMN, polymorphonuclear leukocyte.

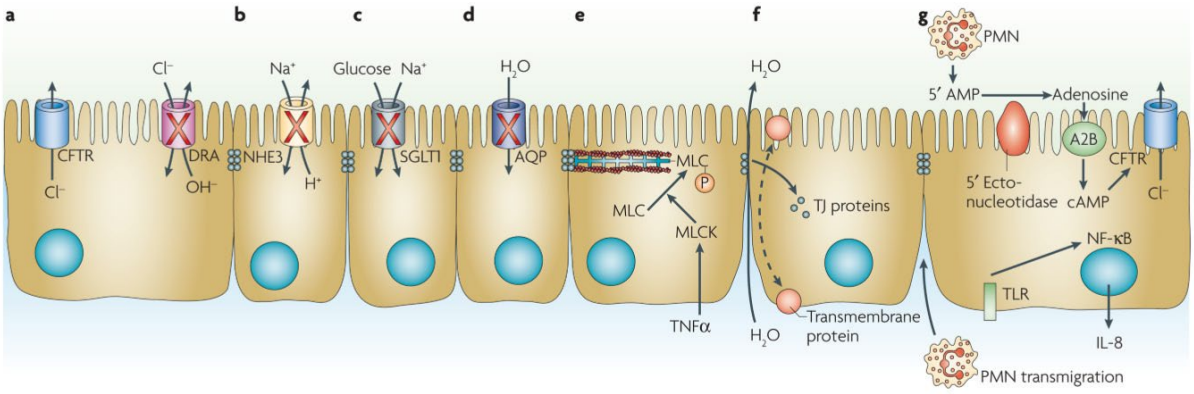


Figure 2.6: Mechanism of diarrhea caused by bacterial enteropathogens [83].

2.4.1.2 Diagnosis of bacterial pathogens

Identification of diarrheal pathogens potentially streamlines the therapeutic options and also helps in management of antimicrobial resistance. There is scarcity of laboratories which are engaged in characterization of diarrheal pathogens, especially in developing countries. Assessment and characterization of etiological agents are mainly based on clinical pathophysiology presented and by application of conventional and molecular approaches.

- **Clinical symptoms of bacterial diarrhea**

Diarrheal disease is defined as the course of three or more loose stools per day. Additionally, disease can be associated with other clinical symptoms of dehydration, fever, vomit and abdominal pain [1]. Dehydration usually results from excessive water and electrolyte loss during episodes of liquid stools, vomit and urine output. Sodium, potassium, bicarbonate and chloride ions are lost during malfunctioned secretory/absorptive process. Clinical assessment of severe dehydration status is performed by visible signs like unconsciousness, sunken eyes, skin pinch, reduced urine output and inability to drink water. Bacterial pathogens like enteroaggregative *E. coli* (EAEC), EIEC and *Shigella* are often associated with acute invasive diarrhea [84].

- **Laboratory diagnosis of bacterial pathogens**

Considering multiple etiology of diarrhea, specific identification of pathogen is very cumbersome process. Various conventional methods like microbial culture, microscopy, and antigen-based tests face noteworthy limitations of specificity, limit of detection, and require other additional confirmations. Nowadays, molecular diagnostics have circumvented

challenges of conventional techniques and have also simplified detection of co-infections [85]. Success of novel approaches relies on careful understanding of the disease pathophysiology exerted by several etiological agents.

Culture based methods often provide low yield of enteropathogens, specifically in case of antibiotic prescription. Microscopic analysis requires training and specialization in handling and identification of pathogens. Molecular methods involve amplification of DNA or RNA, with polymerase chain reaction (PCR). PCR is the most common amplification approach which offer rapid recognition with greater sensitivity and specificity. After extraction of nucleic acid, either singleplex or multiplex PCR can be performed for detection of multiple enteropathogens. Identification of specific targets needs sequence specific probes/primers, which can be differentiated either by agarose gel electrophoresis or by melting curve analysis of amplicons [86, 87].

In this direction, "real-time" PCR offers detection of number of copies of gene after each

amplification cycle. The detection limit of real-time PCR can be accessed by experiment involving lower limit of detection of 10^2 spores/mL stool in RT-PCR, versus 10^6 spores/mL via microscopy [88]. Inability to correlate identified pathogen with specific clinical pathophysiology raises concern about 'What is a pathogen' and also poses limitation to molecular method. At these points, pathogen with greater abundance can be significantly correlated with disease and hence quantitative approaches come into play.

Next generation sequencing platforms provide massive parallel sequencing of thousands of samples at a time. Various NGS techniques including pyrosequencing, Illumina (HiSeq and MiSeq) have been utilized in detection of enteropathogens [89, 90]. Presently, application of high throughput techniques has explored previously unknown enteropathogens and helped in prioritizing the diagnostic and therapeutic approaches. Overall, combination of conventional and molecular methods warrant specific and higher detection of pathogens [91]. However, molecular methods are mostly confined to resource rich settings and are very limited in settings with low infrastructure.

Major bacterial pathogens and their pathophysiological features are discussed below.

2.4.2 Diarrheagenic *E. coli*

Escherichia coli are rod shaped, gram-negative, facultative anaerobes which commonly inhabit the GI tract of humans and other warm blooded animals (Figure 2.7) [92]. *E. coli* strains colonize host at the time of birth and are generally non pathogenic. Pathogenic *E. coli* are capable of causing diarrhea, meningitis, septicaemia and urinary tract infections [93]. In 1885, German pediatrician Theodor Escherich firstly isolated *E. coli* from stool specimen of a diarrheal child and observed the dual role of bacterium as a commensal inhabiting intestinal microflora of healthy individuals and also as a potent pathogen, capable of causing intestinal and extra-intestinal infections [94]. Studies recognizing potential pathogenic role of *E. coli* were reported in later 1940s [95, 96]. Presently, several outbreaks and reports have deciphered role of *E. coli* as causative agent of moderate to severe diarrhea among all age groups [7, 65, 93, 97]. In 2011, DEC caused an estimated 0.12 million deaths under five years age group globally [49].

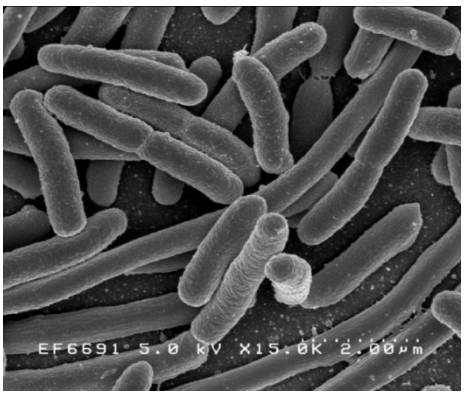


Figure 2.7: Scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip. (Source: Creative commons License no CC BY 2.0). [98].

Diarrheagenic *E. coli* strains have acquired specific virulence factors through horizontal gene transfer and are evolved into more pathogenic phenotypes [97]. The diarrheagenic *E. coli* strains are categorized into various pathotypes on the basis of presence of specific virulence genes. Six different pathotypes associated with diarrhea illness include; enterotoxigenic *E. coli* (ETEC), Shiga toxin–producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). EPEC is frequently associated with diarrhea incidences from both community and healthcare settings. Enterotoxigenic *E. coli* (ETEC) has been reported to be a virulent

bacterium responsible for diarrhea in travelers and population inhabiting endemic regions globally [18, 99, 100]. Since the last decade, several reports have been published for identification of adherent Enteroaggregative *E. coli* (EAEC) as emerging enteropathogens responsible for adult and childhood diarrhea worldwide [101-104]. Another molecular pathotype of diarrheagenic *E. coli*: Enterohemorrhagic *E. coli* (EHEC) is a subgroup of Shiga toxin (Stx) producing *E. coli.* Enteroinvasive *E. coli* (EIEC) strains cause devastating form of gastrointestinal infections and lead to severe life threatening complications like hemolytic uremic syndrome (HUS). EHEC colonize large intestine and secretes toxins [14]. EIEC invade small bowel enterocytes and are regarded a true intracellular pathogens. However, both EIEC and EHEC are associated with large outbreaks and generally display low levels of incidences in routine surveillance studies [10, 105].

Sequelae of ETEC, EPEC, and EIEC infection are not well described. Transmission of DEC occurs via fecal-oral routes as bacteria are shed into feces. Humans and animals are recognized as main reservoir of DEC pathotypes. There is no formal surveillance system for diarrheagenic *E. coli* and most laboratories are unable to identify these pathotypes due to lack of a simple and rapid diagnostic technique.

2.4.2.1 Enteropathogenic *E. coli* (EPEC)

EPEC is an important cause of pediatric endemic diarrhea and were firstly found to be associated with severe nursery epidemics in 1940s [95]. EPEC strains are implicated as one of the important causes of persistent diarrhea and this pathogen is quite prevalent in community and healthcare settings [106].

- **Pathogenesis of EPEC**

Enteropathogenic *Escherichia coli* (EPEC) belong to group of attaching and effacing (A/E) pathogens [107]. EPEC intimately attach to intestinal epithelium and lead to effacement of microvilli. Pathogenesis of EPEC have been described in three different stages

A. Localized adherence, **B.** Signal transduction, and **C.** Intimate adherence (Figure 2.8).

A. Localized adherence

EPEC show attaching and effacing type of pathogenicity in intestinal epithelium. In 1991, researchers for the first time identified bundle forming pilus responsible for localized

adherence (LA) pattern in EPEC [108]. Bundle forming pilus is encoded on *Escherichia coli* (EPEC) adherence factor (EAF) plasmid and is regulated by plasmid encoded regulator (Per). Bundle-forming pili adhere to other EPEC, as well as to host cell surfaces via *N*-acetyl-lactosamine-containing receptors. Adherence of EPEC lead to phosphorylation of protein kinase C and MLC kinase which cause disrupted ion and water absorption and higher permeability of tight junctions [109].

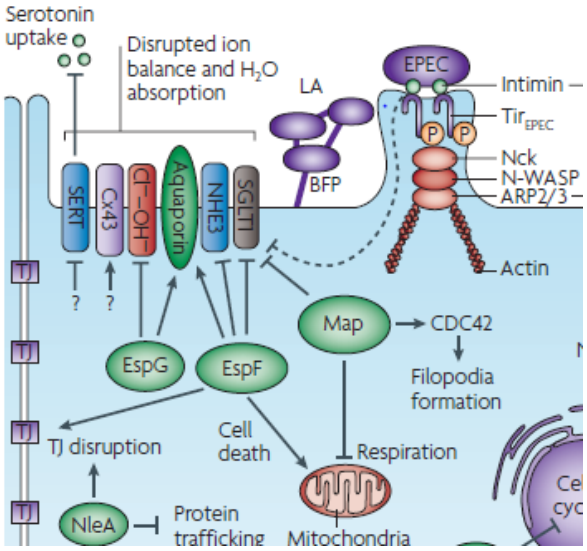


Figure 2.8: A schematic representation of EPEC pathogenesis in epithelial cells [107].

B. Signal transduction

After exhibiting localized adherence, EPEC secrete various proteins on host epithelium which in turn induce various signal transduction pathways in host mucosal cell. Locus of enterocyte effacement (LEE) encodes various genes for a type III secretion system (T3SS), secreted proteins and intimin adhesin. EPEC secreted proteins (ESP) (*espA, espB, espD, espF* and *espJ*) subvert actin polymerization in host epithelium and manifest signal transduction and cytoskeletal changes. In addition to F-actin, the composition of the A/E lesion includes other cytoskeletal components such as α-actinin, talin, ezrin, and myosin light chain.

C. Intimate adherence

For intimate attachment of EPEC, a 94 kDa intimin adhesion and translocated intimin receptor (Tir) are required. Tir protein is phosphorylated at tyrosine residues and is translocated to epithelial cell surface to act as receptor for intimin adhesion. Phosphorylation of Tir recruits host cell receptor protein non catalytic region of tyrosine kinase adaptor

protein (Nck) and also activates neural Wiskott–Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (ARP2/3) [110]. These events lead to formation of pedestal beneath attached bacterium. The ESPs can affect Cl^-/OH^- and Na^+-H^+ exchanger activity, mislocalization of aquaporins and inhibition of sodium-glucose co-transporter 1 (SGLT1). Moreover, mitochondrial-associated protein (Map) regulates actin polymerization and results in filopodia formation. Map and espF proteins are associated with disruption of mitochondrial structure and function.

All above mentioned molecular events leads to disruption of tight junctions and effacement of microvilli resulting in seepage of cellular and intracellular contents into intestinal lumen.

- **Diagnosis of EPEC**

EPEC has been categorized into typical (intimin and bundle forming pili) and atypical (intimin only) classes depending upon possession of characteristic virulence factors. As per recent reports, atypical EPEC is more prevalent than typical EPEC [106, 111]. Moreover, attaching and effacing phenotype associated with the 60-MDa plasmid has been utilized in diagnosis of EPEC. 1-kb fragment from this plasmid was developed as a diagnostic marker for EPEC [112, 113].

2.4.2.2 Enterotoxigenic *E. coli* (ETEC)

Worldwide importance of ETEC is recognized as most common cause of traveler's diarrhea and frequent cause of moderate to severe childhood diarrhea [65, 114]. Clinical manifestations of ETEC infections are similar to the clinical feature of cholera which is caused by *Vibrio cholerae* [93].

- **Epidemiology of ETEC**

ETEC causes an approximately 79,420 infections in United States per annum [84] and is reported as an etiological agent of 20% of diarrheal infections in developing countries [114]. Travelers visiting endemic countries are at higher risk of getting ETEC infections and are often presented with severe form of gastroenteritis.

ETEC also known to cause diarrhea among young animals and repertoire of virulence including toxins and colonization factors is a bit different from human host. It has been observed that ETEC mediated infection rates are higher among younger children and

progressively decrease in 5-15 years age groups [114], this may be attributed to acquisition of immunity against pathogen. It has also been seen that hospitalized adult and elderly patients are presented with more severe forms of ETEC diarrhea.

- **Pathogenesis of ETEC**

ETEC is a non-invasive pathogen and causes secretory diarrhea in the host (Figure 2.9). ETEC strains colonize small intestinal mucosa with various colonization factors. About one third of ETEC isolates, harbor colonization factor antigen I (CFA/I) as major colonization antigen and CFA/I specific IgY antibodies are significantly known to abolish ETEC adherence in HT-29 cell line [115]. Intimate attachment to intestinal mucosa is mediated by outer-membrane proteins Tia and TibA [116]. ETEC strains secrete two enterotoxins namely heat labile (LT) and heat stable toxins (ST) which bind to small intestinal mucosa and interfere with normal absorption and secretion process (Figure 2.6a). ETEC strains possessing either one or both toxin can lead to diarrhea in infected host. Heat stable toxin (ST) binds to guanylyl cyclases and leads to secretion of sodium and activation of CFTR channels. While, heat labile toxin (LT) binds to the monosialoganglioside (GM1) and activates adenyl cyclases, which also leads to activation of CFTR [117].

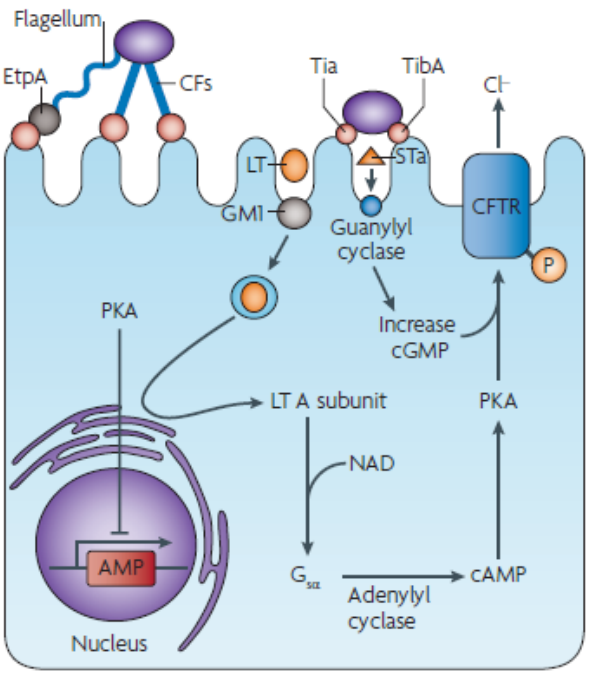


Figure 2.9: Mechanism of enterotoxigenic *E. coli* pathogenicity in intestinal epithelial cell [107].

- **Diagnosis of ETEC**

ETEC in the stool specimen can be confirmed by amplification of two marker genes *estA* and *eltB*, which encode heat stable and heat labile secretory enterotoxins, respectively [93, 118]. Interestingly, host genotypic feature like non-synonymous single nucleotide polymorphisms (SNPs) in *FUT2* gene was well correlated with increased possibility of symptomatic ETEC infection [119]. Efflux pumps and point mutations in *gyrA* have been explored as the mechanisms by which ETEC exhibit resistance against quinolones and azithromycin antibiotics [120]. A recent outbreak of CTX-M-15 harboring ETEC O159:H20 was observed to be resistant against third generation cephalosporins [121].

2.4.2.3 Enteroaggregative *E. coli* (EAEC)

In 1987, EAEC was firstly recognized as pathogen associated with acute childhood diarrhea and in the following years, EAEC have been regarded as an emerging cause of acute and persistent diarrhea among young children and adult populations, globally [104, 122, 123]. EAEC is a foodborne pathogen which produce typical aggregative adherence (AA) pattern on cultured epithelial cells [124, 125]. In AA pattern, bacteria adhere to each other, to epithelial surface and as well as to the abiotic surfaces.

- **Epidemiology of EAEC**

Prevalence of EAEC in asymptomatic young children is associated with impaired and cognitive health [126]. In addition, EAEC is also significantly associated with traveler's diarrhea (19-33%) [127, 128] and can cause chronic diarrhea among HIV infected patients [126]. Several EAEC outbreaks have been reported from low and high income regions of the world and are primarily associated with uptake of contaminated food [129]. In 2011, a hybrid hypervirulent Stx expressing EAEC caused massive outbreak in Germany, which affected 3,842 individuals including 54 deaths and many with severe complications like hemolytic uremic syndrome (HUS) [130, 131]. Massive outbreak was associated with imported fenugreek seeds. Long term survival and carriage of bacteria in food is associated with production of higher amount of colanic acid and biofilm formation [124].

In India, hospital surveillance studies have shown detection of genetically heterogeneous EAEC strains with infection rates ranging from 2-26% [18, 132-134]. Multi-drug resistant (MDR) EAEC strains have been isolated from adult and childhood diarrhea cases from

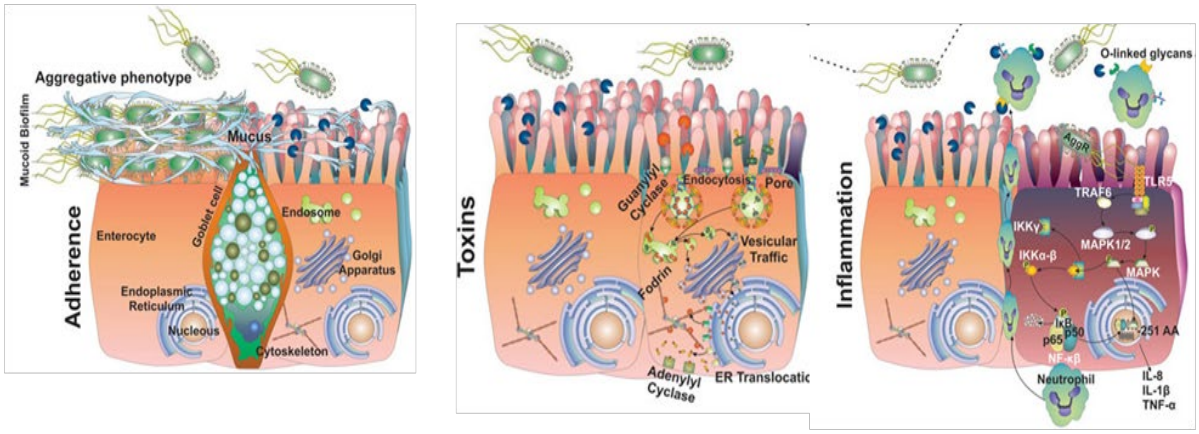
different parts of the country [135]. During travelers diarrhea, carriage of EAEC has been reported between 25% to 48% [127, 136]. Clinical and laboratory diagnosis of traveler's have also showed presence of MDR and CTX-M-15 producing EAEC strains in patient's stool specimens [137, 138].

- **Pathogenesis of EAEC**

Three stages model of EAEC pathogenesis is defined as **A.** Adherence of bacteria to gastrointestinal mucosa particularly to colonic region [104] via aggregative adherence fimbriae (AAF) (AAF/I-AAF/II) and dispersin protein followed by biofilm production intervened by *AAF*, *shf, aatA, yafK*, *fis* and *set1A* genes **B.** Toxin (serine protease autotransporters of the *Enterobacteriaceae* SPATEs) production **C.** Inflammation (Figure 2.10).

A. Aggregative adherence

EAEC strains colonize different parts of gastrointestinal tract (GI) tract in stacked-brick pattern and produce mucoid biofilms [139]. Biofilm formation helps bacterium in invasion of host immune system. The aggregative adherence pattern is encoded on large molecular weight plasmids known as aggregative virulence plasmids or pAA [140]. The prototype strain EAEC 17-2 (serotype O3:H2) harbors plasmid pAA1 having CVD32 probe which has been widely utilized for epidemiological investigations.



A **B** **C**

Figure 2.10: Three stages model of EAEC pathogenesis which involve A. adherence B. toxin production C. inflammation [104].

B. Toxin Production

Toxins secreted by bacteria usually result in secretary diarrhea which is one of the hallmark clinical symptoms of EAEC infection [140]. In vitro organ culture (IVOC) (duodenum, ileum, and colon) studies have shown EAEC toxin mediated cytotoxic effects like microvilli vesiculation, enlarged crypt openings, and increased epithelial cell extrusion [141].

C. Inflammation

Bacterial counterparts like dispersin, flagellar protein FliC have been shown to elicit an immune response through interleukin-8 (IL-8) production resulting in inflammation [142]. IVOC systems also showed upregulated expression levels of IL-8, IL-1β, IL-6, TNF-α and TLR-5 in diarrhea patients [132]. Likewise, host factors like polymorphism in interleukin-8 promoter region is related to increased vulnerability to EAEC infections [143]. In addition, different pathogenicity islands (PAIs) i.e. *she* of *Yersinia* high-pathogenicity island, *hly* PAIs have been found in various EAEC isolates which play important role in the pathogenicity of EAEC [144, 145]

- **Diagnosis of EAEC**

The plasmid encoded gene probe *pCVD* which elucidates aggregative adherence phenotype is utilized for identification of EAEC in diagnostic and epidemiological studies [14, 100, 146]

2.4.2.4 Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* is mainly characterized on the basis of array of molecular signatures, histological features and clinical symptoms [93]. EHEC belongs to group of Shiga toxin producing *E. coli* and secretes Shiga toxin encoded by *Stx* genes. EHEC possess approximately 380 various serotypes and among which serotype O157:H7 is known to be most pathogenic one [147]. Among non-EHEC O157 serotypes, the big six (O26:H11, O45:H2, O103:H2, O111:H8, O121:H19 and O145:H28) are known to have moderate levels of virulence against humans [147].

- **Epidemiology of EHEC**

EHEC infections are common in developed countries like United States (US) and have become endemic in Latin America. In US, 16% of outbreaks have been accounted to EHEC

serotype O157 and only 7% correspond to EHEC non-O157 [148]. Globally, EHEC infection risk is greatest in children <5 years of age. Season wise distribution analysis showed that EHEC infections are more common in summer months and are often associated with undercooked meat, vegetables and unpasteurized milk. Person-to-person transmission occur with a very low infective dose (<100 organisms).

- **Pathogenesis of EHEC**

EHEC generally colonizes human colon and similar to EPEC intimately attach to colonic epithelium with the help of intimin and translocated intimin receptor (Tir) (Figure 2.11). In addition to general *E. coli* pilus (ECP), EHEC possesses a type IV pili i.e. hemorrhagic coli pilus (HCP) which facilitates EHEC adherence and biofilm formation during pathogenesis [149].

Actin rearrangement for pedestal formation is triggered by Tir cytoskeleton-coupling protein (TccP) and is Nck independent [107]. Insulin receptor tyrosine kinase substrate (IRTKS) links Tir to the TccP. EHEC possesses pathogenic island (PAI) encoding a Type III, subtype a, secretion system (T3aSS) which injects bacterial proteins into host cell and produces attaching and effacing (A/E) lesions in *in-vitro*. Association of PAI is more common in pathogenic strains and non-O157:H7 EHEC strains essentially do not carry LEE PAI system [93, 150].

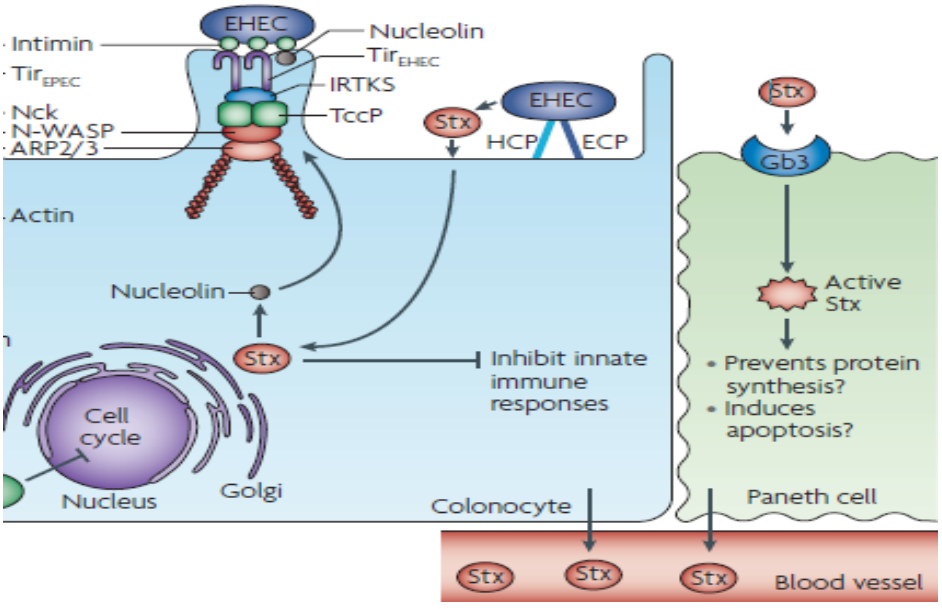


Figure 2.11: Molecular mechanism of enterohaemorrhagic *E. coli* pathogenicity [107].

EHEC microorganisms mainly exert pathogenesis through Shiga-like toxin which is responsible for manifestations like hemolytic-uremic syndrome (HUS) and secondary neuronal complications [151]. Shiga toxin binds to the globotriaosylceramides (Gb3s) and induces internalization of toxin. Shiga toxin is trafficked into golgi and cleaved toxin leads to cell necrosis and death. EHEC can secrete either one or both variants of Stx *i.e.,* Stx1 and Stx2. Stx2 is observed to be more potent toxin than Stx1 [93, 152]. EHEC can sense hormone and quorum-sensing molecules and can trigger various mechanisms like T3SS expression and motility [153].

In EHEC infections, use of antibiotics is not recommended as this can lead to more cellular damage. Antibiotics are known to activate bacterial SOS system and result in secretion of more of Shiga toxins [154]. In severe cases, Shiga toxin can lead its way into blood stream and develops triad of HUS characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure [155].

- **Diagnosis of EHEC**

Laboratory diagnosis of EHEC strains is accomplished by biochemical, serological and molecular assays [93]. Molecular approach presents more precise method of characterization of EHEC. Shiga toxin encoding genes *stx1/vt1* and *stx2/vt2* has been utilized as gene probes for identification in outbreaks and surveillance studies [118].

2.4.2.5 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) is an intracellular pathogen which colonizes colonic epithelium and results in inflammation and ulceration of colonic mucosa. EIEC was firstly described in 1944 and was designated as paracolon bacillus [156].

- **Epidemiology of EIEC**

EIEC infections are observed predominantly in tropical countries and EIEC strains are endemic in countries with poor hygiene and sanitation systems. However, sporadic outbreaks have been reported in developed countries like US, Europe and Italy. Recently, a rare serotype O96:H19 has been found associated with EIEC pathotype in developed countries [157]. Incidence rates of approximately 1.2% have been reported among children <5 years of

age [85]. Although infections of EIEC and EHEC pathotypes are infrequent [18, 158] but often result in severe clinical symptoms.

- **Pathogenesis of EIEC**

EIEC and *Shigella* manifest similar molecular events of pathogenicity and cause bacillary dysentery or bloody diarrhea. EIEC are devoid of adherence factors and virulence is exclusively carried out by 220 kb pINV plasmid which encodes a type 3 secretion system (T3SS) required for enterocyte invasion, cell survival and macrophage apoptosis [159, 160]. These pathogens reach mucosa via microfold (M) cells and multiply inside macrophages (Figure 2.12).

Plasmid encoded invasion plasmid associated (Ipa) proteins further help in survival inside macrophages and basolateral movement of EIEC. Bacterial effector protein IpaC mediates actin polymerization and ruffle formation in epithelium for bacterium uptake. Other proteins, IpgD, IpaA and VirA are also involved in mediating entry of bacteria via ruffle and invasion into a phagosome. IpaB, IpaC, IpaD and IpaH help in bacterial escape from phagosomes.

Translocation of bacteria to adjacent cells occurs via basolateral sides of submucosa. Various effector proteins secreted by EIEC type III secretion system help in intracellular survival, cell to cell transmission and protection from host immune system. Suppression of immune system is carried out by OspG which prevents activation of nuclear factor κ B (NF κB). OspG, OspF and IpaH interact with various cellular components to inhibit production of inflammatory cytokines [107].

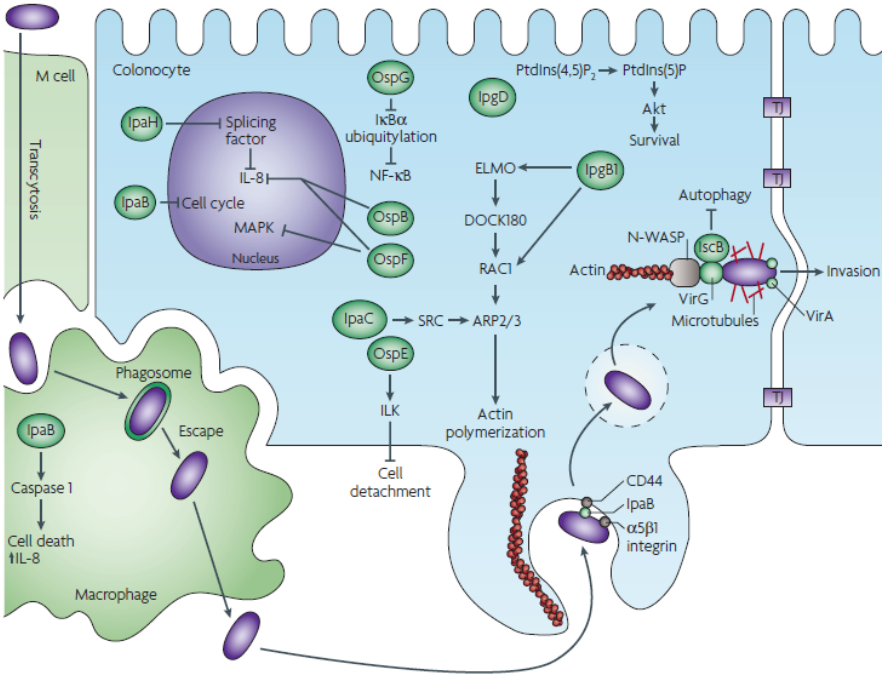


Figure 2.12: Various steps involved in pathogenesis of EIEC and *Shigella.* ARP2/3, Actin-related protein 2/3; DOCK180, Dedicator of cytokinesis protein 1; ELMO, Engulfment and cell motility; IκBα, Inhibitor of NF-κB subunit-α; IL-8, Interleukin-8; ILK, Integrin-linked kinase; MAPK, Mitogen-activated protein kinase; NF-κB, Nuclear factor-κB; N-WASP, Neural Wiskott–Aldrich syndrome protein; PtdIns (5) P, Phosphatidylinositol-5-phosphate; PtdIns, P2, Phosphatidylinositol-4,5-bisphosphate; TJ, Tight junctions [107].

- **Diagnosis of EIEC**

Laboratory diagnosis of EIEC strains is performed by conventional microbiological and molecular methods. EIEC strains have been characterized by various genomic probes like *ipaH, ial, set1A, sen, virF, invE, sat, sigA, pic, and sepA* in different studies [18, 161].

Other causative agents associated with infectious diarrhea include *Vibrio* spp., *Shigella* spp. and *Salmonella* spp. and are discussed below:

2.4.3 *Vibrio* spp. mediated diarrhea

Filippo Pacini, an Italian physician firstly isolated *Vibrio* as a causative agent of cholera [162]. *Vibrio* is a comma shaped gram negative rod which causes secretory diarrhea in host. *Vibrio* mediated cholera is a global health threat and an indicator of discrimination and lack of access to safe water and hygiene [23].

➢ **Epidemiology of *Vibrio* spp.**

Incidences and outbreaks of cholera are reported globally including recent cholera epidemics in Haiti and Yemen [163-165]. Cholera is predominantly observed in the Indian subcontinent, South East Asia, Africa and South America (Figure 2.13) [166-168]. *V. cholerae* and *V. parahaemolyticus* species are the main potential virulent species of *Vibrio* which are capable of causing acute gastrointestinal illness in humans [25, 53]. These foodborne pathogens are primarily transmitted by raw, uncooked, seafood and most common clinical manifestations of *Vibrio* infection involve rice watery stool with or without blood, vomit, muscle cramps and dehydration [169].

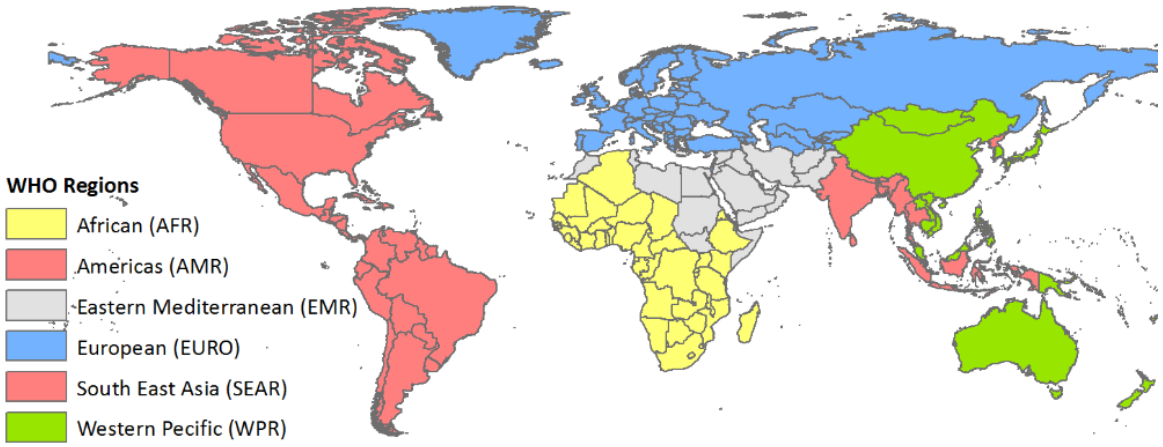


Figure 2.13: Countries endemic for Cholera [168].

Vibrio are classified into O1 or non-O1 serogroups based on whether they undergo agglutination by antisera against the O1 antigen (cell wall polysaccharide) [170]. In 1992, epidemic caused by O139 *Vibrio* appeared in southern India and Bangladesh and later spread to other regions of Southeast Asia [166]. However, incidences of *Vibrio* spp. have also been observed from different parts of the developed countries [171]. However, *V. parahemolyticus*, is more common than *V. cholerae* in developed countries and sporadic outbreaks have been reported from time to time. People with vibriosis become infected by consuming raw or undercooked seafood or exposing a wound to seawater. Most infections occur from May through October when water temperatures are warmer.

➢ **Pathogenesis of *Vibrio* spp.**

The mechanism of infection of *Vibrio* is similar to ETEC labile toxin (Figure 2.14). However, cholera toxin is processed in a retrograde manner from golgi to endoplasmic reticulum (ER).

Cholera toxin (CT) possesses specific amino acid sequence (KDEL) which mimics proteins of ER. CT is translocated from golgi to ER via ER lumen protein-retaining receptor protein (ERD2). From here, CT enters ER-associated degradation (ERAD) pathway and moves out into the cytosol. The A1 peptide of CT leads to cAMP mediated CFTR secretion.

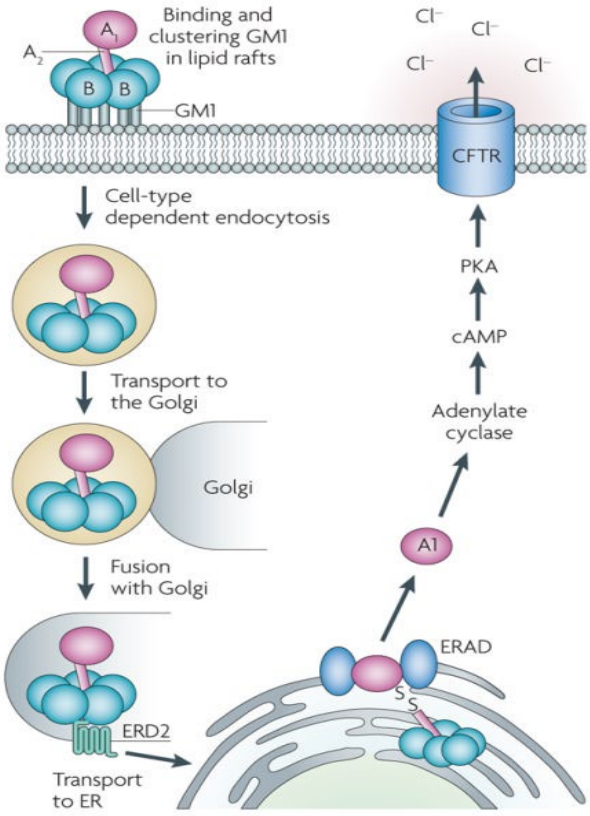


Figure 2.14: Mechanism of *Vibrio cholerae* toxin mediated diarrhea [83].

- **Diagnosis of *Vibrio* spp.**

Laboratory diagnosis of *Vibrio* spp. is performed on the basis of biochemical and molecular approaches. Biochemical assays like, growth on selective media i.e TCBS, cholera red test and string test have been utilized to screen and characterize *Vibrio* from stool specimens [172]. In addition, dipstick test has been employed in remote areas during investigation of outbreaks [173].

2.4.4 *Shigella:* The causative agent of bacillary dysentery

Shigella is a member of family *Enterobacteriaceae* and is identified as the causative organism of bacillary dysentery/shigellosis [174]. *Shigella* is a gram-negative, non-motile rod. *Shigella* and enteroinvasive *E. coli* are related genetically and exhibit same mode of

pathogenesis [107]. The name *Shigella* was given in honor of Kiyoshi Shiga, who examined and isolated bacteria in dysentery stool specimens [175].

- **Incidences of *Shigella* in population**

Among cases of infectious diarrhea caused by invasive pathogens, shigellosis is common and accounts for 10–20% of cases of enteric infections throughout the world. Historically, pandemic waves of *Shigella* mediated dysentery have been observed in sub-Saharan Africa, Central America and South-East Asia [176]. *Shigella* is a foodborne endemic pathogen in India and accurate estimates for incidences and etiology are still lacking [177].

- **Pathogenicity of *Shigella***

Mechanism of infection of *Shigella* is similar to EIEC pathotype (Figure 2.12) [107]. *Shigella* has been classified into four different species; *S. flexneri, S. sonnei, S. dysenteriae* and *S. boydii*. Globally, *S. flexneri* is significant cause of shigellosis. Incidences of *S. sonnei* predominate in developed countries whereas hyper-virulent Shiga-toxin producing *S. dysenteriae* is known to cause epidemics in developing countries.

Shigellosis is transmitted by fecal-oral route but person-to-person spread is also important due to very low infective dose (10-100 organisms). Clinical presentation of shigellosis becomes evident after an incubation period of 2-3 days, and includes abdominal pain, fever, malaise and anorexia and watery diarrhoea which may progress to bloody diarrhea or mucous in stool. In severe cases, complicated symptoms may include febrile convulsions, hyponatraemia, hypoglycaemia, toxic megacolon, encephalitis, reactive arthritis and HUS [79]. *Shigella* outbreaks have also been associated with higher rates of antibiotic resistance, including isolates that are resistant to most commonly utilized antibiotics like ciprofloxacin and azithromycin [79].

- **Diagnosis of *Shigella***

Laboratory diagnosis of *Shigella* includes a variety of microbiological assays which include culturing on differential media, biochemical assays (Indole, methyl red, Voges Prausker's, urease agar test, motility test etc.) and serological assays [178]. Presently molecular methods utilizing invasion plasmid antigen H gene sequence (*ipaH*) gene probe have also been utilized to characterize *Shigella* in diarrhea cases [179].

2.4.5 Non typhoidal *Salmonella* (NTS) induced diarrhea

In 1855, Theobald Smith firstly isolated bacterium from intestine of an infected swine and later bacterium was named *Salmonella* in honor of Dr Daniel Elmer Salmon an American pathologist [180]. According to Centre for Disease Control (CDC), genus Salmonella is categorized into two species *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further classified into six species [180] and among them non typhoidal *Salmonella* Typhimurium is predominant cause of human gastroenteritis worldwide [181].

- **Epidemiology of non typhoidal *Salmonella***

Despite significant advancement in hygienic conditions, NTS incidences are escalating in developed and developing settings of world [29, 148]. However, incidence rates of invasive NTS are higher in low income countries especially in a Saharan Africa and individuals infected with Human Immune Virus [182]. Non typhoidal *Salmonella* infects gastrointestinal tract and infections are transmitted by contamination of food and water resources with fecal matter. Food products of animal's origin like milk, poultry and eggs, and processed food like chocolate and peanut butter have also been reported as source of infection.

NTS gastroenteritis or 'stomach flu' is characterized by GI tract inflammation associated with symptoms of non-bloody diarrhoea, vomiting, nausea, headache, abdominal cramps and myalgias. Incubation period of NTS infections is shorter and range from 6-12 hours and symptoms can usually last for 1-2 weeks. However, NTS can also mediate severe complications like cholecystitis, pancreatitis and appendicitis [183]. Infants, young children, elderly people and immunocompromised patients are highly susceptible to NTS infections and often develop more severe symptoms than normal individuals [184].

- **Pathogenesis of non typhoidal *Salmonella***

After ingestion, *Salmonella* attaches to apical surface of enterocytes via various cell adhesions and stimulate membrane ruffles to initiate engulfment of bacterial cells (Figure 2.15). Microfold (M) cells are the major route of entry of bacteria into host cell and M cells transport the bacteria to the lymphoid tissue [185]. NTS induces only a local inflammation reaction and also causes infiltration of PMNs into intestinal lumen and cause diarrheal illness.

Salmonella Typhimurium alters TLR signaling and phosphorylation in host epithelium and damages epithelial cell function [83]. NTS infection in humans leads to induction of T_H1 response along with elevated levels of inflammatory cytokines [186, 187]. Over expression of various cytokines allows the recruitment of dendritic cells, macrophages and neutrophils into intestinal lumen [188].

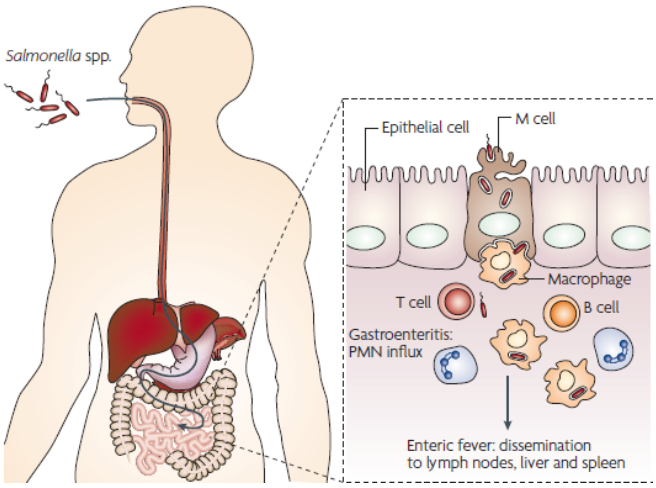


Figure 2.15: Mechanism of *Salmonella* Typhimurium induced diarrhea [185].

- **Diagnosis of non typhoidal *Salmonella***

Salmonella is a member of *Enterobacteriaceae* and laboratory diagnosis is based upon routine microbiological assays which include culturing on differential media, biochemical assays (Indole, methyl red, Voges Prausker's, urease agar test, motility test, etc.) and serological assays [178].

2.5 Risk factors predisposing to diarrhea

GBD estimated malnutrition, unsafe drinking water and suboptimal hygiene conditions as leading risk factors responsible for diarrheal mortality and morbidity, globally [8]. Several other factors predisposing to diarrheal illness include suboptimal breastfeeding, vitamin A deficiency, zinc deficiency, population growth and population ageing (Figure 2.16). Moreover, immunocompromised individuals are more susceptible to diarrheal diseases. For example, human immunodeficiency virus infected child is eleven times more susceptible to persistent diarrhea as compared to a healthy child [189]. In addition, bouts of diarrheal episodes cause impaired childhood growth and cognition ability.

Environmental factors and natural calamities like recurrent floods, stagnant water, earthquakes, and droughts also lead to an increased risk of developing diarrheal disease. Consequences of a humanitarian crisis such as disruption of water and sanitation systems, or the displacement of populations to inadequate and overcrowded camps also contribute significantly to diarrhea. Diarrheal diseases threaten public health and impose more economic burden due to hospitalization and management of disease.

2.6 Treatment of diarrhea

According to CDC, therapeutic measures to control infectious diarrhea rely on use of oral rehydration therapy, anti-motility agents and antibiotics depending upon etiology of infection. In cases of severe dehydration and shock, rehydration can also be done with intravenous therapy (Figure 2.16).

Moreover, in childhood diarrhea cases, it is important to break vicious circle of malnutrition by providing adequate nutritious diet. Furthermore, a fourteen day zinc supplement course (20 mg) also known to reduce duration of disease and prevents future relapse of diarrheal episodes. Subsequent rehydration during diarrhea illness maintains fluid and electrolyte balance and is known to circumvent critical complications like oligoanuric renal failure and hemolytic uremic syndrome. Nowadays, prevention strategies are multifactorial and have been focused on rotavirus vaccination, breastfeeding, improved hygiene and safe drinking water. Overall, these strategies have been proven to be cost effective in terms that INR 65 invested in rotavirus vaccination yield an average return of INR 1666 [1].

Antibiotics recommended for treatment of traveler's diarrhea included fluoroquinolones (ciprofloxacin) and macrolides (azithromycin, rifaximin) [92]. Antibiotic administration causes collapse of indigenous microbes of gastrointestinal tract and makes host vulnerable to subsequent infections. Knowledge regarding antibiotic susceptibility patterns exhibited by region specific etiology is essential, if treatment interventions are to be considered. However, use of antibiotics and anti-motility reagents needs to be evaluated in cases of antibiotic resistance and Shiga toxin producing *E. coli* infections [92].

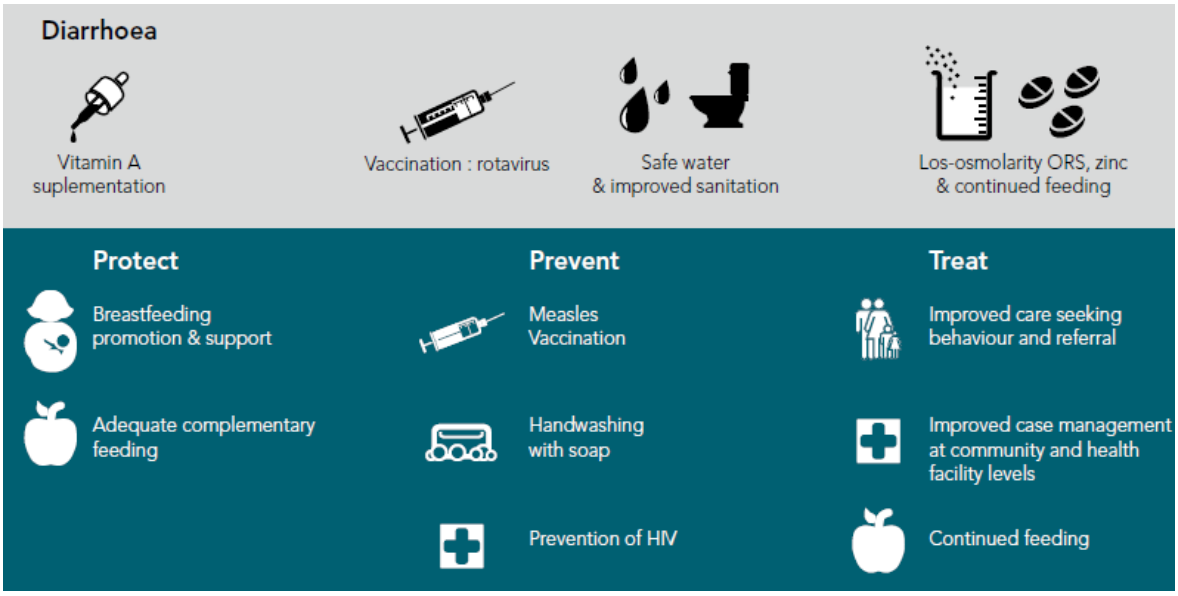


Figure 2.16: Treatment and prevention methods of diarrheal disease [190].

2.7 Current obstacles in management of infectious diarrhea

Despite improvement in global sanitation and hygiene, diarrhea still remains a significant cause of morbidity and mortality worldwide. Following are the critical factors that we need to address with utmost importance and priority.

2.7.1 Scarcity of drinkable water and inadequate hygiene

Diarrheal diseases are transmitted by intake of contaminated food and water. Globally, approximately 780 million people don't have access to clean drinking water and 2500 million are living under suboptimal hygienic conditions [1]. Approximately, 88% of diarrhea related deaths are due to unsafe water, suboptimal hygienic conditions. Oral and intravenous fluid therapy constitutes the first line of treatment for diarrhea, irrespective of the disease etiology. These interventions mainly focus on restoring nutritional deficiencies and fluid loss. Overall, owing to high disease burden, rise of resistant strains, complications of EHEC mediated infections; there is an urgent need to refine health polices and surveillance for greater effectiveness.

2.7.2 Antibiotic resistance

Gastrointestinal infections caused by enteropathogen like *E. coli, Salmonella*, *Shigella,* and *Campylobacter* species are usually treated with antimicrobial agents [191]. Prescription of anti-diarrheal agents usually relies upon clinical symptoms and practical therapy may not be

useful for all diarrheal pathogens [192]. In addition, use of certain antibiotics has also been recommended for self-treatment of traveler's diarrhea caused by ETEC and EAEC pathogens. Presently, global burden of alarming antibiotic resistance prevents administration of accurate treatment and eradication of pathogenic microbes. From last two decades, most of the pathogens have developed resistance against first line of antibiotics [191]. Frequent use of antibiotics against EAEC and ETEC pathogen is one of the causes for acquisition of resistance [193]. Use of antibiotics by travelers for a short period of time leads to prolonged disruption of gut microbiome and frequent use appear to diminish rejuvenation [194]**.** Apart from *E. coli*, other causative agents of diarrhea like *Salmonella* spp. and *Shigella* spp. have rapidly acquired resistance within over a period of five years [195, 196]. Moreover use of antibiotics especially in traveler's and invasive diarrhea will affect the resistance arsenal and hence, endorse geographic spread of resistance. Therefore, it is important to manage proper resistance surveillance data so that society and individuals reside safe from high risk of resistance.

2.8 Future prospects in diarrheal therapeutics

Considering diarrhea illness related to poor hygiene, management of disease can't be assured by only use of good sanitation practices. Currently recommended interventions for case management of diarrhea (rehydration therapy, continued feeding, and zinc supplementation) have been very effective in management of rotavirus mediated diarrhea. However, these strategies have limited effectiveness against bacterial infections which lead to intestinal injury, persistent diarrhea, and growth stunting [7, 197, 198]. Effectiveness of antibiotics advised by WHO for the treatment of dysentery is threatened by the global spread of MDR strains. It is also uncertain whether, antibiotics benefit children with watery diarrhea associated with *Shigella* or ST-ETEC or some specific therapy is required.

To combat evolution and spread of antibiotic resistance, several studies are focused on development of specific vaccine candidates (Figure 2.17). Owing to the severity and duration of infection, predominant bacteria considered for vaccine development include ETEC, EPEC and EHEC pathotypes. In case of ETEC, antisera hyperimmune bovine colostrum (HBC) has been proven effective against ETEC mediated diarrhea [199]. HBC mainly contains IgG and IgA antibodies against O-polysaccharide 78 and colonization factor antigen I. Easy and inexpensive diagnostic methods are needed in order to define optimal treatment and

prevention of childhood diarrhea in endemic areas. Investigations carried out by various research groups have explored several potent vaccine candidates (in current use/in progress) and these are summarized in (Figure 2.17) [152]

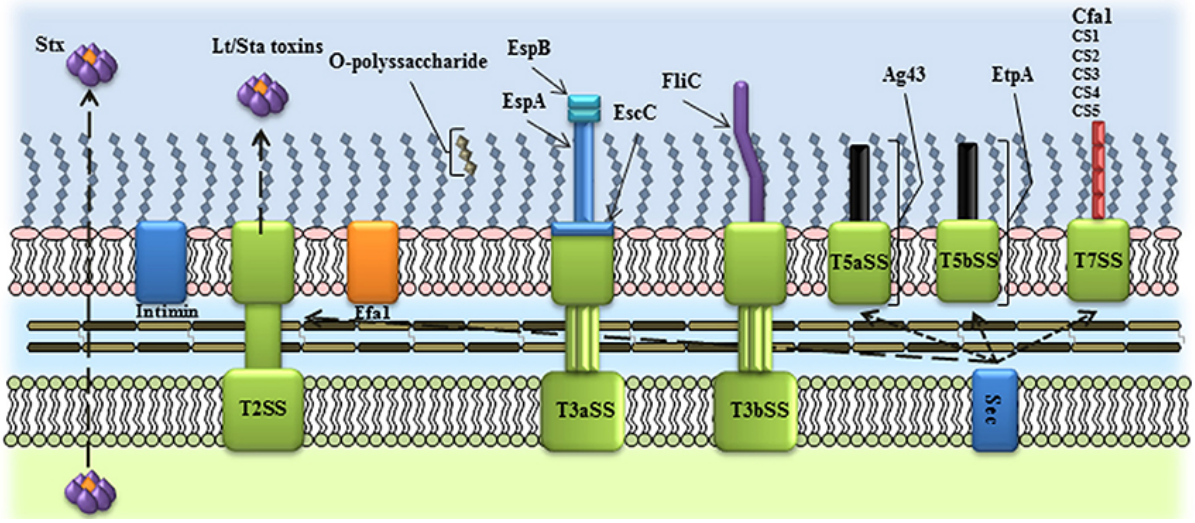


Figure 2.17: Illustration of various virulence factors of DEC pathotypes targeted for vaccine development (Source: Creative common License No. CC BY 4.0). [152].
https://www.frontiersin.org/files/Articles/333650/fmicb-09-00440-HTML/image_m/fmicb-09-00440-g001.jpg

- **Additional preventive measures for diarrhea**

Other preventive measures for diarrhea include safe drinking-water, hand washing with soap, exclusive breastfeeding for the first six months of life, education about good personal and food hygiene. Implementing rotavirus vaccination at national level can also reduce rotavirus mediated diarrhea cases. National and state level programs should be conducted for educating community health workers and caretakers about diarrhea treatment and need for medical assistantship. Laboratories must be setup and equipped for identification and characterization of causative agents.

Rationale of the study

For effective treatment of diarrhea, elucidation of etiological agent is of utmost importance to implement better health services and planning interventions. Present study is an attempt to characterize predominant bacterial pathogens and classify their specific clinical symptoms and resistance profiles which will lead to better treatment options.

CHAPTER 3

OBJECTIVES

3. OBJECTIVES

In India, estimates for childhood and adult diarrhea cases are available as case control studies and incidences of adult diarrhea are not studied uniformly. Incidences and prevalence of diarrheal illness is known to vary from different parts of the nation and there are still no surveillance reports on bacterial etiology of diarrhea from regions of Himachal Pradesh. Therefore, taking these research gaps into consideration we have designed following objectives.

1. To collect diarrheal samples from different regions of Himachal Pradesh and to correlate epidemiological details and medical history of hospitalized diarrhea patients.
2. To isolate and identify different bacterial pathogens from diarrheal samples using standard microbiological and biochemical methodologies.
3. To characterize most prevalent pathogen at their molecular pathotype levels by molecular and serological methods and establishment of these pathogens prevalence in Himachal Pradesh.
4. To estimate the level of resistance among prevalent diarrheal pathogens against different antibiotics (commonly used in diarrheal therapies) using MIC determination and antibiogram formation.

CHAPTER 4

MATERIALS & METHODS

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Microbiological Media

The selective, differential and routine culture media- MacConkey agar, eosin methylene blue agar, thiosulphate citrate bile salt (TCBS) agar, Muller Hinton agar, nutrient agar, LB broth, agarose, and peptone were purchased from Hi-Media Laboratories Limited, India. Media utilized in biochemical assays; MR-VP (glucose phosphate) medium, triple sugar iron agar, Simmon's citrate agar, Christensen's urease agar were also bought from Hi-Media, India.

4.1.2 Antibiotic disks and E-strips

Commercially available antibiotic disks i.e. ampicillin (10 g), cefixime (5 g), cotrimoxazole (75 g), norfloxacin 10 g and nalidixic acid 30 g, cefepime (30 g), ceftazidime (30 g), ceftriaxone (30 g), imipenem (10 g), netilmicin (30 g), amikacin (30 g), kanamycin (30 g), tobramycin (10 g), streptomycin (10 g), ofloxacin (5 g), ciprofloxacin (5 g), levofloxacin (5 g), and vancomycin (30 g) and E-strips were also purchased from Hi-Media, India.

4.1.3 Chemicals

Sodium chloride, Tris base, glacial acetic acid, ethylene diamine tetracetate (EDTA), ethidium bromide and other chemicals of analytical grade were purchased from Merck Pvt. Ltd., India. 2X PCR Master Mix was purchased from Promega, India and New England Biolabs (NEB). DNA ladder of 100 bp and 1 kb were bought from New England Biolabs (NEB) and Promega, India. The primers used in the study were procured from Integrated DNA Technology, India. Sequencing of amplified PCR products was carried out commercially at Xcelris Genomics, India and Eurofins Genomics India Pvt Ltd. The constituents and composition of media, buffers, and dyes used in current study are listed in Appendix.

4.1.4 Primers sequences

Diarrheagenic *E. coli* and *Vibrio* spp. were observed as the most prevalent bacterial pathogens in the current study. Therefore, these pathogens were taken further for molecular

characterization. Diarrheagenic *E. coli* was firstly characterized by amplification of 16S rRNA gene and confirmed isolates were molecularly typed for various DEC pathotypes by amplification of specific virulence genes. Primer sequences for DEC virulence specific genes were taken from previously published studies [118, 200]. Following are the primer sequences utilized for molecular characterization of DEC (Table 4.1).

Table 4.1: Primer sequences utilized for molecular identification of genus *Escherichia* and characterization of diarrheagenic *E. coli* pathotypes.

Genus/ pathotype	Gene	Primer sequences for *E. coli*	Significance
E. coli	16S rRNA	F- 5'-GGAAGAAGCTTGCTTCTTTGCTGAC-3' R-5'- AGCCCGGGGATTTCACATCTGACTTA-3'(542bp)	For 16S rRNA gene of *E. coli*
Enterotoxigenic *E. coli* (ETEC)	*eltB* [118, 200]	F- 5'-CACACGGAGCTCCTCAGTC-3' R-5'- CCCCCAGCCTAGCTTAGTTT-3' (508bp) F- 5 -TCTCTATGTGCATACGGAGC-3 R-5'-5 -CCATACTGATTGCCGCAAT-3 (322 bp)	Either both or one of *estA* & *eltB* genes confirm presence of ETEC
	estA [118]	F-5'-GCTAAACCAGTAGAGGTCTTCAAAA-3' R-5'-CCCGGTACAGAGCAGGATTACAACA-3'(147bp)	
Enterohemorrhagic *E. coli* (EHEC)	*Vt1* [118]	F-5'-GAAGAGTCCGTGGGATTACG-3' R-5'-AGCGATGCAGCTATTAATAA-3' (130bp)	*vt1* and *vt2* can be present alone in atypical EHEC or can be present with *eae* gene in typical EHEC
	Vt2 [118]	F-5'-ACCGTTTTTCAGATTTTGACACATA-3' R-5'-TACACAGGAGCAGTTTCAGACAGT-3' (298bp)	
Enteropathogenic *E. coli* (EPEC)	*eae* [118] [200]	F-5'- CCCGAATTCGGCACAAGCATAAGC-3 R-5'- CCCGGATCCGTCTCGCCAGTATTCG-3'(881bp) 5 -CACACGAATAAACTGACTAAAATG-3 5 -AAAAACGCTGACCCGCACCTAAAT-3 (376 bp)	Typical EPEC have both *eae* and *bfpA* genes, but atypical possess only *eae* gene.
	bfpA [118]	F-5'-TTCTTGGTGCTTGCGTGTCTTTT-3' F-5'-TTTTGTTTGTTGTATCTTTGTAA-3' (367bp)	
Enteropathogenic E. coli (EIEC)	*ial* [118]	F-5'-CTGGTAGGTATGGTGAGG-3' R-5'-CCAGGCCAACAATTATTTCC-3' (320bp)	EIEC amplify *ial* gene only.
Enteroaggregative E. coli (EAEC)	*pCVD* [118]	F-5'- CTGGCGAAAGACTGTATCAT-3' F-5'-CAATGTATAGAAATCCGCTGTT-3' (630bp)	EAEC amplify *pCVD* gene probe

The another prevalent bacterial pathogen, *Vibrio* was also confirmed at molecular level by amplification of *Vibrio* specific 16S rRNA gene (Table 4.2).

Table 4.2:16S rRNA gene primer sequence utilized for characterization of *Vibrio* spp.

Gene	Primer Sequence	Amplicon
16S rRNA gene [201]	Forward 5'- CGGTGAAATGCGTAGAGAT- 3' Reverse 5'- TTACTAGCGATTCCGAGTTC- 3'	663bp

4.2 Methodology

Present study was comprised of four different objectives for estimating incidences and characterization of bacterial pathogens from Indira Gandhi Medical College, Shimla and regional Govt. hospital Solan of Himachal Pradesh. These hospital settings have been established as tertiary and zonal health care centres accessible for people from wider areas. Therefore, we have adopted and designed following methodology to achieve objectives of present study (Figure 4.1).

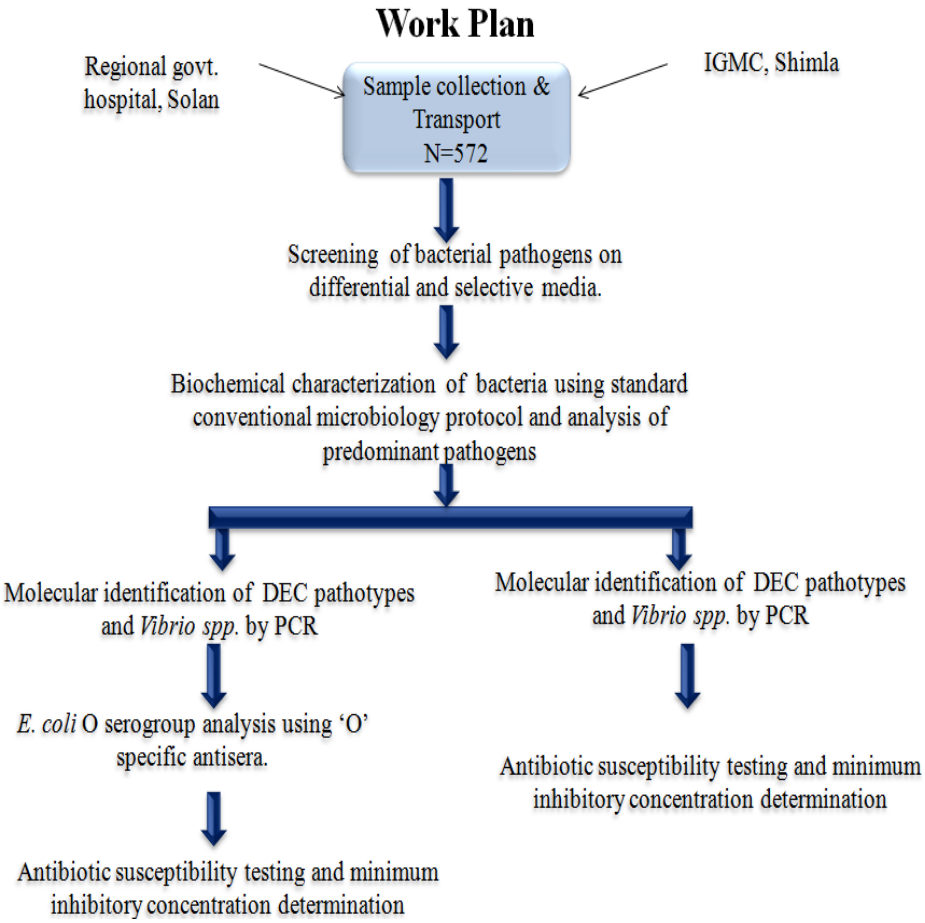


Figure 4.1: Plan of work showing different steps involved in the study.

4.2.1 Collection of diarrheal samples and study subjects

From February 2013 to April 2016, a total of 572 stool specimens of diarrheal patients aged between 13 days to 85 years were collected from selected hospital settings. Samples were collected from diarrheal patients with primary complaint of three or more loose stools/day who were admitted to Regional hospital, Solan and tertiary care hospital Indira Gandhi medical college Shimla. All patients were presented with loose stool as chief complication but also reported to have other clinical manifestations like dehydration, vomit, fever, abdominal pain, mucus, in common. Information on gender, age, geographic origin and clinical symptoms was obtained by means of standard questionnaire. Written informed consents were taken from patients or patient's parent or legal guardians in case of children. Stool specimens from diarrheal patients were collected in sterile containers and transported immediately to the laboratory after collection. All experiments included in the study were authorized by the Institutional Ethics Committee (IEC/Project no-04-2014) (Appendix).

4.2.2 Microbiological and biochemical characterization of bacteria

Collected diarrheal fecal specimens were analyzed firstly by culturing on selective and differential media and then by standard biochemical assays.

4.2.2.1 Microbiological characterization of bacteria

The stool specimens were inoculated into Luria Broth and alkaline peptone water for enrichment of *E. coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. After incubation, bacterial cultures were streaked onto MacConkey agar, eosin methylene blue agar, thiobacillus citrate bile salt agar (TCBS), xylose lysine deoxycholate (XLD) agar and incubated for 24 to 48 hours at 37°C. Different bacterial species were identified by colony characteristic produced on various differential and selective media. For example, typical lactose fermenting pink colored colonies from MacConkey agar were selected for *E. coli* followed by sub-culture on Luria Bertani agar. For *Vibrio* spp. golden yellow colonies from TCBS agar were picked and inoculated into alkaline peptone water. Red and red colonies with black centers were chosen from XLD media for *Shigella* spp. and *Salmonella* spp., respectively.

4.2.2.2 Biochemical characterization of bacteria

Colonies selected from various media were further subjected to standard biochemical assays. Various biochemical assays included IMViC (indole, methyl red, Voges Prausker, citrate), triple sugar iron agar, urease agar and motility tests [200, 202]. Bacterial strains with characteristic IMViC pattern were biochemically characterized as *E. coli, Vibrio* spp., *Shigella* spp.,*Salmonella* spp. respectively. Following is the workflow of different biochemical assays (Figure 4.2).

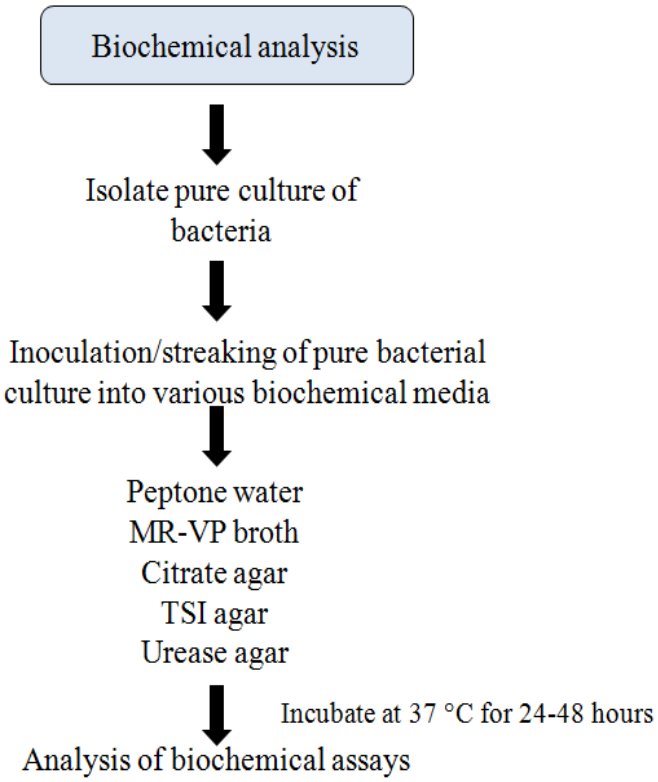


Figure 4.2: Workflow for biochemical assays to differentiate various members of *Enterobacteriaceae* [202].

Description and details of different biochemical methods is provided in Appendix**.**

4.2.3 Molecular characterization

From microbiological and biochemical characterization, diarrheagenic *E. coli* and *Vibrio* spp. were observed as the predominant bacterial pathogen in the current study. Therefore, both of the pathogens were further molecularly characterized as follow;

4.2.3.1 Characterization of diarrheagenic *E. coli*

A. DNA extraction & 16S rRNA gene characterization

From biochemical analysis, confirmed *E. coli* isolates were subjected to molecular analysis by amplification of 16S rRNA gene. DNA extraction of *E. coli* strains was performed by phenol chloroform method as described previously by Sabat and coworkers [203]. PCR thermo cycling conditions for *E. coli* 16S rRNA gene were standardized at following condition (Table. 4.3).

Table 4.3: PCR thermo cycling conditions for *E. coli* 16S rRNA gene.

PCR reaction steps	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	1 cycle
Cyclic denaturation	94°C	30 sec	x 35 cycles
Primer annealing	52°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	7 min	1 cycle

PCR reaction was set up with 25µl reaction mixture having 12.5 µl 2X PCR Master Mix, 0.2 µM of each primer, 300 ng/µl of template DNA and nuclease free water.

B. Molecular characterization of DEC pathotypes

Various DEC molecular pathotypes were characterized by amplification of virulence specific genes. Primer sequences for DEC molecular pathotypes were adopted from previously published research [100, 118]. Firstly, DEC molecular pathotypes were amplified in a multiplex PCR followed by single gene PCR for identification and reproducibility of specific DEC pathotypes. Various molecular pathotypes were recognized on the basis of amplification of following amplicons of genes; ETEC encoded heat stable (*estA* 147bp) and heat labile toxin (*eltB* of 322bp or 508 bp) genes, EPEC encoded bundle pilus forming gene (*bfpA* 367bp) and intimin gene (*eae* of 830 or 376 bp amplicon), EHEC encoded verocytotoxins (*vt1* 130bp and *vt2* 298bp), EIEC encoded invasion gene (*ial* 320bp) and EAEC plasmid encoded aggregative phenotype specific (*pCVD* 630bp) were targeted in the PCR.

During EPEC pathotype detection, *eae* and *bfpA* genes amplify at 367bp and 376bp respectively [118], which could not be determined by agarose gel electrophoresis, therefore, *eae* gene of (830bp) and *bfpA* gene (367bp) were stringently amplified by single gene PCR.

PCR thermocycling conditions for pathotypes were similar as described above for amplification of 16S rRNA gene (Table 4.3). Upon amplification of various DEC molecular pathotypes, PCR products were further confirmed by commercial Sanger sequencing at various time intervals during study. Sequenced DEC pathotypes were taken as positive control in subsequent PCR reaction.

C. Serological characterization of DEC molecular pathotypes

Molecularly confirmed diarrheagenic *E. coli* strains were further characterized for different serogroups. Identification of bacterial somatic 'O' antigen was done by standard agglutination tests using 176 'O' specific antisera [204]. For serogroups characterization, biochemically and molecularly confirmed *E. coli* isolates were screened at National *Salmonella* and *E. coli* Centre (NSEC) at Central Research Institute (CRI), Kasauli (H.P.), India. Pure bacterial cultures were inoculated into 5ml of nutrient broth and incubated at 37°C for overnight with continuous shaking. Bacterial culture was heated at 100°C for one hour and formalin was added to a final concentration of 0.3% (Test antigen). For testing with pooled sera, 50 l of sixteen pools of O antisera were added to 96 well plate. Then 50 l of test antigen was added to each well. A negative control was put with 50 l each of antigen and saline, respectively. Plates were incubated at 37°C overnight and observed for agglutination reaction. Bacterial isolates which showed agglutination in all wells including negative control were characterized as 'rough'. If, agglutination was seen with a single pool, then next agglutination test was setup with factor sera constituting the pool. But, if agglutination was seen with more than one pool, then antigen was titrated against all sera constituting the pools. The test antigen which even didn't show agglutination following antigen preparation at 121°C for 2½ hours was regarded as 'untypeable'.

4.2.3.2 Antimicrobial susceptibility test (AST) and minimum inhibitory concentration determination (MIC) for DEC pathotypes

Antibiogram patterns exhibited by predominant diarrheagenic *E. coli* pathotypes were also explored through AST and MIC assays. The antimicrobial susceptibility of the PCR positive *E. coli* pathotypes was determined by standard Kirby Bauer's disk diffusion method [205] against ampicillin (10µg), cefixime (5µg), co-trimoxazole (25µg), nalidixic acid (30µg) and norfloxacin (10µg) according to CLSI and ICMR guidelines [31, 206]. Break points for similar antibiotics were determined by minimum inhibitory concentrations for ampicillin

(0.016-256 µg), cefixime (0.016-256 µg), co-trimoxazole (0.016-256 µg), nalidixic acid (0.016-256 µg) and norfloxacin (0.016-256 µg) by using the E-test. *Escherichia coli* ATCC 25922 was used as reference strain for quality control in AST & MIC tests. Results were interpreted according to Clinical Laboratory Standard Institute (CLSI) and Indian Council of Medical Research (ICMR) guidelines [31, 206].

4.2.4 Molecular characterization of *Vibrio* spp.

The molecular characterization of another prevalent bacterium, *Vibrio* spp. was performed by PCR amplification of 16S rRNA gene. DNA was extracted by colony boiling method as described previously [207]. 2 l of DNA extracted by colony heat boiling method was PCR amplified in a 15 l reaction mixture; 7.5 l of 2 PCR master mix, 0.25 l of each primer and nuclease free water. PCR amplification was accomplished using following conditions (Table 4.4):

Table 4.4: PCR thermo cycling conditions for *Vibrio* spp.

PCR reaction steps	**Temperature**	**Time**	
Initial denaturation	94°C	5 min	1 cycle
Cyclic denaturation	95°C	30 sec	x 35 cycles
Primer annealing	50°C	30 sec	
Extension	72°C	40 sec	
Final extension	72°C	7 min	1 cycle

Single PCR without template was run as a negative control for each reaction. The amplified PCR products were analyzed with ethidium bromide stained 1.5% agarose gel (in 1X TAE buffer) and visualized using ultra violet (UV) trans-illuminator.

4.2.5 Antimicrobial susceptibility test (AST) and minimum inhibitory concentration determination (MIC) for *Vibrio* spp.

Kirby Bauer's disk diffusion on Mueller Hinton agar was also used to evaluate *Vibrio* spp. antibiogram patterns against fifteen different antibiotics; cefepime (30g), ceftazidime (30g), ceftriaxone (30g), imipenem (10g), netilmicin (30g), amikacin (30g), kanamycin (30g), tobramycin (10g), streptomycin (10g), ofloxacin (5g), ciprofloxacin (5g), levofloxacin (5g), norfloxacin (10g), nalidixic acid (30g) and vancomycin (30g).

Results were interpreted according to manufacturer's instruction for *Enterobacteriaceae* family.

4.2.6 Statistical analysis

The patients were classified into five various age groups; 0-2 years, 3-5 years, 6-17 years, 18-65 years and >65 years. Elderly patients of >65 years were used as a reference category to explore significant risk group among the study population. Fisher's exact test was carried out to ascertain mutual relatedness between different age groups and predominant bacterial pathogens; DEC and *Vibrio* spp. Clinical symptoms observed among diarrhea patients were also correlated with distinct DEC pathotypes and *Vibrio* spp. by using Chi square bivariate analysis. P values of 0.05 were considered as statistically significant and odds ratio (OR) at the 95% confidence interval (CI) were calculated.

CHAPTER 5

RESULTS

5. RESULTS

5.1 Collection of fecal specimens and study population

Between February 2013 to April 2016, a total of five hundred seventy two diarrheal stool specimens were collected from hospitalized patients admitted to regional (Govt. hospital Solan) and tertiary care hospital (IGMC, Shimla) in Himachal Pradesh. The age group of diarrheal patients consisted of a very wide window ranging from 13 days to 85 years (Figure 5.1). The patients were stratified into five various age groups, infant (0-2 years), children (3-5 years), adolescent (6-17 years), adult (18-65 years), elderly (>65 years). In statistical analysis, >65 years of age represented the most normative group because elderly from developing countries are more prone to diarrheal infection due to immunocompromised status [77]. Therefore, elderly age group comprising study subjects >65 years of age were taken as reference for comparative statistical analysis similar to previous study [18].

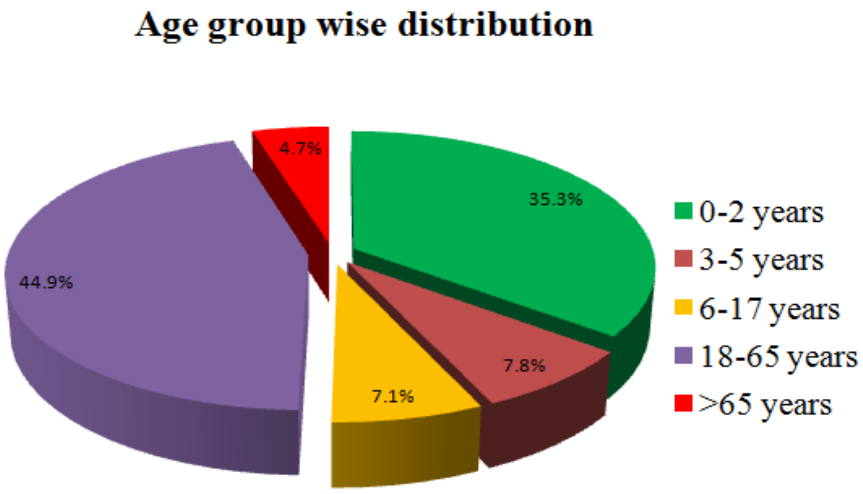


Figure 5.1 Prevalence of diarrheal incidences among different age groups. 0-2 years= infants, 3-5= children, 6-17= adolescent, 18-65 years= adult, >65 years elderly.

We collected a total of 247 stool specimens from children under the age of five and 325 fecal specimens from patients with >5 years of age. Highest diarrheal incidences were observed among infant and adult age groups, while children and adolescent followed approximately similar trends of infections.

In addition, to loose stools several other clinical symptoms of dehydration, fever, vomit, abdominal pain, rice watery stools and mucus were also observed among study population

(Figure 5.2). Among these, clinical symptoms of dehydration, vomit and fever were mainly observed among hospitalized patients.

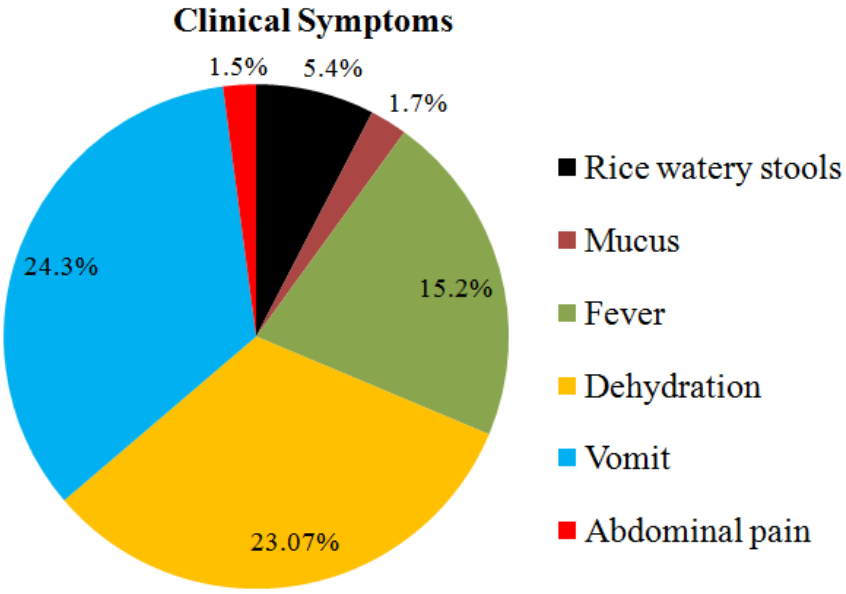


Figure 5.2: Different clinical symptoms associated with diarrheal illness among hospitalized patients.

5.2 Identification & biochemical characterization of bacteria

Fecal specimens were screened for the presence of bacterial pathogens through standard microbiological and biochemical approaches. Screening of fecal specimens provided various bacteria i.e. *E. coli*, *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. on differential and selective media (Figure 5.3). For *E. coli*, bacterial isolates showing lactose fermenting pink colonies and colonies with metallic sheen were chosen from MacConkey and eosin methylene blue agar, respectively (Figure 5.3 A & B). Colonies with red color or red colonies with black centre on XLD media were identified as *Shigella* spp. and *Salmonella* spp., respectively (Figure 5.3 C). For *Vibrio* spp., isolates with yellow golden yellow golden colonies from TCBS were taken further for characterization (Figure 5.3 D).

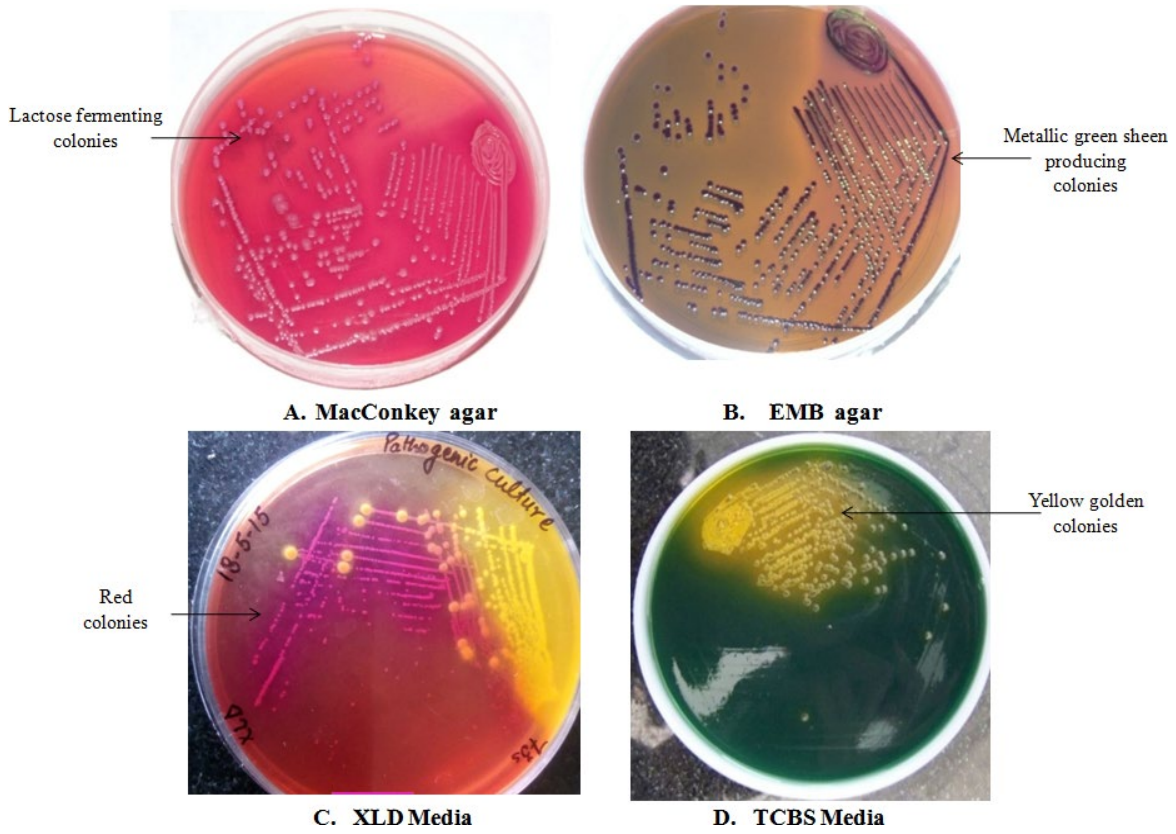
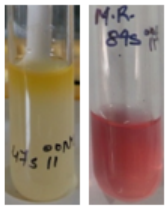
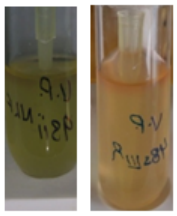
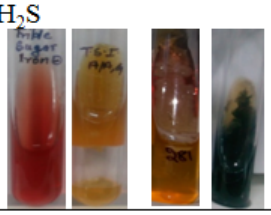


Figure 5.3: Screening of bacteria on differential and selective media. *E. coli* was identified by lactose fermenting colonies on MacConkey agar (A) and metallic green sheen producing colonies on EMB agar (B). *Shigella* isolates identified by red colonies on XLD agar (C). *Vibrio* spp. showed yellow golden colonies on TCBS agar (D).

Following microbiological screening, bacterial isolates were analyzed by standard biochemical assays (IMViC, triple sugar iron agar and urease tests). Bacterial isolates with characteristic IMViC patterns were characterized biochemically as in following image (**Table 5.1**). Overall, biochemical characterization elucidated diarrheagenic *E. coli* as the predominant pathogen (n=136, 23.7%) followed by *Vibrio* spp. (n=29, 5%), *Salmonella* spp. (n=5, 0.8%) and *Shigella* spp. (n=2, 0.3%) among all diarrheal age groups.

Table 5.1 Results and interpretations of various biochemical assays. - negative. + positive. D different.

S. No.	Name of test	Media	Analysis	*E. coli*	*Shigella*	*Salmonella*
1.	Indole test	Peptone water	Pink color 	+	**D**	-
2.	Methyl red test	MR-VP broth	Red color 	+	+	+
3.	Voges Prausker test	MR-VP broth	Red color ring 	-	-	-
4.	Citrate utilization test	Simonn's citrate agar	Color changed to Blue 	-	-	-
5.	Triple sugar iron agar test	Triple sugar iron agar	A/A, gas, K/A, gas, K/A, H_2S 	**A/A, gas**	**K/A, gas**	**K/A, H_2S**
	Urease agar	Christensen's urease agar	Color change to pink 	-	-	-

Present study elucidated diarrheagenic *E. coli* and *Vibrio* spp. as the abundant bacterial pathogens. Therefore, both bacteria were further confirmed and characterized at molecular levels to estimate incidences of different molecular pathotypes.

5.3 Incidences of DEC molecular pathotypes in study population

Characterization of DEC was stringently ascertained on the basis of standard biochemical, molecular and serological methods. Biochemically identified *E. coli* were further characterized for genus *Escherichia* by using 16S rRNA gene. A characteristic 542bp amplicon was amplified validating the presence of *E. coli* in diarrheal cases (Figure 5.4).

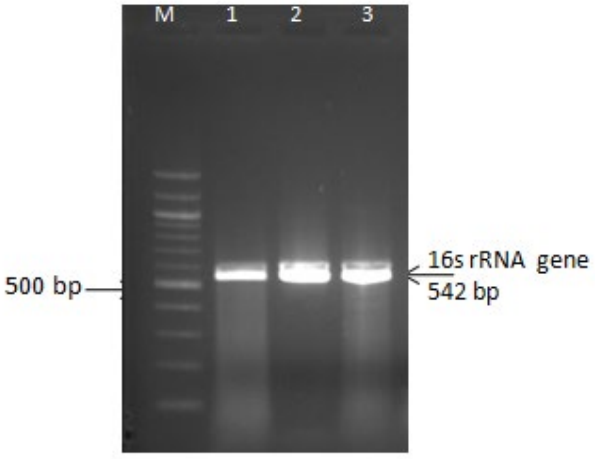


Figure 5.4: PCR agarose gel (1.5% in 1 X TAE) for genus *Escherichia* 16S rRNA gene (542 bp). Lane M represents 100 bp DNA marker (NEB). Lanes 1, 2, 3 show amplified 16S rRNA gene (542 bp), confirming *E. coli* strains.

Molecularly identified diarrheagenic *E. coli* isolates were further categorized into different pathotypes by screening of particular virulence genes selected from studies by Nataro & Kaper [93]. By using molecular approach, a total of three DEC molecular pathotypes i.e. enteropathogenic *E. coli*, enterotoxigenic *E. coli* and enteroaggregative *E. coli* were identified in the current study (Figure 5.5). We couldn't identify any enterohemorrhagic *E. coli* and enteroinvasive *E. coli* pathotypes by amplification of specific virulence genes in analyzed samples. Overall, we observed approximately 21% (n=120/572) DEC incidence rates among hospitalized diarrheal patients.

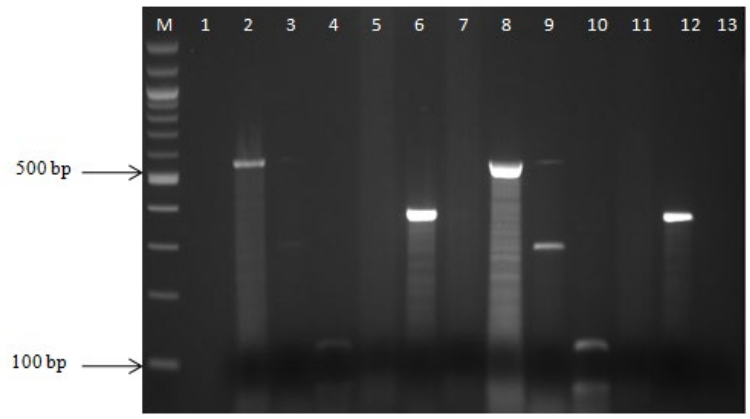


Figure 5.5: Represntative PCR agarose gel (1.5% in 1 X TAE) of diarrheagenic *E. coli* pathotypes. Lane M reprsents 100 bp marker (NEB) , lane1 shows negative control, lane 2 & 8 reprsent 16S rRNA gene (542 bp), lane no 3 and 9represent ETEC (*eltB* gene 322bp) and lanes 4 and 9 represent ETEC (*estA* gene 147bp). Lane no 6 & 12 represent EPEC (*eaeA* gene 376 bp). Lane no 7 & 13 showed no amplification.

Following multiplex PCR characterization, DEC molecular pathotypes were further confirmed by single gene PCR (Figure 5.6 and Figure 5.7). Among various DEC pathotypes, EPEC (n=79/572, 13.8%) was found to be predominant pathotype followed by ETEC (n=33/572, 5.8%) and EAEC (n=8/572, 1.4%).

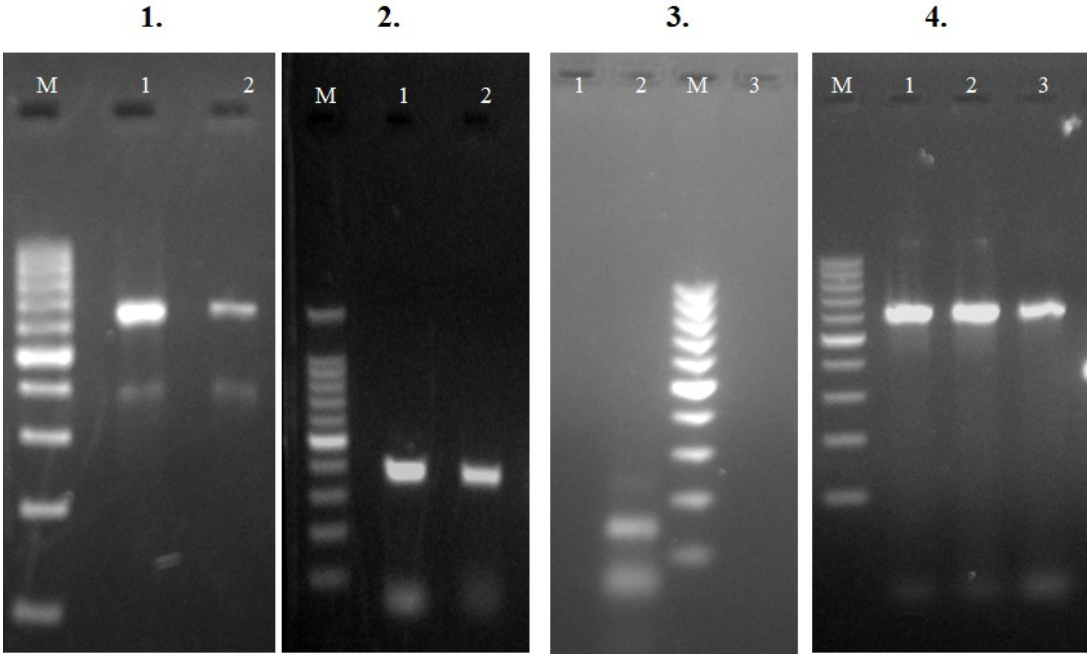


Figure 5.6: PCR characterization of DEC molecular pathotypes using virulence specific genes in 1.5% agarose gel. **Gel1;** lane M represent 100bp DNA ladder, lane 1and 2 typical EPEC (*bfpA* 369 bp &*eae* 881bp). **Gel2** Lane M shows 100bp ladder, lane 2 and 3 represent atypical EPEC (*eae* 881bp) **Gel3;** Lane 1 & 3 are empty, lane 2 shows ETEC (*estA* 147bp). **Gel4; Lane** M represents 100bp DNA ladder, lanes 1, 2, 3 show EAEC pathotype with amplified *pCVD* gene (630bp) .

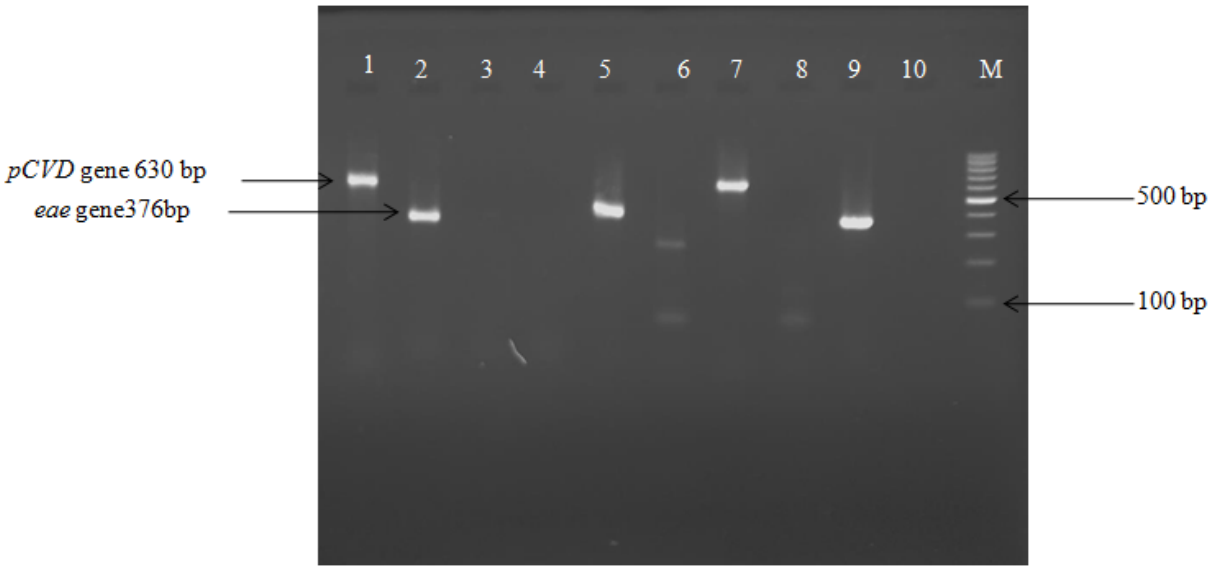


Figure 5.7: PCR gel of diarrheagenic *E. coli* pathotypes run on 1.5% agarose gel run in 1X TAE buffer. Lane 1 and 7 represents EAEC (*pCVD* gene 630 bp). Lanes 2, 5 and 9 represent EPEC (*eae* gene 376bp). Lane 6 represents ETEC (*eltB* gene 322 bp). Lane 8 represents ETEC (*estA* 147bp). Lane 10 is a negative control (*E. coli* DH-5α) and Lane M represents 100 bp marker (NEB). Lane 3 4 represents sample which did not amplify any virulence genes of *E. coli* pathotypes.

5.3.1 Sequencing of PCR products

The amplified products of PCR were further confirmed by commercial Sanger sequencing and partial coding sequences obtained were found to be 100% similar to targeted reference genes. The gene sequences were submitted to NCBI database (NCBI accessions: KX911251, KX911252, KX911253, KX911255) and were utilized as positive control in subsequent PCR analysis. Following are the description for various NCBI accessions

- GenBank: KY794916.1 (*Escherichia coli* strain E12579 16S ribosomal RNA gene, partial sequence).
- GenBank: KX911251 (*Escherichia coli* bundle forming protein gene, partial cds)
- GenBank: KX911252 (*Escherichia coli* intimin gene, partial cds)
- GenBank: KX911253 (*Escherichia coli* ATP binding cassette transporter gene, partial cds)
- GenBank: KX911254 (*Escherichia coli* heat-labile enterotoxin protein beta chain OS gene, partial cds)

5.4 Clinical symptoms Vs DEC molecular pathotypes

Clinical symptoms of DEC pathotypes mediated infection vary from acute to persistent diarrhea, fever and moderate to severe symptoms of dehydration. Besides, loose stools as a common illness among study population, features of fever, vomit, dehydration, mucus & abdominal pain were also observed. To ensure pathogen-specific symptoms, chi square analysis was carried out and P values at 95% confidence interval (CI) and odd ratio (OR) were calculated (Table 5.2).

Table 5.2: Correlation analysis of clinical symptoms related with DEC positive and DEC negative patients by chi square test.

Clinical symptoms Observed	DEC positive (n=120)	DEC negative (n=452)	P value (at 95% CI)
Vomiting	30 (25%)	109 (24.1%)	0.840
Fever	25 (20.8%)	62 (13.7%)	0.0536
Dehydration	25 (20.8%)	107 (23.6%)	0.5117
Watery diarrhea	20 (16.6%)	11 (2.4%)	0.0001*
Mucus	6 (5%)	4 (0.8%)	0.0022*
Abdominal pain	3 (2.5%)	6 (1.3%)	0.3589

(*) Mark shows statistically significant values

For comparative analysis, DEC positive patients (n=120) were taken as infected group and patients without DEC infection (n=452) were considered as negative control. In our study, clinical symptoms of watery stools, visible mucus were found statistically associated with DEC pathotype infection. Other pathophysiological features like vomiting, severe dehydration and fever were also observed with higher frequency in EPEC and ETEC pathotypes however, similar proportion of symptoms were also seen in DEC negative population, therefore statistically insignificant. EAEC infection was not statistically correlated with various clinical symptoms however infection was presented with much severity. EAEC pathotype was primarily found associated with clinical symptoms of frequent bowl movements (>6 episodes of watery stool), fever, vomiting and dehydration.

5.5 Distribution of virulent genomic elements among various DEC molecular pathotypes

Identification of DEC molecular pathotype was confirmed by amplification of either distinct gene or combination of genes. In the present study, *eae* gene of atypical EPEC (62.5% *eae* gene, n=75/120) was the most prevalent as compared to typical EPEC (3.3% *eae* and *bfpA,* n= 4/120). In case of ETEC infected patients, isolates harboring *estA* gene (18.3%, n=22/120) were more common than isolates possessing both *estA* and *eltB* genes (10%, n=12). All EAEC strains (n=8) possessed *pCVD* (6.6%, 8/120) gene probe.

5.6 Age group distribution of DEC molecular pathotypes

For exploring high risk age groups, study population was stratified into five various age groups viz. children 0-2 years (n=202) and 3-5 years (n=45), adolescent 6-17 years (n=41), adult 18-65 years (n=257) and elderly >65 years (n=27). Our study revealed uniform abundance of EPEC & ETEC infections in all age segments however, children less than five years (<2 years & 3-5 years) of age showed higher incidence rates as compared to any other age group (Figure 5.8). Although EAEC pathotype was detected with low frequency but enteropathogen was predominantly found in children population (5.2%, 7/572) as compared to adult diarrheal patients (0.3%, 1/572) (Figure 5.8).

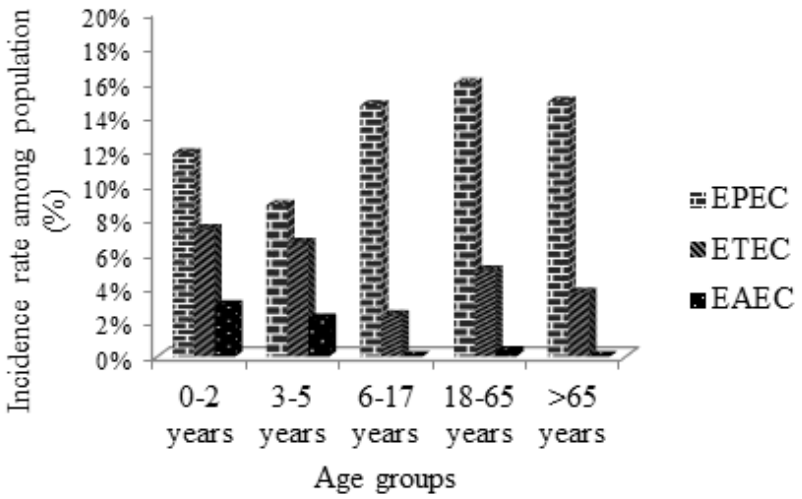


Figure 5.8: Prevalence of diarrheagenic *E. coli* (DEC) pathotypes among different age groups. X- Axis represents different age groups under study and Y-axis represent proportions of different pathotypes. EPEC= Enteropathogenic *E. coli,* ETEC= Enterotoxigenic *E. coli,* EAEC= Enteroaggregative *E. coli.*

In order to recognize specificity of identified DEC pathotype to particular age groups, bivariate Fisher analysis was performed (Table 2). Statistically significant correlations were observed for EPEC, ETEC & EAEC pathotypes with those of children <2 years of age. However, in adult age group (17-65 years), only EPEC & ETEC prevalence was correlated significantly.

Table 5.3: Bivariate analysis of age wise distribution of diarrheagenic *E. coli* pathotypes using Fisher's exact test.

Age group	DEC pathotype	Odd Ratio (at 95% CI)	p- value
0-2 years	EPEC (24/79)	8.182 (2.684-24.94)	0.0001*
	ETEC (15/33)	26.67 (3.248-219)	0.0001*
	EAEC (6/8)	44.20 (1.794-1089)	0.0070*
3-5 years	EPEC (4/79)	1.00 (0.2411-4.418)	1.0000
	ETEC (3/33)	3.1 (0.3050-31.50)	0.6312
	EAEC (1/8)	3.4 (0.1194-96.78)	1.0000
6-17 years	EPEC (6/79)	1.541 (0.4175-5.688)	0.7480
	ETEC (1/33)	1.000 (0.05988-16.70)	1.0000
	EAEC (0/8)	ND	
18-65 Years	EPEC (41/79)	20.23 (6.743-60.69)	<0.0001*
	ETEC (13/33)	20.80 (2.522-171.5)	0.0005*
	EAEC (1/8)	3.400 (0.1194-96.78)	1.0000
	>65 years as reference category		

(*) Mark shows statistically significant values

5.7 Serogroup analysis of DEC pathotypes

Molecular pathotypes of DEC were characterized for *E. coli* somatic O antigen and were found associated with at least twenty three different O serogroups (Figure 5.9). Serologic analysis revealed 60.8% (73/120) of *E. coli* isolates as diarrhea associated serotypes. Serogroups O2, O26, O35 and O41 were the most commonly characterized with a prevalence of 41% (30/73).

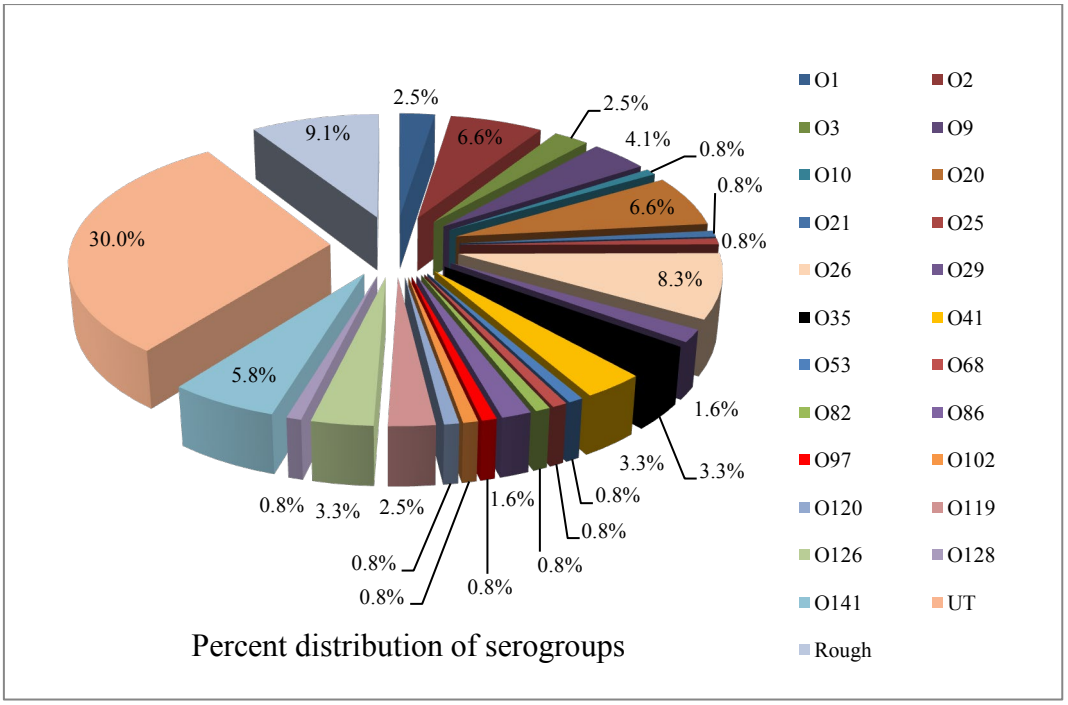


Figure 5.9: Distribution of different O serogroup among diarrheagenic *E. coli* (DEC) isolates. UT (untypeable), Rough (Non-agglutinable with antisera).

Figure 5.10 shows O26 and O2 as most commonly isolated among EPEC and ETEC pathotypes, respectively. O26 is often associated with classical attaching & effacing group (EPEC) and non O157- EHEC strains. We observed serogroups O2, O25 & O128 in ETEC strains only. Apart from classical serogroups, EAEC predominantly belonged to serogroup O3 (2.5%). However, higher proportions of strains belonging to EPEC, ETEC and EAEC pathotypes remained un-typeable (30%) and didn't agglutinate with O antiserum (9%).

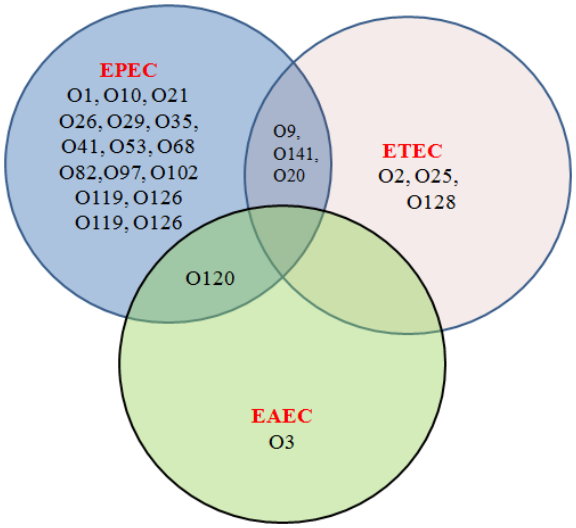


Figure 5.10: Interactions based upon serogroup sharing between various diarrheagenic *E. coli* pathotypes. The overlapping area is showing mutual serogroup in respective molecular pathotypes.

Correlation between DEC virulent genes and O serogroups revealed *eae* gene of EPEC was most commonly associated with O serogroups (Figure 5.11). *estA* and *eltB* genes of ETEC toxins were observed with O2, O20, O25, O102 and O141 serogroups while, serogroup O9 was observed with *estA* only. The *pCVD* gene of EAEC was found to be associated majorly with one serogroup O3.

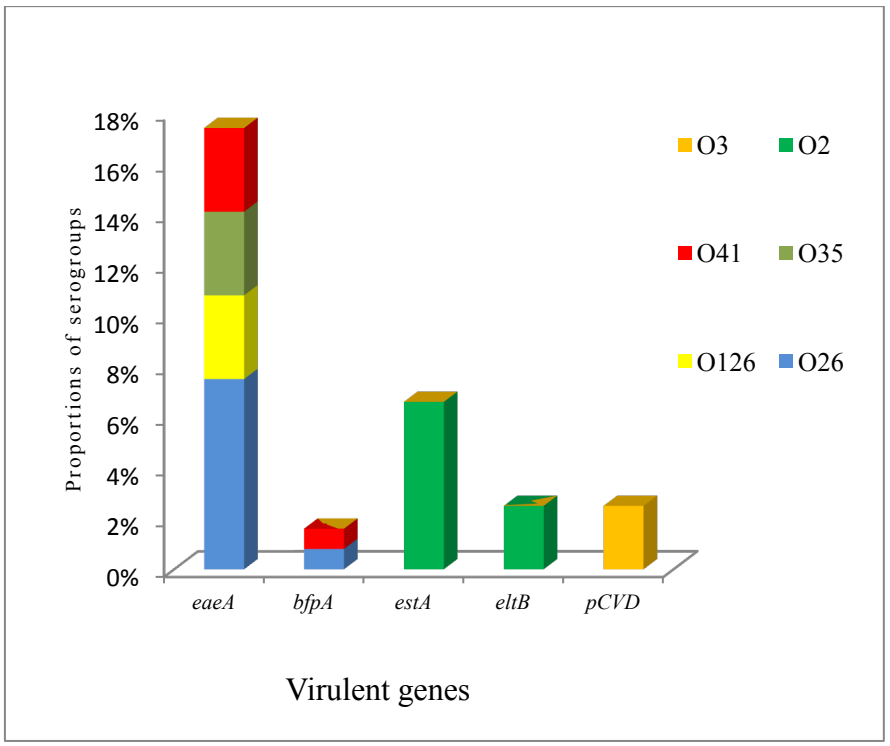


Figure 5.11: Representing association of diarrheagenic *E. coli* (DEC) virulent genes with top six O serogroups.

5.8 Seasonal variations in DEC pathotypes

Incidences of various DEC molecular pathotypes were analyzed for year wise distributions (Figure 5.12). Incidences of different DEC pathotypes were observed throughout the years, however seasonal peaks were seen during rainy and winter seasons. Among DEC pathotypes, infection rates of ETEC strains were escalated during rainy season up to 15%, however, EPEC infections varied between 15-25% during different seasons. EAEC pathogen was observed with a low incidence below 5%.

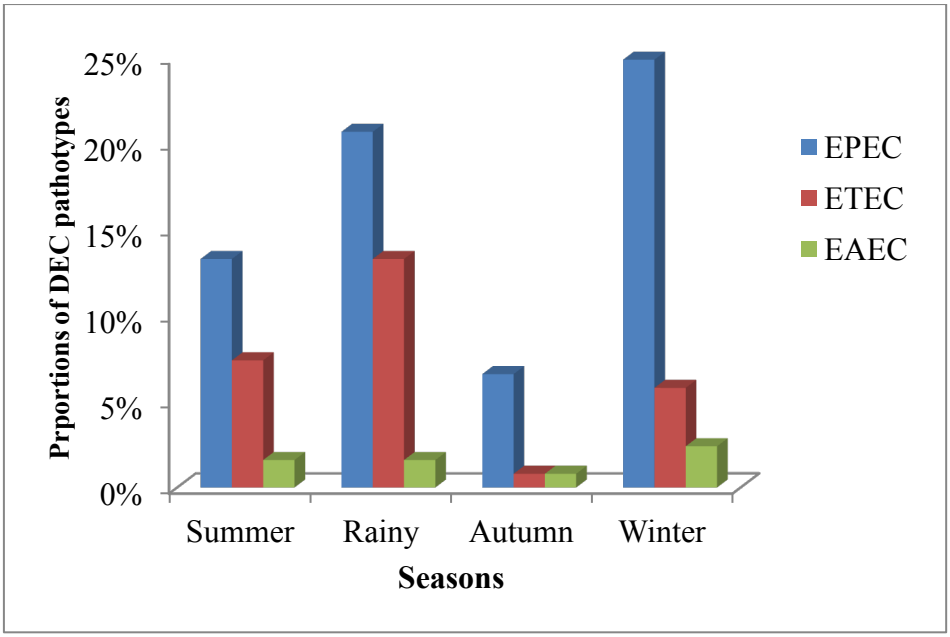


Figure 5.12: Season wise distribution analysis of diarrheagenic *E. coli* molecular pathotypes.

5.9 *Vibrio* incidences among population

Molecular characterization of biochemically confirmed *Vibrio* isolates showed amplification of *Vibrio* genus specific 16S rRNA gene in 5% (n= 29/572) of isolates (Figure 5.13). Mixed enteric infections of *Vibrio* spp. with *E. coli* and with rotavirus were observed to be 1.2% and 0.03%, respectively. Amplified product of *Vibrio* genus specific 16S rRNA gene (663bp) was sequenced and the obtained sequence was submitted to GenBank NCBI (Accession no. KX891575).

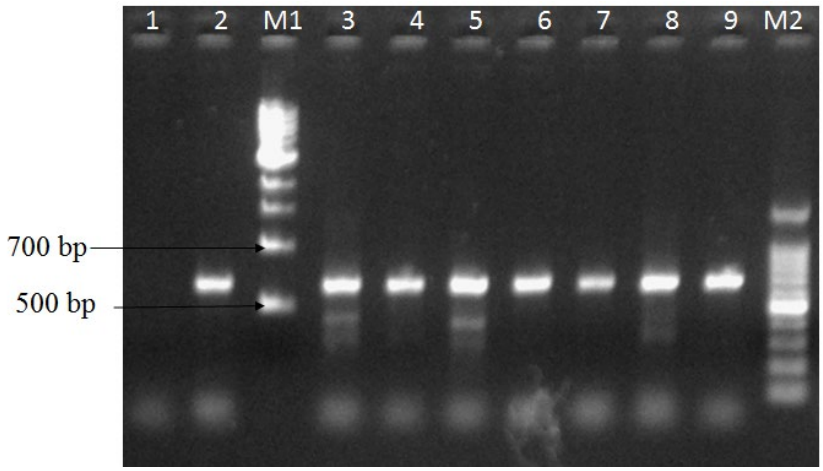


Figure 5.13: PCR assay for characterization of *Vibrio spp.* through amplification of 16S rRNA gene. Lane 1 shows a no template negative control. Lane 2 and lanes 3-9 show amplified PCR product for *Vibrio* genus specific 16S rRNA gene (663bp). Lane M1 & M2 show 1Kb and 100 bp DNA ladder (NEB) respectively.

5.10 Pattern of *Vibrio* infections among different age groups

Incidences of *Vibrio* spp. were analyzed among all five age groups i.e. infants (0-2 years), children (3-5 years), adolescents (6-17 years), adults (18-65 years) and elderly patients (>65 years) (Figure 5.14). Highest frequency of *Vibrio* spp. infection was observed in adolescents (7.3%) and adult age (6.6%) groups while, lowest infection rates observed among infants (2.9%) followed by elderly (3.7%) and children population (4.4%).

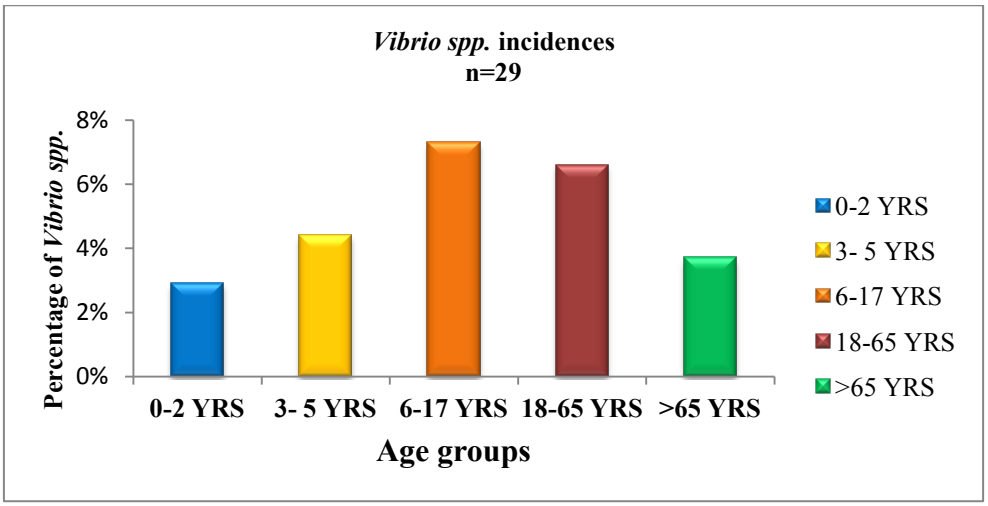


Figure 5.14: Incidences of *Vibrio* spp. among different age groups of moderate to severe diarrhea infected patients. 0-2 years: infant, 3-5 years: children, 6-17 years: adolescent, 18-65 years: adult, >65 years represents elderly population.

Fisher's exact test was calculated to decipher highest risk age group among *Vibrio* infected population (Table 5.4). There was no significant correlation between *Vibrio* spp. infections with classified age groups.

Table 5.4: Identification of high risk age group among diarrheal patients by Fisher's exact test.

Age groups	P –value at 95% confidence interval (CI)	Odd ratio (OR)	Significance
0-2 yrs	0.59	0.79	NS
3-5 yrs	1.00	1.20	NS
6-17 yrs	0.92	2.05	NS
18-65 yrs	1.00	1.84	NS

> 65 years used as reference category, NS= Not significant

5.11 Seasonal frequency of *Vibrio* incidences

Screening of *Vibrio* species was carried out throughout the year to analyze season wise distribution (Figure 5.15). Season wise classification of months was performed according to Indian seasons: summer (March to May), rainy (June to September), autumn (October to November) and winter (December to February). Highest incidences of *Vibrio* were observed for rainy season (34.3%) followed by winter (27.5%) and summer seasons (23.9%). Among different age groups, infants were highly susceptible to *Vibrio* species during winter and summer seasons while, no case was reported among infants in rainy season. Among adolescents and adults who is the most active and productive group, highest infection rate was observed during rainy season followed by winter and summer. Overall, seasonal peaks were observed to have a rise in summer, reached at a maximum in rainy season and a final decline during autumn.

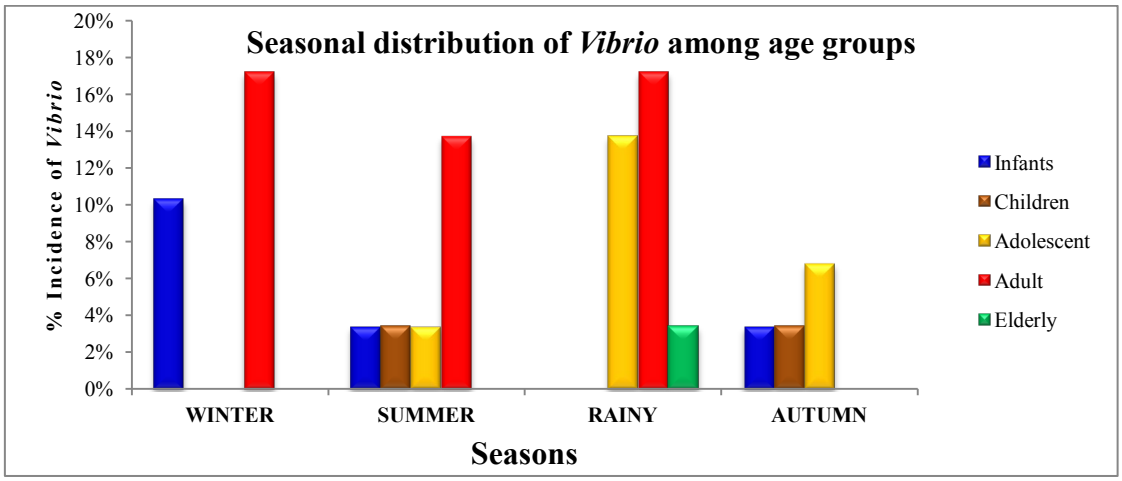


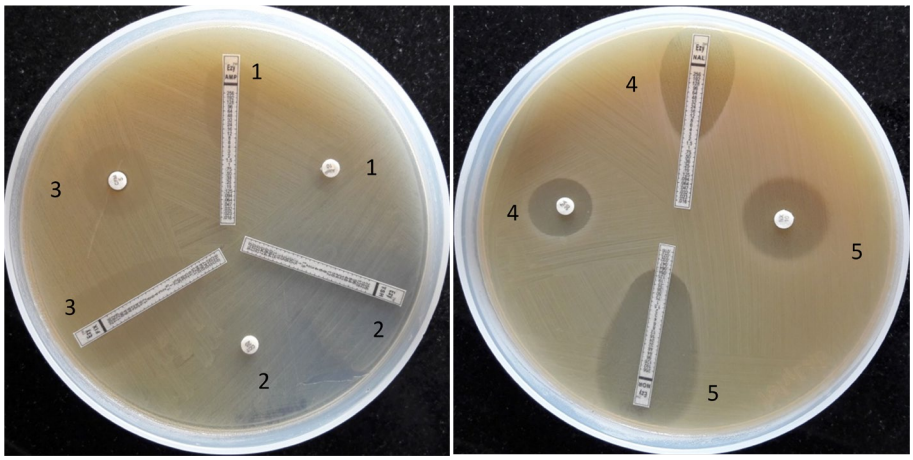
Figure 5.15: Seasonal trends of *Vibrio* species incidences among different age categories of diarrheal patients.

5.12 Antimicrobial resistance among bacterial pathogens

The major bacterial pathogens were further screened for antibiotic resistance levels against commonly utilized treatment regimens. Several studies have been performed for analyzing antibiotic resistance patterns among diarrheal pathogens [31, 208, 209].

5.12.1 Antimicrobial resistance in DEC molecular pathotypes

Molecular pathotypes of DEC were screened for antibiogram patterns by antimicrobial susceptibility test (AST) and minimum inhibitory concentration (MIC) according to CLSI guidelines [206]. Antibiotics utilized in screening were chosen on the basis of ICMR recommendations (Figure 5.16 A & B). Minimum inhibitory concentrations of DEC pathotypes were also performed to determine MIC breakpoints for different antibiotics (Table 5.5)



A B

Figure 5.16: Antibiotic susceptibility test and minimum inhibitory concentration test for diarrheagenic *E. coli* pathotypes. Zones of inhibition were seen against co-trimoxazole, nalidixic acid, norfloxacin, cefixime and ampicillin in AST and MIC tests. 1 Ampicillin (10µg), 2. Co-trimoxazole (75µg), 3. Cefixime (5µg), 4. Nalidixic acid (10µg), 5. Norfloxacin (30µg).

AST and MIC assays revealed that majority of diarrheagenic *E. coli* strains were sensitive for co-trimoxazole (36%), while <20% were sensitive for cefixime, norfloxacin, ampicillin and nalidixic acid (Table 5.5). Proportions of intermediate strains against all five antibiotic were <5%. DEC pathotypes exhibited alarming resistance rates against widely used antimicrobials; ampicillin, cefixime, nalidixic acid and norfloxacin (approximately 80%).

On correlating resistance pattern with distinct DEC pathotype, EPEC strains were observed to be most resistant against all tested drugs. ETEC strains were found 18-49% sensitive against all tested antibiotics. EAEC pathotypes showed highest sensitivity for co-trimoxazole (62.5%), norfloxacin (37.5%), nalidixic acid and cefixime (25% in both).

Table 5.5 showed statistical analysis using chi square test performed to evaluate pathotype specific antibiotic resistance level. All molecular DEC pathotypes were found to possess significant levels of antibiotic resistance against all antibiotics (Table 4).

Table 5.5: Antibiotic resistance among the different diarrheagenic *E. coli* groups in patients with diarrhea. AMP=ampicillin, COT= Cotrimoxazole, CPM= cefixime, NOR=norfloxacin and NAL=nalidixic acid. * P< 0.05, ** P<0.01 (chi Square test), for the comparison of the resistance percentage among the different *E. coli*.

Antibiotics	Characteristics (Resistant)	P value	OR (95% CI)
AMP	EPEC 82.2% (65)	0.0026**	2.792 (1.456-5.353)
	ETEC 78.7% (25)	<0.0001**	3.473 (1.866-6.462)
	EAEC 75% (6)	<0.0001**	4.895 (2.668-8.979)
CPM	EPEC 77.2% (61)	0.0311*	2.052 (1.108-3.801)
	ETEC 81.8% (26)	0.0004**	3.329 (1.721-6.440)
	EAEC 75% (6)	<0.0001**	4.895 (2.668-8.979)
NAL	EPEC 79.7% (67)	0.0078**	2.452 (1.299-4.627)
	ETEC 81.8% (27)	<0.0001**	4.205 (2.209-8.005)
	EAEC 62.5% (5)	<0.0001**	3.807 (2.078-6.974)
NOR	EPEC 79.7% (67)	0.0078**	2.452 (1.299-4.627)
	ETEC 69.6% (22)	0.0135*	2.154 (1.205-3.849)
	EAEC 62.5% (5)	<0.0001**	3.807 (2.078 -6.974)

*COT was utilized as reference category

5.12.2 Antimicrobial resistance patterns of *Vibrio* species

According to Indian Council of Medical Research different antibiotic classes; tetracycline, β–lactam and quinolones are prescribed for treatment of severe diarrhea cases [31]. Treatment regimen involving norfloxacin, nalidixic acid, co-trimoxazole, tetracycline and ampicillin are specific for *Vibrio* mediated diarrhea (24). Other antibiotics were also screened in the study to investigate the common resistance profiles of *Vibrio spp.* Accordingly Kirby Bauer's disk diffusion test was performed against fifteen different antibiotics (Figure 10 a-d).

In β–lactam class, alarming resistance levels were analyzed for ceftazidime (65.5%), cefipime (62%) and ceftriaxone (55.1%) antibiotics (Figure 5.17 a). The proportion of intermediate isolates was found from 6.8% to 20.6% for all β–lactam antibiotics. However, lowest resistance was observed for imipenem (13.8%). Therefore, elucidating imipenem as most effective β–lactam antibiotic against *Vibrio* infections.

Among aminoglycosides, highest resistance rate was observed against streptomycin (34.4%) and kanamycin (31%) (Figure 5.17b). Percentage of intermediate isolates for aminoglycoside group ranged from 6.8% to 31%. *Vibrio spp.* exhibited minimum resistance levels for netilimicin (6.8%) followed by tobramycin (13.7%) and amikacin (20.6%).

For quinolones, alarming resistance rates were observed for nalidixic acid (72.4%) followed by ofloxacin (37.9%) and ciprofloxacin (37.9%), and norfloxacin (27.4%) (Figure 5.17c). *Vibrio spp.* exhibited least resistance against levofloxacin (30.1%). Proportions of intermediate isolates for quinolones were observed to range between 3.4% - 20.6%.

Overall, *Vibrio* isolates were most sensitive for imipenem, netilmicin, tobramycin, levofloxacin and norfloxacin hence, these antibiotics could be priority antibiotics of choice for treatment of *Vibrio* infections. Moreover, continuous monitoring of antibiotic resistance is re uired to access β-lactam resistance trends as third generation cephalosporin resistant *Vibrio* incidences are rare and anxiously alarming.

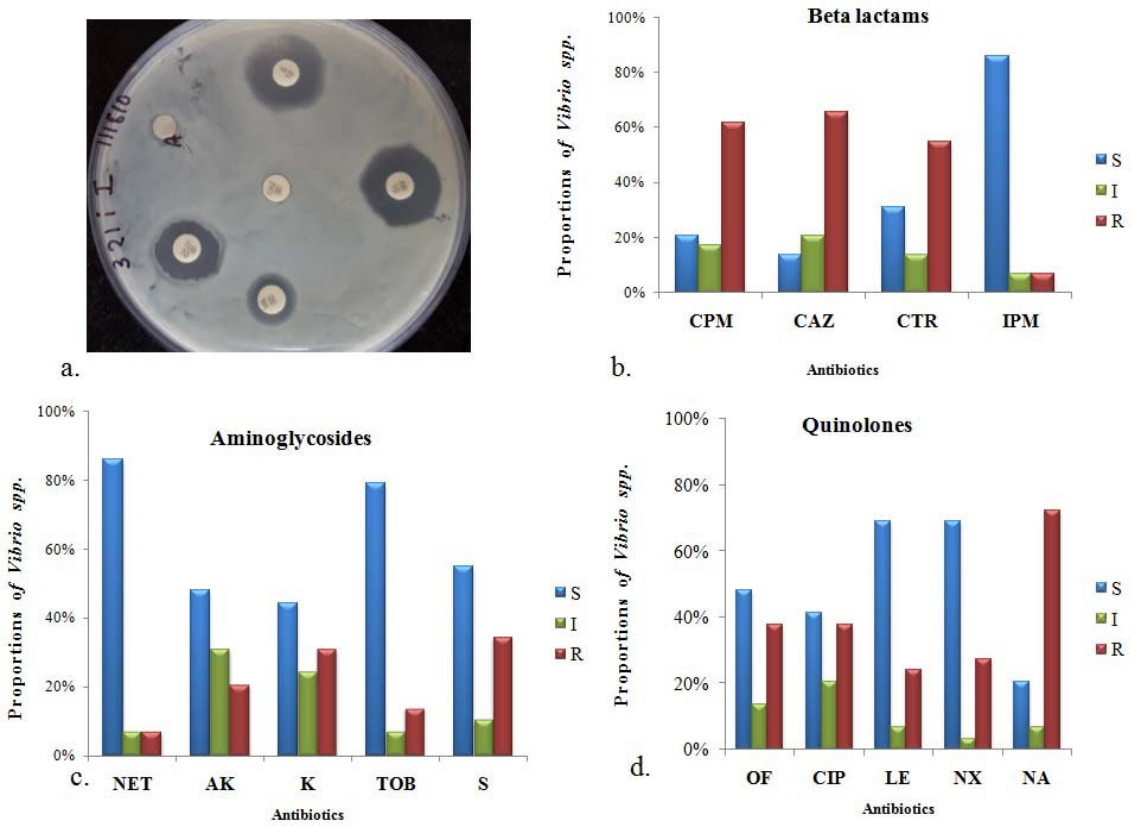


Figure 5.17: Antibiotic susceptibility patterns of *Vibrio spp.* a: Antibiotic susceptibility performed by Kirby Bauer's disk diffusion method. Proportions of sensitive (S), intermediate (I) and resistant (R) phenotypes for β lactams (b), aminoglycosides (c) and quinolones (d). CPM- Cefepime, CAZ- Ceftazidime, CTR- Ceftriaxone, IPM- Imipenem, NET- Netilmicin, AK- Amikacin, K- Kanamycin, TOB- Tobramycin, S- Streptomycin, OF- Ofloxacin, CIP- Ciprofloxacin, LE- Levofloxacin, NX- Norfloxacin, NA- Nalidixic acid.

CHAPTER 6

DISCUSSION

6. DISCUSSION

Diarrhea is gastrointestinal illness of multifactorial etiology and symptoms, generally transmitted by fecal-oral route. Effective and specific interventions in management of enteric illnesses require specific characterization of the etiological agent and treatment of clinical symptoms presented during the disease. Diarrhea illness is caused by vast spectrum of pathogens including bacteria, viruses, and parasites [6, 210]. Amalgam of conventional microbiological techniques with molecular method incredibly increases reproducibility and scalability of etiological agent's characterization [211]. However, in perspective of diarrhea, the viral causative agents have been explored to a remarkable extent as compared to others [212-214]. There are several reports suggesting predominance of bacterial pathogens in surveillance studies and outbreaks. Incidences of bacterial pathogens vary across different regions of India due to varied geographical conditions. Lack of uniform surveillance system for bacterial pathogens is accountable to underestimation of their role in diarrhea incidences. Our group is engaged in exploring diarrhea etiology from regions of Himachal Pradesh and in our previous study, we have investigated role of viral agents of diarrhea [215]. Present work is exclusively focused on deciphering frequency of bacterial pathogens using microbiological and molecular approach in hospitalized moderate to severe diarrhea population of Himachal Pradesh.

The diarrhea study cases involved in comprehensive investigation belonged to a broad window of age from 13 days to 85 years. Therefore, the study population was stratified into five different age groups (0-2 years, 3-5 years, 6-17 years, 18-65 years, >65 years) and age group >65 years was taken as reference group for statistical analysis.

From microbiological and biochemical analysis we observed *E. coli* (23.7%), *Vibrio* spp. (4.7%), *Salmonella* spp. (0.5%) and *Shigella* spp. (0.03%) as causative agents of diarrhea in regions. Similar, observations have been reported from India and world, indicating prevalence of diarrheagenic *E. coli* in diarrheal stool specimens [210, 216]. *Vibrio* spp. is generally associated with large outbreaks and epidemics which have origins in Indian subcontinent and then disseminated to various parts of the world [97, 217]. Incidences of non typhoidal *Salmonella* spp. and *Shigella* spp. commonly occurs during foodborne outbreaks worldwide [148, 218].

From microbiological and biochemical findings, we found *E. coli* and *Vibrio* spp. as predominant pathogens among all diarrhea cases and were taken further for molecular and

serological characterization. *E. coli* elaborates abilities from important gut commensal to pathogen of intestinal as well as extra-intestinal infections by virtue of heterogeneous phenotypic and genotypic attributes [97, 219]. *E. coli* possesses an inventory of virulent elements which leads to segregation of this bacterium into diverse kinds of pathotypes/genotypes.

Frequency of DEC pathotypes was determined using virulence gene markers in moderate to severe diarrhea population. Prior to this study, there have been no reports from present region addressing bacterial pathogen incidences. Therefore, it is of utmost importance to address DEC associated infections and clinical symptoms to provide a comprehensive view of diarrhea etiology within the region which will facilitate further epidemiological and therapeutic prospects.

From molecular characterization, diarrheagenic *E. coli* pathotypes incidences were observed up to 21%. Our study showed moderate DEC infection rates, similar to the reports from developing world [220-223]. However, reports from different parts of India and neighboring countries showed 10-35% variation in DEC incidence rates [18, 19, 224-226]. However, sporadic outbreaks with 42% to 65% of infection rates have also been reported from regions of India [20, 87, 227]. Moreover, prevalence of diarrheagenic *E. coli* as an etiological agent of diarrhea is well reported between 30%- 40% cases [228, 229].

Approximately, 6% co-infection of DEC and rota virus was observed in study population. Co-infection with other enteric pathogens is greatly known to aggravate symptoms and duration of diarrhea [77, 225]. Following age group categorization, our observations indicate higher proportions of DEC pathotypes associated with childhood diarrhea than any other age set. In a recent study conducted in Mexico, Canizalez-Roman and coworkers [223] also reported higher DEC incidences in children population. In addition, higher frequencies of DEC pathotypes in moderate to severe cases of childhood diarrhea are reported all over the globe [18, 211, 229-231]. Previous studies established that DEC preponderance among children may be due to their compromised immune level and intimate attachment of pathogens to the tender epithelial mucosa. DEC infection induced alterations to the intestinal physiology and microbiota composition remain restricted to the postnatal period also [232]. Therefore DEC infection might predispose children less than five years to sequelae of diarrheal episodes.

By molecular identification approach, DEC molecular pathotype EPEC was observed with highest frequency among all diarrheal patients. Recurrent isolation of EPEC from severe diarrhea cases is implicated especially in pediatric populations [106]. Persistent diarrhea is the most common clinical presentation in EPEC infection and this enteropathogen possess an innate propensity to persist longer in intestine than other pathotypes.

EPEC is typically categorized into two classes, atypical EPEC having *eae* gene and typical EPEC possess combination of *bfpA* and *eae* genes [233]. High frequencies of the *eae* gene in current study underpin the importance of atypical EPEC as predominant diarrheal pathogen in the region. The Low frequency of *bfpA* observed in present study suggests its limited pathogenic role [234, 235]. Both *eae* and *bfpA* genes are responsible for intimate attachment to the surfaces via intimin and bundle forming pilus. In addition, EPEC also possesses different combination of fimbriae and type III secretion system proteins for producing attaching and effacing phenotypes. On global level, EPEC alone contributes for 5-10% of pediatric diarrhea [230, 233, 236]. Our observations coincide with various epidemiological studies from different parts of world which reported EPEC as the main DEC pathotype affecting children and adults with similar frequency [237-239].

Another DEC molecular pathotype ETEC specific clinical outcomes rely upon the secretion of two enterotoxins viz heat labile (*estA* gene) and heat stable (*eltB* gene) toxins. These toxins result in secretory diarrhea via Cl^- secretion through the cystic fibrosis transport receptor (CFTR) and cyclic guanosine mono-phosphate (cGMP) [93]. Among the ETEC positive patients, *estA* gene was more frequently isolated than *eltB* alone or *estA* and *eltB* in combination which similar to what has been observed in other studies [18, 85]. In the present study ETEC showed varying prevalence among all ages and similar observations were reported from the northern part of the country [240, 241]. For past many years, ETEC has been implicated as the major cause of traveler's diarrhea [242]. However, the DEC positive patients in Himachal Pradesh did not report foreign travel and infections were apparently locally acquired.

The EAEC enteropathogen was identified by using *pCVD* gene probe. Pathogen is known to cause disease via multiple mechanisms; adherence to mucosa, secretion of toxins & mucosal inflammation [104]. We observed EAEC predominantly in children (n=7/8) followed by elderly age group (1/8). Other studies have also shown prevalence of *pCVD* positive *E. coli* in the stool specimens of adults and childhood diarrhea cases and this can be as high as 11%

[243]. Current findings strengthen evidences that EAEC is an emerging diarrheal agent in the South East Asian children population [244].

Different DEC molecular pathotypes exhibit surface to invasive pathophysiology resulting in different clinical outcomes. We evaluated clinical characteristics of DEC infections statistically among DEC positive and DEC negative population. We found that clinical symptoms of watery stools, blood and mucus were significantly associated with DEC pathotype infection. EPEC and ETEC infected patients showed acute invasive diarrhea symptoms of blood and mucus in stool. Present observations reinforce the conviction that EPEC & ETEC pathotypes are considerably responsible for severe gastrointestinal infections associated with childhood and adult diarrhea [211].

Characterization of *E. coli* somatic 'O' antigen still appears to be useful technique for presumptive identification of certain DEC pathotypes [93, 245, 246]. In present study, the serogroup O26 was most commonly observed followed by O2, O41, O35, O126 and O1. Our findings were in agreement with previous reports in which predominant diarrheagenic *E. coli* serogroups O26, O2 were predominant and could be associated reproducibly with severe clinical symptoms of blood and mucus in diarrhea stool specimens [234]. Interestingly, few isolates were found as untypeable or rough strains in various categories of DEC pathotypes. From literature, *E. coli* serogroups have been much related to identification of clonal variant of DEC pathotypes rather than precise identification [93, 97, 246, 247].

Annually, Himachal Pradesh is fed by snow and rainfall however, owing to its mountainous slopes water is drained into two main basins; Indus and Gangetic river systems. Season wise distribution analysis of DEC showed prevalence throughout the years. Seasonal variations for bacterial incidences are driven by various factors like recurrent floods, stagnant water and suboptimal hygienic conditions [217, 261].

The second majorly observed pathogen *Vibrio* spp. was also further characterized by molecular method. In past few years, increased incidences of *Vibrio* spp. have been reported from various parts of the globe with coastal regions more prone to this infectious disease [23, 248]. Historically, *Vibrio* spp. has caused many pandemic and epidemics along with several explosive outbreaks which continue to thrive in regions with contaminated water and suboptimal hygiene practices [169, 249, 250]. By amplification of *Vibrio* genus specific 16S rRNA gene, infection rate of *Vibrio* was found to be 5 % (29/572) among diarrheal

population (Figure 6). In a previous study, Bora & coworkers reported 16.5% of *Vibrio* infections in an outbreak from remote areas of Himachal Pradesh [251]. In addition, such higher *Vibrio* spp. incidences have also been reported from surrounding areas of state [252, 253]. Another study, deciphered higher proportion of *Vibrio spp.* in water samples collected from different river systems [254]. Similarly, *Vibrio* incidence rates up to 4% have also been reported from surrounding regions of Himachal Pradesh [255, 256]. In contrast to our observation, higher *Vibrio* infection rates ranging from 10% -15% are well reported from different parts of India [257, 258]. Furthermore, surveillance studies from Bangladesh have reported up to 18% of *Vibrio* spp. infections among diarrheal populations [259, 260]. Overall, our study depicts low levels of *Vibrio* spp. incidences and similar trends were observed for active surveillance studies in the country.

Uniform seasonal distribution of *Vibrio* spp. were seen throughout the years however, slight peaks were observed during rainy, winter and summer seasons. Similar, seasonal trends have been observed in coastal area but frequency is much higher as compared to current study [217, 250, 260-263]. This may be due to lack of stagnant water reservoirs in the region and direct drainage of rain water into major river systems.

The global spread of antimicrobial resistant strains threatens the effective prevention and treatment of enteric infections caused by gram negative bacteria. *E. coli* has become increasingly resistant to conventional and commonly used antibiotics in hospital and community settings [264, 265] and certainly poses serious threat to the management of infectious diseases. Since, diarrheal disease is generally self-limiting and antidiarrheal agents are not usually recommended for treatment of diarrhea [80]. However, in traveler, persistent and acute invasive diarrhea cases high severity of infection and extended recovery periods reinforce the use of antimicrobials [80, 209, 232].

We examined the DEC pathotypes resistance against five antibiotics: ampicillin, cefexime, norfloxacin, nalidixic acid and co-trimoxazole. These antibiotics are mostly prescribed as the first line of treatment in the country and are also advised by ICMR & CLSI [206, 266]. In the present study, EPEC was found as most resistant pathotype and highest levels of antibiotic resistance were observed against ampicillin. These observations are in concordance with previous studies analyzing DEC resistance patterns [267, 268]. We observed lowest resistance rates against co- trimoxazole among all DEC pathotypes. However, Sadeghabadi and coworkers reported approximately 80% resistance against co- trimoxazole in

diarrheagenic *E. coli* [269]. Similar reports across the globe also elucidated high levels of resistance against DEC pathotypes [241, 268-271]. In our study, proportions of DEC as diarrheal pathogen is limited to a moderate level, however current study revealed high levels of resistance in DEC pathotypes among hospitalized patients. Currently observed high resistance rates against commonly used antibiotics could be a result of extreme disease severity and persistence of infections among hospitalized patients.

Moreover, *Vibrio* strains showed rising levels of resistance against beta lactam group (cefepime, ceftazidime and ceftriaxone) and nalidixic acid. *Vibrio* strains exhibiting resistance against cephalosporins have been reported scarcely [272] while, the current study elicited alarming resistance rates against cephalosporin class in the region. Previous studies reported very higher levels of resistance against nalidixic acid, streptomycin and ciprofloxacin antibiotics [254, 255, 273, 274] however, our study showed relatively low levels of resistance against these antibiotics.

Diarrhea is an illness which is largely preventable through safe drinking water and hygiene practices; however relevance of such interventions is not applicable due to explosive population growth and limited resources. Present study is to our knowledge, the first comprehensive research in the region addressing associations of molecular DEC pathotypes with clinical outcomes and antibiogram patterns. In conclusion, in nearly one out of five moderate to severe diarrhea cases in Himachal Pradesh one DEC pathotype was isolated, in which EPEC was the most predominant pathotype. Our findings highlight the importance of continuous DEC pathotype surveillance programs for therapeutic approaches and not the least benefit of employing comprehensive inspection of antimicrobial resistance in the region.

In relation to treatment, a very few studies have evaluated comprehensive importance of drugs for the management of DEC pathotype infection. After introduction of the rotavirus vaccines into national immunization program of India, as well as thrust for development of ETEC and *Shigella* vaccines, Enteropathogenic *E. coli* should be the next priority for vaccine development owing to its high morbidity and mortality rates. The study would help in prioritizing and strategizing therapeutic and prophylactic measures against predominant DEC pathotypes in the country. Exploring the resistant phenotypes would aid in management & preventing spread of multidrug resistant strains.

CHAPTER 7

CONCLUSIONS

7. CONCLUSIONS

Diarrhea is a major gastrointestinal illness worldwide which affects millions of people each year. Diarrhea is associated with multiple etiological agents and effective treatment procedures require region wise information on causative agents, specific clinical symptoms and resistance profiles.

Present study was an attempt to generate baseline data regarding etiology of bacterial pathogens in unexplored regions of Himachal Pradesh. We determined the prevalence, antimicrobial susceptibility patterns, and association of these patterns with specific class of major bacterial pathogens. Following are the major and minor conclusions from present study:

Major Conclusion

- This is the first study elucidating role of major bacterial pathogens in diarrhea from regions of Himachal Pradesh. A total of five hundred seventy two diarrheal stool specimens from hospitalized patients were screened by microbiological and molecular methods. Biochemically, diarrheagenic *E. coli* (23.7%), *Vibrio spp.* (4.7%), *Salmonella spp.* (0.8%), and *Shigella spp.* (0.3%) were observed as etiological agents of diarrhea.
- Molecular characterization of major pathogens i.e. diarrheagenic *E. coli* and *Vibrio* spp. showed incidence rates of 21% and 4.7% respectively, in analyzed samples.
- On pathotype levels of molecular classification, EPEC was detected as predominant *E. coli* pathotypes (13.7%), followed by ETEC (5.7%) and EAEC (1.3%) pathotypes.
- Serological characterization of DEC elucidated 23 serogroups consisting of O26, O2 and O41 as predominant one.
- DEC molecular pathotypes infection rates were higher among children under the age of five, while *Vibrio spp.* infection rates were higher among adolescent and adult age groups.
- Incidences of DEC and *Vibrio spp.* infections were observed throughout the year, however infection peaks were seen during summer and rainy seasons.
- Predominant pathogens showed alarming resistance against first line of antibiotic classes like beta lactams and quinolones.

Minor conclusion

- Atypical EPEC pathotype was observed as predominant bacterial pathogen in the region.
- DEC pathotypes were found to be susceptible for co-trimoxazole and hence could be utilized in case of multidrug resistant pathogens

Present study provides information which will be useful both in discouraging inappropriate use and guiding physicians to more appropriate choices when therapy is necessary. In case of children, use of specific antimicrobials should be limited to well-defined bacterial agents due to small time frame in which treatment choices must be made.

CHAPTER 8

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8. REFERENCES

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CHAPTER 9

APPENDICES

9. APPENDICES

9.1 Media & Broth

MacConkey agar

Composition	Grams per litre
Peptone (Meat & Casein)	3
Pancreatic digest of gelatin	17
Lactose monohydrate	10
Bile Salts	1.5
Sodium chloride	5
Crystal violet	0.001
Neutral red	0.03
Agar	13.5
pH after sterilization (at 25 °C)	7.4±2

Eosin methylene blue agar

Composition	Grams per litre
Peptic digest of animal tissue	10
Dipotassium phosphate	2
Lactose	5
Sucrose	5
Eosin-Y	0.4
Methylene blue	0.065
Agar	13.5
pH after sterilization (at 25 °C)	7.4±2

Peptone water

Composition	Grams per litre
Peptic digest of animal tissue	10
Sodium chloride	5
Final pH (at 25°C)	7.2±0.2

Thiosulfate-citrate-bile salts-sucrose agar

Composition	Grams per litre
Proteose peptone	10
Yeast extract	5
Sodium thiosulphate	10
Sodium citrate	10
Oxgall	8
Sucrose	20
Sodium chloride	10
Ferric citrate	1
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15
pH after sterilization (at 25 °C)	8.6±2

Xylose lysine deoxycholate agar

Composition	Grams per litre
Yeast extract	3
L-lysine	5
Lactose	7.5
Sucrose	7.5
Xylose	3.5
Sodium chloride	5
Sodium deoxycholate	2.5
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	15
pH after sterilization (at 25 °C)	7.4±2

Triple sugar iron agar

Composition	Grams / Litre
Beef extract	3
Peptone	20
Yeast extract	3
Lactose	10
Sucrose	10
Dextrose monohydrate	1
Ferrous sulphate	0.2
Sodium chloride	5
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12

MR-VP (Glucose phosphate broth)

Composition	Grams per litre
Buffered peptone	7
Dextrose	5
Dipotassium phosphate	5
Final pH (at 25°C)	6.9±0.2

Simmon's citrate agar

Composition	Grams per litre
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15
Final pH (at 25°C)	6.8±0.2

Luria Bertani agar

Composition	Grams per litre
Casein enzymatic hydrolysate	10
Yeast extract	5
Sodium Chloride	10
Agar	15
pH after sterilization (at 25 °C)	7.4±2

Urea agar base

Composition	Grams per litre
Peptic digest of animal tissue	1
Dextrose	1
Sodium chloride	5
Di-sodium phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15
Final pH at 25° C)	6.8±0.2

Mueller Hinton agar

Composition	Grams per litre
Meat, infusion solids from	2
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17
Final pH (at 25°C)	7.3±0.1

9.2 Reagents and Buffer

All the reagents and buffer utilized for molecular work were prepared in milli Q nuclease free water.

Glycerol for preservation of culture

Composition	
Glycerol	30%

Kovac's indole reagent

Composition	**Per 100 ml**
p-dimethylamino benzaldehyde	5gm
Amyl alcohol	75ml
Hydrochloric acid, concentrated	25 ml

Methyl red

Composition	**Per 500 ml**
Methyl red	0.1 gm
95% ethyl alcohol	300 ml
Distilled water	200 ml

Sodium deoxycholate reagent

Composition	
Sodium deoxycholate	0.5%

SDS lysis buffer for DNA extraction

Composition	
Sodium dodecyl sulphate	0.5mM
Tris base	20mM
Ethylene Diamine tetraacetate	1% W/V
Final pH (at 25°C)	7.4±0.2

Sodium citrate

Composition	
Sodium citrate	0.5M

Phenol: choloroform: isoamyl alcohol

Composition	
Phenol	25 parts
Chloroform	24 parts
Isoamyl alcohol	1 part

Phosphate Buffer saline

Composition	Grams per litre
Potassium dihydrogen phosphate	0.34
Di-potassium hydrogen phosphate	1.21
Sodium chloride	8.0
Final pH (at 25°C)	7.4±0.1

TAE buffer (50X)

Composition	Per litre
Tris base	242 gm
Glacial acetic acid	57 ml
0.5M EDTA	8.0 ml
Final pH (at 25°C)	7.4±0.1

6X loading dye

Composition	
Bromothymol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

9.3 Biochemical tests

Standard biochemical tests were utilized to characterize bacterial pathogens and description is given below.

Indole test

Kovac's indole test was used to detect ability of members of Enterobacteriaceae to degrade tryptophan into indole via tryptophanase enzyme [275]. Indole test is one of battery of test utilized to distinguish members of *Enterobacteriaceae*. Briefly, pure culture of bacterial isolate was inoculated into 5 ml of peptone water and incubated at 37°C for 24-48 hours. After incubation, five drops of Kovac's indole reagent were added to the culture. A positive indole test is shown by development of cherry red color and absence of cherry red color indicates a negative reaction.

Methyl red test

Methyl red test was used to detect mixed acid fermentation (succinic acid, lactic acid and acetic acid) by *Enterobacteriaceae*. Pure culture of test strains were inoculated into MR-VP (glucose phosphate) broth and incubated for 24-48 hours at 37°C. Methyl red indicator was added to the incubated cultures and development of red color indicated a positive result, while yellow color or no color indicated a negative test results.

Voges Prausker's test

Voges Praukser's test was performed to verify butyric acid fermentation in members of *Enterobacteriaceae*. Similar to methyl red test, pure bacterial culture was inoculatedinto MR-VP (glucose phosphate) broth and was incubated for 24-48 hours at 37°C. After ambient incubation, 1 ml of 40 potassium hydroxide and few drops of 5 α-naphthol were added to culture. Culture tubes were incubated aerobically at room temperature for 20-25 minutes. Development of red colored ring indicated a positive result.

Simmon's citrate test

Simmon's citrate test was used to check ability of bacteria to utilize citrate as sole source of carbon. Pure culture of bacteria were streaked onto Simmon's citrate slants and incubated for 24-48 hours at 37°C. In case of citrate utilization, color of media was changed from green to blue.

Triple sugar iron agar test

Triple sugar iron agar test detects differential utilization of three sugars present in the media namely; glucose, sucrose and lactose via aerobic and anaerobic processes. Members of *Enterobacteriaceae* were identified from different patterns produced after incubation.

Table 9.1: Interpretation of triple sugar iron agar test for *Escherichia coli*, *Salmonella* spp. and *Shigella* spp.

Organism	**Slant**	**Butt**	**Gas**	**H_2S**
Escherichia coli	Acid	Acid	+	-
Shigella	Alkaline	Acid	-	-
Salmonella	Alkaline	Acid	+	+

Christensen's urease agar test

Urease agar test detects presence of enzyme urease in bacteria. Pure culture of bacteria was streaked on to urease agar medium and slants were incubated at 37 °C for 24 hours. Change of urea media into pink color indicated a positive test.

Cholera red reaction

Yellow golden colonies from TCBS media, were picked and inoculated into alkaline peptone water and incubated at 37 °C for 24 hours. Few drops of concentrated sulphuric acid were added into culture and formation of red color indicated a positive cholera red reaction.

String test

Yellow golden colonies from TCBS media were emulsified in 0.5% sodium deoxycholate solution and bacterial colonies forming a string were designated as positive for string test.

9.4 Interpretive criteria for antimicrobial susceptibility tests

9.4.1 Table Showing antibiotics concentrations on disk, E- strip and interpretive criteria utilized for diarrheagenic *E. coli* study according to ICMR, SOP.

S. No.	Antibiotics	Concentration on disk in µg	Concentration of E-strip in µg	Sensitive S (in mm)	Intermediate I (in mm)	Resistant R (in mm)
1.	Ampicillin	10	0.016-256	17	14-16	13
2.	Cefixime	30	0.016-256	18	15-17	14
3.	Norfloxacin	10	0.016-256	19	14-18	13
4.	Nalidixic acid	30	0.016-256	17	13-16	12
5.	Co-trimoxazole	25	0.016-256	16	15-11	10

9.4.2 Table Showing antibiotics concentrations on antibiotic disk and interpretive criteria utilized for *Vibrio* spp. as per CLSI guidelines for *Enterobacteriaceae* in the present study.

S. No.	Antibiotics concentration in µg)	Phenotypes Zones in mm S	I	R
1.	Cefepime (30)	18	15-17	14
2.	Ceftazidime (30)	21	18-20	17
3.	Ceftriaxone (30)	23	20-22	19
4.	Imipenem (10)	23	20-22	19
5.	Netilmicin (30)	15	13-14	12
6.	Amikacin (30)	17	15-16	14
7.	Kanamycin (30)	18	14-17	13
8.	Tobramycin (10)	15	13-14	12
9.	Streptomycin (10)	15	12-14	11
10	Ofloxacin (5)	16	13-15	12
11	Ciprofloxacin (5)	21	16-20	15
12	Levofloxacin (5)	17	14-16	13
13	Norfloxacin (10)	17	13-16	12
14	Nalidixic acid (30)	19	14-18	13
15	Vancomycin (30)	17	15-16	14

9.5 Gene sequences submitted to NCBI

***Escherichia coli* strain E12579 16S ribosomal RNA gene, partial sequence**

GenBank: KY794916.1 633 bp

FASTA Sequence:

>KY794916.1 *Escherichia coli* strain E12579 16S ribosomal RNA gene, partial sequence

GATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGG
TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTT
CGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCC
AGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACA
CACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTA
TGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAACTCG

***Escherichia coli* bundle forming protein gene, partial cds**

GenBank: KX911251.1 389bp

FASTA sequence:

>KX911251.1 *Escherichia coli* bundle forming protein gene, partial cds

CTTGGTGCTTGCGTGTCTTTTTTAGTTTTAAGATTATTCCGTGACCTATTAATACGGGGGTTTTATA
AGGAAAACAGTTTTTATGGTTTCTAAAATCATGAATAAGAAATACGAAAAAGGTCTGTCTTTGATT
GAATCTGCAATGGTGCTTGCGCTTGCTGCCACCGTTACCGCAGGTGTGATGTTTTACTACCAGTCTG
CGTCTGATTCCAATAAGGCGCAGAATGCTATTTCAGAAGTAATGAGCGCAACGTCTGCAATTAATG
GTTTGTATATTGGGCAGACCAGTTATAATGGATTGAACTCAAATGTTTTGCTTAACACATCTGCTAT
TCCGGATAATTACAAAGATACAACACACAAAAAGACACGCAGCCCC CACGAAAAAA

***Escherichia coli* intimin gene, partial cds**

GenBank: KX911252.1 952bp

FASTA Sequence:

>KX911252.1 *Escherichia coli* intimin gene, partial cds

TATCTTTTCGCTTTTTAACGTCCCCCCCCGAATTTGTGGGCAACATAAGCTAAAAAAAACATTTAT
TATGCTTAGTGCTGGTTTAGGATTGTTTTTTTATGTTAATCAGAATTCATTTGCAAATGGTGAAAAT
TATTTTAAATTGGGTTCGGATTCAAAACTGTTAACTCATAATAGCTATCAGAATCGCCTTTTTTATA
CGTTGAAAACAGGTGAAACTGTTGCCGATCTTTCTAAATCGCAAGATATTAATTTATCGACGATTT
GGTCGTTGAATAAGCATTTATACAGTTCTGAAAGCGAAATGATGAAGGCCGCGCCAGGTCAGCAG
ATCATTTTGCCACTCAAAAAACTTCCCTTTGAATACAGTGCCTTACCACTTTTAGGTTCGGCACCTC
TTGTTGCTGCAGGTGGTGTCGCTGGTCATACAAATAAAATGACTAAAATGTCCCCGGACGTGGCCA
AAAGCAACATGACCGATGACAAGGCATTAAATTATGCGGCACAACAGGCGGCGAGTCTCGGTAGC
CAGCTTCAGTCGCGATCTCTGAACGGCGATTACGCGAAAGATACCGCTCTTGGTATCGCTGGTAAC
CAGGCTTCGTCACAGTTGCAGGCCTGGTTACAACATTATGGAACGGCAGAGGTTAATCTGCAGAGT
GGTAATAACTTTGACGGTAGTTCACTGGACTTCTTATTACCGTTCTATGATTCCGAAAAAATGCTG
GCATTTGGTCAGGTCGGAGCGCGTTACATTGACTCCCGCTTTACGGCAAATTTAGGTGCGGGTCAG
CGTTTTTTCCTTCCTGAAAATATGTTGGGCTATAACGTCTTCATTGATCAGGATTTTTCTGGTGATA
ATACCCGTTTAGGTATTGGTGGCGAATACTGCAGGGGAACACCGCGAGGAGTGTTTTTTTTTATTT
ATTGTAAAAAAGGAGGAGAAGAGGA

***Escherichia coli* ATP binding cassette transporter gene, partial cds**

GenBank: KX911253.1 589bp

FASTA Sequence:

>KX911253.1 *Escherichia coli* ATP binding cassette transporter gene, partial cds

AGACTGTATCATTGATAATTTCTTTCAGAAAAGCATCCAGTTTAATTCTTATTCTCTTGATATCGAA
GAGTTAGATATTAATAAACATAACAATATAAAAACGATGTTACCAGATATAAATATAGGGTTAGG
GCAGTATATAAACAACAATCAATGGTTCTCATCTATTACAGACAGCCATTTTTATTTATCATTATCC
TATAATCTTCTATCGGCTTATGAAGCAAAAATGCAGAATAATAAATTGGATATTGCTAATTATTTA
AAATATATTGAAATGCTTAGTGAGAGGAACAACTACATAATTAATTTGTTCTCGGAAATTATTAAC
TATAAGATAAAAAAATCTCACCTGATGTTGATGCTCGAGAGATATAGGAAGCTCAATAAAGAATA
CGAAATTGCAAAGCGTAAAATGTCAATTGGATTAATATCTGTTCTTGATGTAGAGATGATATATAA
TATTTTACAAAAAATCAGGTTTGATATTGATGTCCTTGAGGAGGAGGAAAGTTTACTGTCAGATAA
AATCTCGAGAGAATATCATGTTCCTGAGAGTGCAATCCCAGACATTACATATCATAAGTTA

***Escherichia coli* heat-labile enterotoxin protein beta chain OS gene, partial cds**

GenBank: KX911254.1 262bp

FASTA Sequence:

>KX911254.1 *Escherichia coli* heat-labile enterotoxin protein beta chain OS gene, partial cds

ACACAAATATATACGATAAATGACAAGATACTATCATATACGGAATCGATGGCAGGCAAAAGAGA
AATGGTTATCATTACATTTAAGAGCGGCGCAACATTTCAGGTCGAAGTCCCGGGCAGTCAACATAT
AGACTCCCAAAAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAATCACATATCTGACCGAGA
CCAAAATTGATAAATTATGTGTATGGAATAATAAAACCCCCAATTCAATTGCGGCAATCAGTATGG

***Vibrio* sp. strain cholera 16S ribosomal RNA gene, partial sequence**

GenBank: KX891575.1 639bp

FASTA Sequence:

>KX891575.1 *Vibrio* sp. strain cholera 16S ribosomal RNA gene, partial sequence

CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTT
GGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGG
TGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAG
ACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGG
GCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCAT
AAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAACTCG

Institutional Ethics Committee (IEC)

Chairman
Professor TC Bhalla
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Member Secretary
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No. IEC/project no-04-2014 Dated: 23.05.2014

Approval of Institutional Ethics Committee

To

Dr.Jitendraa Vashistt Ph.D.
Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology,
Solan, Himachal Pradesh – 173 234

The Institutional Ethics Committee of Jaypee University of Information Technology, Waknaghat reviewed and discussed your application to conduct the research work titled **"Identification and characterization of diarrhoeagenic pathogens in Himachal Pradesh",** hereby assigned IEC/project no-04-2014" in the meeting of the committee held on 23.05.2014 at 11:00 AM in the in the board room at JUIT Wakanaghat Solan. The following Members of the Ethics Committee were present in the meeting.

Professor TC Bhalla; Chairman
Ms. Ambika Sharma, Media Correspondent
Dr. SS Kanwar, Department of Biotechnology, HPU, Shimla; Member
Dr. Omesh Bharti, Epidemiologist (M.A.E), Directorate of Health, Kasumpti, Shimla; Member
Dr. Meenakshi F Paul, Center of Evening Studies, HPU, Shimla; Member
Dr. RS Chauhan Head, Professor & Head, Department of BT & BI JUIT, Waknaghat; Member Secretary

Decision: The committee members after due consideration and discussion approved the project.

The institutional Ethics Committee needs to be informed at regular intervals about:

- Copy of all consent forms, filled and signed
- Any serious adverse event occurring during the study
- A copy of the final report of the study
- The Institutional Ethics Committee has to be informed and permission taken before any changes in the protocol
- Please note that members of IEC have the right to monitor the trial with prior information

Member Secretary

(253)

Patient Consent form

Patient Name- Lakshay s/o Tek chand Registration no. / ID- 20150111 9875

Age - One year Sex- Male ☑ Female ☐

Origion/ Place of residence- Chakkhar, Arki, Solan

Recent visits to places and when- /

Initial diagnosis- C/O- loose stools (10-15 episodes) x1 day
Vomiting CB-4 episodes
No H/O fever, No signs of dehydration
child was apparently well 1 day back when he developed vomitting not blood stained, loose stool watery in consistency not blood stained

Type of diarrhea/ symptoms- Acute GE C No dehydration Period of illness-
No H/O fever
No H/O any abnormal body movement, cough cold

No of diarrheal episodes/ day-

Other clinical presentation-

Type of diet- Breast fed ☑ other ☑

other substituted liquid diet.

Mention other dietary constituent-

Source of water- dalija, daal &
Boiled water

Polio vaccination..... ...yes..✓... no..... .....if yes then when? 18th January, 2015.

Treatment undergone (If any) ~~N/A~~ undergone one operative surgery for ortho.

a) List all medications you are currently taking
ORS, Zn-20, Taxpn, Calpol

Reenu Devi
Signature of patient or patient's representative

Date
19th January 2015

24s

Patient Consent form

Patient Name- Raka

Registration no. / ID- 201408219555

Age - 42 days.

Sex- Male ☑ Female ☐

Origion/ Place of residence- Chaupal, Shimla

Recent visits to places and when-

Initial diagnosis- Acute gastroenteritis no dehydration.

Type of diarrhea- loose motion ✗ watery stools (20-30 times) w/o blood
fever (103°F)

Period of illness- ✗2 days

No of diarrheal episodes/ day- Vomiting (15 times), decreased acceptance of feed ✗1 day,

Other clinical presentation- mild cough after birth.
No similar previous history observed in past

Type of diet- Breast fed ☑ other ☐

Mention other dietary constituent- /

Source of water- Boiled.

Polio vaccination..... ...yes.✓... no.....if yes then when?..... After birth.

Treatment undergone (If any) .....

a) List all medications you are currently taking

.....

.....

.....

Signature of patient or patient's representative

Date 22nd August 2014.

PUBLICATIONS

1. **Thakur N**, Jain S, Changotra H, Shrivastava R, Kumar Y, Grover N, Vashistt J (2018). *"Molecular characterization of diarrheagenic Escherichia coli pathotypes: association of virulent genes, serogroups and antibiotic resistance among moderate to severe diarrhea patients"*. **Journal of Clinical Laboratory Analysis.** Vol. 32(5):e22388. DOI: 10.1002/jcla.22388.
2. **Thakur N**, Changotra H, Grover N, Vashistt J (2018). *"Elucidation of bacterial species during childhood diarrhea through 16S rRNA Illumina Miseq approach"*. **MetaGene**. vol. 16, pg. 234-240. DOI:10.1016/j.mgene.2018.03.012
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2. **Thakur N**, Jain S, Changotra H, Shrivastava R, Vashistt J (2015). Metagenomic Studies in Relation to Diarrheal Diseases. Proceedings of the International Congress on Friedreichs Ataxia and DNA Structure in Health & Disease [All India Institute of Medical Sciences, [New Delhi, India] 11-13 April, 2015].
3. Thakur A, Vaid K, Saxena S, **Thakur N**, Vashistt J, Shrivastava R (2015). Aminoglycosides resistance pattern of *Escherichia coli* isolated from urine samples in various hospital settings of Himachal Pradesh. Proceedings of the National Conference on Emerging Trends in Host-Microbe Interactions [DAV University, Jalandhar (Punjab): 17- 18 April, 2015].
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WORKSHOPS

1. *"Innovation & intellectual property rights"* at Jaypee University of Information Technology, Waknaghat. 9th December, 2016.

2. *"Patent filing and geographical indication"* at Jaypee University of Information Technology, Waknaghat. 27th February, 2017.

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RESEARCH ARTICLE

WILEY

Molecular characterization of diarrheagenic *Escherichia coli* pathotypes: Association of virulent genes, serogroups, and antibiotic resistance among moderate-to-severe diarrhea patients

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Background: Diarrheagenic *Escherichia coli* (DEC) signifies as an important etiological agent of moderate-to-severe diarrhea. This study was primarily focused on molecular identification of DEC pathotypes; their association with serogroups and estimates of resistance profiles against different antibiotics regime.

Methods: Five hundred seventy-two stool specimens from diarrhea patients were investigated for DEC pathotypes. Molecular pathotypes were identified by amplification of virulence genes associated with distinct pathotypes followed by sequencing. Diarrhea is a self-limiting disease, however, severity and persistence of infection suggest antibiotic use. Therefore, AST and MIC were determined against common antibiotic regimen. Correlations between molecular pathotypes and serogroups were analyzed by somatic "O" antigen serotyping.

Results: The present findings reveal incidence of DEC as an etiological agent up to a level of 21% among all diarrheal age groups. DEC infection rate was higher in children. Enteropathogenic *E. coli* EPEC, a molecular pathotype of DEC, was found as a predominant pathotype with highest frequency of 13.7%. Two other molecular pathotypes enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) accounted for 5.7% and 1.3%, respectively for all diarrhea incidences. Serological analysis deciphered somatic antigens O26, O2, and O3 as major serogroups identified among EPEC, ETEC, and EAEC pathotypes, respectively. All DEC pathotypes exhibited high levels of antibiotic resistance except for cotrimoxazole and norfloxacin.

Conclusion: Comprehensive molecular characterization of DEC pathotypes, their incidence estimates, and antibiogram patterns will help in ascertaining better diagnostic and therapeutic measures in management of diarrheal diseases.

KEYWORDS
antimicrobial resistance, childhood diarrhea, diarrhea, diarrheagenic *E. coli*, serogroups

1 | INTRODUCTION

Diarrheal diseases are major cause of morbidity and mortality in low- to middle-income countries and estimated to be second leading cause of mortality among children < 5 years of age, resulting in 0.5 million deaths globally.[1] Sub-Saharan and South East Asian regions account for highest burden of the disease (>72%).[2] Unfortunately, India bears highest toll of the disease which demands acceleration in interventions

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Research Article

ESTIMATION OF *VIBRIO* SPECIES INCIDENCES AND ANTIBIOTIC RESISTANCE IN DIARRHEA PATIENTS

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ABSTRACT

Objectives: Diarrheal diseases are globally important public health concern due to high morbidity and mortality rates among all age groups. Incidences and antimicrobial resistance patterns of *Vibrio* species are either underreported or overlooked in low-to-middle income countries. The present study is a hospital surveillance conducted to estimate the incidences of *Vibrio* infections in diarrhea.

Methods: A total of 572 diarrheal stool specimens were collected from two major hospitals and investigated using bacteriological tests coupled with molecular assays for characterization of *Vibrio* species. Kirby-Bauer's disk diffusion method was utilized to monitor antibiotic resistance against different antibiotic classes.

Results: Biochemical and molecular analysis of isolates revealed that *Vibrio* species accounted for about 5% of diarrheal incidences. Higher rates of *Vibrio* spp. infections were observed among adolescent and adult age groups than children and elderly. Seasonal distribution analysis showed uniform *Vibrio* spp. infections throughout the year; however, frequency peaks were observed during rainy seasons. *Vibrio* spp. showed sensitivity for most antibiotics classes: Quinolones, aminoglycosides, and carbapenems; however, alarming resistance rates were observed against cephalosporins and nalidixic acid.

Conclusion: The present study provides credible estimates of *Vibrio* incidences and resistance pattern in diarrheal patients. Our findings will help in establishing trends in diarrhea etiology and management of severe diarrhea cases.

Keywords: *Vibrio* spp., Secretory diarrhea, Antimicrobial resistance, Childhood diarrhea, Seasonal variations.

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INTRODUCTION

Diarrhea is a multifactorial disease associated with numerous etiological agents which may cause high disease severity and persistence. A vast array of diarrhea associated pathogens includes bacterial, viral, and parasitic agents of which rotavirus and diarrheagenic *Escherichia coli* have been reported as main diarrheal pathogens among children [1]. Increased diarrhea incidences have been reported from the past decade, which implicated recurrent floods and natural catastrophe as main drivers of the communicable diseases [2]. *Vibrio* species signify as important public threat responsible for watery diarrhea in infected humans and are transmitted by fecal- oral route [3]. Globally, *Vibrio* is known to cause approximately three million cases per annum [4]. However, according to the World Health Organization (WHO), officially reported cases represent only 5–10% of the total incidences [4]. Recent cholera outbreaks in Yemen alleged a very high morbidity and mortality incidences in the country [5]. Due to high morbidity and mortality rates associated with *Vibrio* spp. in epidemics and pandemics, continuous surveillance studies should be accelerated in developing nations. However, the national level surveillance for *Vibrio* species is limited to certain outbreaks in India, and there are no reports from most parts of the country [6].

Vibrio, member of family *Vibrionaceae* is a comma shaped, Gram-negative, aquatic bacterium which colonizes small intestine of human [7]. Infections caused by *Vibrio* are typically categorized into *Vibrio* cholera and non-cholera *Vibrio* infections. *Vibrio* spp. causes profuse watery diarrhea and are historically known for worldwide epidemics and pandemics [8]. Symptoms of *Vibrio*-mediated gastroenteritis include rice watery stools, severe dehydration, fever, and vomit. *Vibrio* species express several virulence factors such as toxins (*ctxA, stn, OmpW*, and *toxR*), colonization factors (lipopolysaccharides, flagellar components, outer membrane proteins, hemagglutinins, and *tcpA*), protease (hemolysins, cytolysins, thermolysins, and metalloproteases), and iron acquisition systems [9]. Cholera toxin encoded by *ctxA* gene has been isolated only from clinical strains and is utilized as an epidemiological marker to access toxigenic potential of *Vibrio* strains [10,11].

Clinical guidelines for the treatment of diarrhea caused by *Vibrio* mainly focus on rehydration therapy and its maintenance. Probiotics and other traditional herbs are also utilized as traditional therapy [12,13]. However, use of antibiotics is mainly known to reduce severity and shedding of the pathogen [14]. Isolation of multidrug resistant *Vibrio* species from clinical sources is a major concern. Recent studies have reported a rise in antimicrobial resistance in *Vibrio* species against major classes of antibiotics (aminoglycosides, tetracyclines, and β-lactams) [14,15].

The National Institute of Cholera and Enteric Diseases works as collaborating body with the WHO and depicted underreporting and negligence in cholera incidence surveillance [6]. Till date, seven cholera pandemics have been reported, and the Bay of Bengal was identified as an epicenter for the most recent one [16,17]. Molecular typing of *Vibrio cholerae* in nearby region of Himachal Pradesh revealed predominant ribotype IV and RIII [18,19]. Our previous study elucidated incidences of viral pathogens among moderate to severe cases [20]. The present research was performed as a part of the study to reveal *Vibrio* incidences and coinfection rates in diarrheal disease. Simultaneously, antibiotic resistance profiles of *Vibrio* were also deciphered to understand the

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Elucidation of bacterial species during childhood diarrhea through 16S rRNA Illumina Miseq approach

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ARTICLE INFO

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Bacterial diarrhea
Metagenomics
16S rRNA gene
Gastrointestinal tract
Childhood diarrhea

ABSTRACT

Diarrhea causes a debilitating infectious illness among children, especially in developing countries with higher mortality and morbidity rates. Gastrointestinal (GI) tract is occupied by complex microbial-communities structures and the composition of indigenous microbial-consortia gets altered during diseases associated with GI tract. However, effect of microbial imbalance and proliferation in childhood diarrhea severity is less understood. Therefore, present study is focused to compare composition and diversity of bacterial communities in healthy and diarrhea infants using metagenomic approach.

Stool specimens were collected from two acute and one persistent diarrhea infected infants. A stool sample from a healthy infant of same geographical region was taken as reference for comparative analysis. All four fecal specimens were subjected to 16S rRNA V3 region metagenomic profiling.

Metagenomic analysis of healthy control revealed enrichment with higher proportion of *Firmicutes*, *Bacteroides* and *Proteobacteria* phyla. In case of acute and persistent diarrhea infected infants, collapse of indigenous anaerobic microbial-communities like *Firmicutes*, *Bacteroides*) and aberrant proliferation of facultative anaerobes (*Proteobacteria*) was observed. Moreover, *Klebsiella*, *Haemophilus*, *Rothia*, *Granulicatella*, *Chelonobacter* and *Vibrio* species were identified as key pathogenic lineages in diarrheal samples.

Our findings reveal significant imbalance between intestinal bacteria of healthy and diarrhea subjects and witnessed occurrence of pathogenic bacteria which may be responsible for diarrheal illness.

1. Introduction

Diarrhea disease is a global health concern compromising child survival with second highest morbidity and mortality rates among under age five (Liu et al., 2012). Symptoms of diarrhea pathophysiology include severe dehydration, intestinal inflammation and malabsorption of nutrients (Hodges and Gill, 2010). Alteration in gut microbial population during diarrheal illness constitutes an important factor and its relationship to disease is needed to be addressed (Manges et al., 2010; Greenblum et al., 2012; Yin et al., 2016).

Key evolution of intestinal microbes passages through paleolithic to neolithic period and primitive lifestyle, geographical conditions translated succession of microbial communities (De Filippo et al., 2010; Quercia et al., 2014). In addition, several factors like age, gestation period, mode of birth, diet, pre or pro- biotic formulations, antibiotic therapy and individual's genetic makeup, also have significant impact in shaping gut microbial structures (Jakobsson et al., 2014; Ursell et al., 2013; Benson et al., 2010; Dominguez-Bello et al., 2011; Wu et al., 2011).

Gut microbiome possess a gene catalogue of approximately 576 gigabases sequence, approximately 150 times larger than human (Kurokawa et al., 2007) and is engaged in housekeeping and putative functions of GI tract (Qin et al., 2010). Human gut microbiome is a complex microbial consortium and it is estimated to have 10 times more cells than human host (Luckey, 1972; Qin et al., 2012). *Bacteriodes* and *Firmicutes* constitute > 90% of gastrointestinal niche (Eckburg et al., 2005; Mariat et al., 2009). GI tract disorders like intestinal bowl disorder, Crohn's disease and other metabolic dysfunctions like obesity, diabetes leads to anomalous reduction of indigenous anaerobes and proliferation of pathogenic microbial communities (Qin et al., 2012; Rosenbaum et al., 2015; Lewis et al., 2015).

Etiological agents of diarrhea comprise vast array of infectious agents; bacteria, viruses and parasites. Custom microbiological methods are routinely utilized for detection and characterization of causative agents of diarrhea. However, conventional methods lack sensitivity and specificity for fastidious and anaerobic pathogen and approximately 40% of the diseased cases couldn't report any pathogen (Suau et al., 1999; Dutta et al., 2001; Huijsdens et al., 2002; Tannock, 2002;

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Research paper

Predominance of unusual rotavirus G1P[6] strain in North India: An evidence from hospitalized children and adult diarrheal patients

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ABSTRACT

Group A Rotavirus remains the leading cause of gastroenteritis in children and accounts for 0.2 million fatalities each year; out of which, approximately 47,100 deaths occur in India. In adults also, rotavirus is reported to be responsible for diarrhea severe enough to require hospitalizations. India has recently introduced rotavirus vaccine in the Universal Immunization Programme and Himachal Pradesh became the first Indian state to implement this project. This study is an attempt to provide the pre-vaccination data on rotavirus gastroenteritis burden and circulating genotypes in Himachal Pradesh, India. A total of 607 faecal specimens (247 children ≤5 years, 50 older children and 310 adults) from hospitalized diarrheal patients from Himachal Pradesh, India were screened for rotavirus using ELISA and RT-PCR. The positive samples were further G/P genotyped using semi-nested PCR. Rotavirus was detected in 25.2% and 28.3% of samples with ELISA and RT-PCR, respectively. In children, rotavirus frequency was significantly high with positivity in 49.0% cases whereas 14.0% adult samples have rotavirus in them. Genotyping of the positive samples revealed predominance of G1 (66.0%) and P[6] (66.7%) genotypes. The most common G and P combination was G1P[6] (62.8%) followed by G1P[8] (16.5%), G9P[6] (7.4%) and G12P[6] (5.0%). Molecular analysis reveals the belonging of P[6] strains in Lineage 1a. This pre-vaccination data on rotavirus prevalence and diversity would be helpful for assessing the affect of vaccination on the disease burden and its comparison with post-vaccination data of circulating genotypes would help in studying the effect on diversity of rotavirus strains possibly due to vaccine selection pressure.

1. Introduction

Group A Rotavirus (RVA) associated illness is responsible for significant morbidity and mortality in infants and children aged below 5 years. Globally, RVA is responsible for around 2 million hospitalizations (Banajeh and Abu-Asba, 2015) and approximately 0.2 million deaths annually (Tate et al., 2016). India is the leading nation in terms of number of RVA associated deaths and accounts for 22% of the global mortality due to rotavirus (Tate et al., 2016). In a recent report from India, John and co-workers have shown that RVA was responsible for 78,583 deaths, 872,000 hospitalizations, 3,270,000 outpatient visits and 11.37 million episodes of diarrhea in children (John et al., 2014). These figures reflect the substantial burden of rotavirus disease in Indian population. Although RVA is recognized as a pathogen responsible for diarrhea associated illness among children at large, but reports have documented its ability to cause diarrhea in adults also. Although, the data related to RVA gastroenteritis in adults is limited, there are some studies which documented the role of RVA in diarrhea in adults also (Anderson and Weber, 2004; Nakajima et al., 2001).

RVA is a RNA virus, member of Reoviridae family and has a segmented genome comprising of 11 segments of double stranded RNA. The genome encodes for six structural (VP1, VP2, VP3, VP4, VP6, VP7) and six non-structural proteins (NSP1-NSP6). Rotaviruses are classified serologically into eight groups (A-H) on the basis of VP6 reactivity (Estes and Greenberg, 2013; Matthijnssens et al., 2012). Group A, B and C rotaviruses are reported to infect humans where group A rotaviruses are accountable for majority of the morbidity and mortality in humans. The VP4 and VP7 genes are used to genotype rotaviruses in the G/P-genotyping system. The VP7 nucleotide sequences specify for G-type (**G**lycoprotein) whereas VP4 sequences specify for the P-type (**P**rotease sensitive protein) (Matthijnssens et al., 2011). According to recent reports, 32 G-types (G1-G32) and 47 P-types (P[1]-P[47]) of RVA have been identified (RCWG, 2016; Li et al., 2016). The most common genotypes isolated from humans are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (da Silva Soares et al., 2014). However, a number of other genotypes have also been detected in significant fractions from time to time (Esona et al., 2010; Gurgel et al., 2014; Piekarska et al., 2015). The

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ORIGINAL ARTICLE

Prevalence of rotavirus, norovirus and enterovirus in diarrheal diseases in Himachal Pradesh, India

Swapnil Jain[1] · Nutan Thakur[1] · Neelam Grover[2] · Jitendraa Vashistt[1] · Harish Changotra[1]

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Abstract Diarrheal diseases are responsible for a significant proportion of mortality and morbidity all around the globe. The contribution of viruses to gastroenteritis incidences in humans is well established. In the present study, we have studied the prevalence of rotavirus, norovirus and enterovirus in Himachal Pradesh, a north Indian state. A total of 287 (111 children and 176 adults) stool samples of gastroenteritis patients were screened for the viruses using RT-PCR method. 34.5 % samples were positive for the viral pathogens of gastroenteritis. Rotavirus was the predominant virus detected in the study with 49.5 and 14.8 % positivity in children and adults, respectively. Enterovirus was present in 5.6 % cases whereas norovirus had least prevalence (1.4 %). Co infection (rotavirus and enterovirus) was witnessed at the prevalence rate of 0.6 %. Among different age groups, the prevalence of studied viruses was highest in the children belonging to the age groups of <5 years. Rotavirus infections were found to be significantly associated with vomiting and trend of higher rates of fever and dehydration was seen in children along with diarrhea. Seasonal distribution shows circulation of diarrheagenic viruses throughout the year. This is the first report of prevalence of various diarrheagenic viruses circulating in this region. The outcome of the study from this cohort provides a baseline data which can be used to design the preventive strategies in the otherwise unexplored state of Himachal Pradesh.

Keywords Viral Gastroenteritis · Diarrhea · RT-PCR · Rotavirus · Norovirus · Enterovirus

Introduction

Diarrhea is a major healthcare concern as it is responsible for a significant proportion of morbidity and mortality with 0.8 million annual fatalities to its account all around the globe [13]. The role of viral pathogens in diarrhea is well established and several diarrheagenic viruses have been reported till date [7, 38]. The disease burden associated with the viral gastroenteritis has been well documented in the studies from all over the world [30, 35]. Rotavirus (RV), the RNA (segmented double stranded) virus belonging to the family of *Reoviridae* is the major viral agent which is responsible for diarrhea basically in children below the age group of 5 years. This deadly virus is responsible for around 0.5 million deaths in this age group [29]. However, it has been found to cause diarrhea in adults and elderly also [2]. The underdeveloped and developing nations of the world bear the highest rotavirus associated disease burden [10, 37]. Noroviruses (NoVs) are also major viral pathogen associated with diarrhea among all age groups in humans. These single stranded positive sense RNA viruses are the leading cause of gastroenteritis outbreaks and are important agents involved in sporadic diarrheal cases [15, 23]. Out of the six (GI–GVI) genogroups of NoVs, GII is accountable for majority of the NoV associated gastroenteritis cases, followed by [14, 40]. Enteroviruses (EVs) are single stranded RNA viruses, belonging to the *Picornaviridae* family and they are

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Computational Tools; Indispensable Armamentarium of Medical Biotechnology

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Abstract

Objective: Computational infrastructure of medical biotechnology provides insight into elements of genomics, proteomics for understanding diseases and biological systems in a more comprehensive and systematic way. The present study exclusively focuses on role of computational and bioinformatics tools employed in troubleshooting heterogeneous data of biology origin. **Method and Analysis:** Methodology adopted for study included analysis of online literature through Google Scholar, Pub-med, and Science Direct by searching with comprehensive input like computational tools, biotechnology, proteomics, genomics, drug design and discovery, metagenomics to include all relevant research/review articles within period from 1980 to 2016. Collection, inclusion and exclusion of published articles, review and reports were critically assessed and discussed within line of available databases. Furthermore, bibliography of relevant research articles was taken into consideration. **Findings:** Major conventional biological databases i.e., National Centre for Biotechnology Information (NCBI) and its subset PubMed, not only provide and exchange biological sequence information but also allow comprehensive analysis for sequence alignment and comparisons to find out disease biomarkers like Single Nucleotide Polymorphisms (SNPs). Likewise, other computational tools and servers are developed to be specific for organism, disease, biological pathway, microbial resistance and drug design. Use of currently available computational techniques provides rapid cross-reference search with higher accession of the sequences with statistical approach. However, a significant challenge in this field is to organize and provide user access, readability and skill sets to heterogeneous data repositories like databases and web servers as well as to keep data privacy. These temporal processes significantly enhanced the vision and understanding about cryptic biological processes. **Novelty/ Improvement:** Present study exclusively targets computational tools employed in deciphering complex biological systems through use of algorithms and software which made available large data repositories in public domain. Current study will enhance and compile amalgam of computational and bioinformatics pipelines engaged in vast perspectives of basic and applied biology.

Keywords: Biotechnology, Bioinformatics, Computational Tools, Genomics, Proteomics, Web-Servers

1. Introduction

Computational tools are invaluable for researchers and scientists indulged in various fields across the globe for data extraction, analysis and integration of complex heterogeneous data sets. Amalgam of classical biological techniques with high throughput computational technologies leads to beginning of a new stratum for precise understanding and answers of complex biological problems. In this regard, advances in computational biology and bioinformatics have made access to vast genome, transcriptome and proteome data which are being utilized for a variety of biomarker identification, nano-device engineering and drug design etc[1,2]. In the present paper we are discussing the usage of different computational approaches which render the information of biological processes systematic and more useful.

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Next Generation Sequencing: An amalgam of disease biology and computer algorithms for comprehensive exploration of Infectious agents.

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***Abstract*— Genome of an organism shapes its phenotype and hence forms the basis of each and every cellular process implicated in well being to diseased one. Traditional diagnosis of an infectious disease was laid on the phenotypic and genotypic expression level of a pathogen. Limiting constrains of these diagnostic approaches include time expenditure, laborious and uncertainty in identification. However, current scope of biological/medical diagnosis is undertaking from phenotype to genotype with the help of rapid speed next generation sequencing platforms. Several sequencing platforms are established to reveal the basis of diseased states by exploring DNA sequence information of disease causing organisms. Next generation sequencing platforms deciphered genome-wide sequence readout to analyze mutations, polymorphism, and total biodiversity of a microbial community of pathogens. The genome analysis toolkit involves algorithms for sequencing read alignment, assembly algorithms and software, coverage calculators and single nucleotide polymorphism (SNP) calling. Advent of software packages and algorithms for next-generation sequencing data keeps changing on a rapid pace. This paper analyzes the currently available standard methods and approaches of next generation sequencing technology employed in infectious disease analysis.**

Keywords—Next generation sequencing, computational algorithms, genome, infection.

I. INTRODUCTION

Emergence of next-generation sequencing (NGS) techniques provided world with objectives of disease diagnosis, pathogen detection and decoded mysteries of infectious biology. The genetic material deoxyribonucleic acid (DNA) makes the basis of organisms and thus establishes DNA as imperial basis of research. Decoding DNA through high speed NGS technology platforms like Roche 454, ABI SOLiD and Illumina/Solexa platforms provides error free short read lengths with high coverage as compared to conventional Sanger sequencing technology. These high throughput technologies enabled numerous groundbreaking discoveries in genetic diseases, pathogen detection and evolutionary relationship among organisms. Before discussing about the algorithms and software packages of NGS, it is important to know history about development of NGS platforms. Fig. .1 describes the various generation of sequencing platforms till date. Basically, DNA sequencing methods combine principles of molecular biology, nucleotide chemistry and computer science.

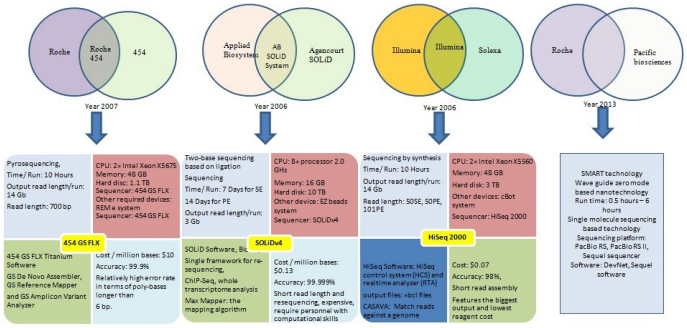

Fig.1. Comparative analysis of various sequencing platforms.

Beginning of this discipline began at the Medical Research Council, Cambridge, to decipher nucleotide sequences in 1950s. Dr. Frederick Sanger's group devised methods for RNA sequencing in 1960s [8] followed by chain-termination method for DNA sequence analysis in 1977. Chain termination method involved addition of chemically modified di-deoxynucleotides (2'-H group instead of 2'-OH group) into reaction mixture resulting in termination of growing nucleotide chain. DNA fragments with different sizes were visualized on thin slab poly-acrylamide gel in adjacent lanes for each base. Following X-ray exposure fragments positions were identified through radio-labeled ^{32}P, initially incorporated into nucleotides. Limitations of Sanger' method comprise being labor intensive, manual errors and high costs of reaction mixtures.

Subsequent advancements like *E. coli* Klenow fragment polymerase and use of radio-labeled ^{35}S resulted in more uniform addition of nucleotides providing longer read lengths, improved separation on gel and sharp visualization of DNA fragments. Further to increase scalability of experiments attempts were made to make certain processes automated i.e. pipetting of sequencing reactions, reading of the autoradiograph banding patterns. Replacing radio labeled nucleotides with fluorescent one was a major breakthrough in sequencing technology. In Leroy Hood's laboratory attempts were made to replace cumbersome process of radiolabel with a fluorescent DNA sequencing instrument. Method

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