

Determination the Effect of ST Enterotoxin Isolated from Enterotoxigenic *Escherichia coli* Strains on Colon Cancer from Diarrhea Patients in Basra Hospitals

Lina Abass Naser , Kawther H. Mahdi , Mohammed Abdul-Imam Almazini

Department of Biology, College of Science, Basra University, Iraq

ABSTRACT: A total of (375) stool samples and rectal swabs were collected from patients in all age group suffering from watery diarrhea , the collection from patients admitted to seven main hospitals in Basra city . From (375) patients, 167 females , and 208 males were involved in this study . Specimens were cultured on Mac Conkey agar plates and incubated at 37°C overnight. From each plate lactose fermenter colonies with typical appearance of *E. coli* were selected and inoculated on Eosin methylene blue (EMB) agar medium for preliminary characterization , the colonies showing characteristic metallic sheen on EMB agar were picked up and considered as presumptive *E. coli*. All presumptive *E. coli* , identified by conventional biochemical tests for *E.coli* bacterial identification, API 20E system .

The results showed that (115) was diarrhoea samples were positive for *E.coli*, 13 out of 115 were positive for Enterotoxigenic *E.coli* (ETEC) .

PCR results revealed that out of 13 positive ETEC isolates, only two isolates showed positive results for heat labile (*Lt*) , 6 positive for heat stable (*St*) and 5 were positive for both genes . Sequencing some DNA fragments of the expected length obtained after PCR were purified.

The toxicity of ST toxin was examined through experimental animals (Balb/c) mice. Four mice from six injected intraperitoneally (I.P.) with 0.25 ml crude ST enterotoxin were died after 24 hours of injection; six mice that have been administered ST crude enterotoxin orally were survived.

The abdomen was opened and the entire intestine all groups were removed for the interpretation of toxin effect and immediately fixed in 10% formalin in overnight at 4°C . The results showed macroscopically changes between normal (control) and injected mice with ST toxin , degenerated features with stomach and intestine compared to normal mouse.

Cell line viability assay using HCT 116 (human colorectal cancer cells) were used to investigate the effect of toxin ST on the viability of the colon cancer cells.

Our results indicated that bacterial enterotoxins could inhibit DNA synthesis in human colon cancer cells and stopped poliferation of the cells. The viability decreased from 94% to 38% when the HCT116 cells in one well were treated by toxin ST directly (1 ml ST in concentrate 0.04 mg/100 ml).

I. INTRODUCTION

The most important causes of intestinal infections in the world's leading Health problems are diarrheal diseases . "Two to four billion episodes of infectious diarrhea have been estimated to occur annually in developed countries, resulting in 3 to 5 million deaths" . {half of all cases of diarrhoea are due to bacteria that cause disease by producing one or more enterotoxins} (Sánchez and Holmgren, 2005).

Among the bacterial pathogens, diarrheagenic *E.coli* is the most caused by a wide range of microbial agents (Asadi *et al.*, 2010) . There are at least six established categories of diarrheagenic (DEC) : { enteropathogenic *E.coli* (EPEC) , enterohemorrhagic *E.coli* (EHEC) , enterotoxigenic *E.coli* (ETEC) , enteroinvasive *E.coli* (EIEC) , enteroaggregative *E.coli* (EAEC) , and diffusely adhering *E.coli*(DAEC)} (Ghilardi *et al.*,2001).

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

The most important cause of diarrheagenic *E. coli* is Enterotoxigenic *E. coli* (ETEC). Infection with {ETEC is the leading cause of traveler's diarrhea and a major cause of diarrheal disease in the developing nations, especially among children} (Rao *et al.*, 2003). {There are two types of enterotoxins, the heat-labile (LT) and the heat-stable (ST)} (Rajkhowa, 2009). Heat-labile enterotoxin produced by ETEC is an immunogenic protein which loses its activity at 60°C for 10 min. while, ST is a heat-stable polypeptide with non-immunogenic properties and is resistant to heat at 100°C for 15 minutes. The ST toxin stimulates guanylatecyclase enzyme leading to increase intracellular cyclic guanosine monophosphate (cGMP) level. This activity leads ultimately to stimulate chloride secretion and / or inhibition of sodium chloride absorption, resulting in net intestinal fluid secretion that leads to diarrhea (Pachauri *et al.*, 2013).

The PCR test is quite sensitive and specific test for identification of ETEC because of there are no suitable biochemical markers by which ETEC could be identified and distinguished from other *E. coli* strains causing diarrhea. PCR can be completed rapidly, giving results such as identification of genes of enterotoxins within one working day (Woodward *et al.*, 1992).

The main causes of death all around the world is colorectal cancer. Many methods and strategies have been used to treat such kind and others of cancer. The traditional methods include{{radiation, chemotherapy and surgery (Pitari *et al.*, 2003)}}. There is approaches have been suggested and developed; method by using *E. coli* toxin, for the treatment of colorectal cancer. It was found that {{*E. coli* produces a toxin stimulates a natural colon process and provoked diarrhea. The toxin also causes a flood of calcium into the affected cells, stopping colorectal cancer cells from replicating rapidly}} (Crawford and Kumar, 2003).

[[There is unexplained inverse relationship between the incidence of colorectal cancer and enterotoxigenic *E. coli* infection (Morgan, 2003)]]. {The ST toxin causes one of the serious forms of food poisoning may be used in the treatment of one of the most deadly types of cancer} (Ferlay *et al.*, 2001; Carrithers, 2003).

Due the importance of these bacteria and the possibility of using the toxin secreted by them in dealing with the most important and the most dangerous types of cancers and due to the lack of studies and research in Iraq, based on that this study designed to:

1. Determining the effect of the ST enterotoxins producing isolates by histological study experimentally using injection mice assays.
2. Clarified the effect of ST enterotoxins on colon cancer cell line viability.

II. MATERIAL AND METHODS

* **Samples collection :** A total of (375) stool and rectal swabs samples were collected from patients in different age suffering from watery diarrhoea. Out of those population 167 were females, and 208 were males. Stool samples were collected from patients admitted to seven main hospitals in Basra city. These hospitals are:

1. Basra General hospital.
2. Al-Sader Teaching hospital.
3. Al-Shefa General hospital.
4. Al-Faaha General hospital.
5. Al-Basra Maternity and Children Hospital.
6. Al-Mawana General hospital.
7. General Child hospital.

* **Isolation of *E. coli* :**

Samples were cultured on MacConkey agar plates and incubated at 37°C for overnight. From each plate seven to ten lactose fermenter colony with typical appearance of *E. coli* was selected and "[inoculated] on Eosin methylene blue (EMB) [[agar medium for preliminary characterization] . " Colonies showing metallic sheen color on EMB agar were picked up and considered as presumptive *E. coli* ".

The purified cultures of *E. coli* were stored in Nutrient agar slant for further

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

identification by biochemical tests. All the isolates were stained by Gram's stain to determine the purity of the isolates (Tsubokura *et al.*, 1995; Forbes *et al.*, 2007). Conventional biochemical identification of *E.coli* isolates dependence by (Bailey and Scott's, 2007).

***Identification by API 20E system:** [Isolates were confirmed by API 20E, test strips were inoculated and incubated according to the instructions provided to the manufacturer's manual (Biomerieux, France)].

*** Serotyping of *E.coli*:**

Serological typing of all positive strains was carried out by slide agglutination with *E. coli* O, H antisera (O169:H41). i.e., [determination of 'O'. The reaction results was recorded (Persson *et al.*, 2007).

*** Genotypic Characterizations of the Isolates :**

*** Genomic DNA extraction:**

Genomic DNA was extracted and purified from bacterial according to the protocol of presto™ Mini gDNA bacteria kit (Geneaid). Procedure of total DNA extraction and purification:

1) Sample preparation :

Bacterial culture (up to 1*10⁶CFU/ml) was transferred to a 1.5 ml microcentrifuge tube. Centrifuge for 1 minute at 15,000 x g discard the supernatant.

Cells lysis (200 µl) of GB Buffer was added to the sediment and mixed by shaking vigorously for 5 second, incubated at 60°C for 10 min or until the sample lysate is clear. During incubation, the tube was inverted every 3 min :

A- DNA binding : '(200 µl) of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously[[Place a GD column was diluted in a 2 ml collection tube .The mixture (including any precipitate) was transferred to the GD column, then Centrifuge at '15,000xg' for 2 minutes" 2 ml collection tube containing the flow place the GD column was discard in a new 2 ml collection tube]].

B-Wash : "(400 µl) of W1 buffer was added to the GD column. Centrifuge at 15,000 xg for 30 second" The flow through was discarded and the GD column was placed again in the 2 ml collection tube .Then add 600 µl of wash to the GD column. Centrifuge at 15,000 xg for 30 second , The flow through was discarded and the GD column was placed again in the 2 ml collection tube Centrifuge again 15,000 xg for 3 minute to dry the column matrix" .

2-DNA Elution :

The dried GD column was transferred to a clean 1.5 ml micro-centrifuge tube . Then add "100 µl" of pre-heated elution buffer to the center of the column matrix , Let stand for minutes to allow elution buffer to be completely absorbed centrifuge at 14-16,000 xg for 30 second to elute the purified DNA[[(DNA was stored at - 20° C)].

*** Visualization of total DNA on agarose gel.** DNA was visualized by agarose gel electrophoresis (Sambrook and Russel, 2001). Agarose gel was prepared as follow:

1. Agarose of was 0.2 gm dissolved with 25 ml of TBE buffer (1 X) in a beaker, the mixture was melting by hot plate agarose, then 0.2 µl of ethidium bromide was added , and mixture was left to cool for 50 - 60°C.
2. Melted agarose gel was poured into the casting tray of the electrophoresis apparatus and the comb was placed at one end of the tray to form wells for loading .
3. After the agarose solidified at room temperature, the cast was pushed gently upwards out of the tray, and the comb was lifted up out of the gel. The gel was gently replaced on the electrophoresis tray.
4. The electrophoresis apparatus was filled with TBE buffer until the entire gel surface was covered with the buffer.
5. The DNA of 9 ml sample was mixed with 3 µl of bromophenol blue and the mixture was transferred carefully in the wells of agarose gel.
6. The gel was subjected to equal electric current by connecting to a power supplier.
7. The cathode was connected to the well sides of the tray while the anode on the other side and the gel was run at 60V until the promo phenol blue tracking dye migrated to the end of the gel.
8. DNA bands were detected and examined under UV. Transilluminator.

*** Polymerase Chain Reaction (PCR) Using *ST* and *LT* Gene Primers:**

Two gene specific primers sets were used for detection of LT and ST genes in *E. coli* strains, sequence of primers specific for LT and ST genes were given In table (1) , Selected primer-sets for PCR of pathogenic *E.coli* (Kim *et al.*, 2010). Table (2): Selected primer-sets for PCR of pathogenic *E.coli* (National Institute of Health ISS, 2013).

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

Target Genes	Primers sequences (5-3)	Amplicon size (bp)	Ref.
StR	TCA CCT TTC CCT CAG GAT GC	179	(Kimata <i>et al.</i> ,2005)
StF	ATA TTA TTA ATA GCA CCC GG		
LtR	GCA CAC GGA GCT CCT CAG TC	218	(Vidal <i>et al.</i> ,2004)
LtF	TCC TTC ATC CTT TCA ATG GCTTT		

Table (1), Selected primer-sets for PCR of pathogenic *E.coli* (Kim *et al.*, 2010).

Target Genes	Primer name	Primers sequences (5-3)	Amplicon size (bp)	Reference
ST	STF	GCTAAACCAGYAGRGTCCTCAAAA	147	(National Institute Of Health ISS,2013)
	STR	CCCGGTACARGCAGGATTACAACA		
LT	LTF	TTCCCACCGGATCACCAA	62	
	LTR	CAACCTTGTGGTGCATGATGA		

Table (2): Selected primer-sets for PCR of pathogenic *E.coli* (National Institute of Health ISS, 2013).

* Sequencing of ST gene

The PCR products sequencing and its preparation were done according to (NICEM / USA) by (ABI3730XL APPLIED BIOSYSTEMS) sample volume [15 ul] and for primer concentration for each (10 pmol/ul) and volum (15 ul) then sending to (NICEM/USA) for sequencing.

* Purification of PCR product:

PCR product was purified by Gel /PCR . DNA Fragments Extraction kit (Geneaid), which is illustrated in table (3).

Table (3): Reagent and volume Gel /PCR DNA Fragments Extraction kit.

Reagent	Volume
Elution buffer	6 ml
DF buffer	80 ml
DF Column	100 column
W1 buffer	45 ml
Wash buffer (add ethanol)	25 ml
2 ml Collection tube	100 tube

Procedure of purification DNA from PCR product:

1- Sample preparation :

A volume of (100 µl) of a reaction product was transferred to a 1.5 ml microcentrifuge tube, and then 5 volume of DF buffer was added to 1 volume of the sample and mix by vortex.

2- DNA binding :

DF column was placed in a 2 ml collection tube. The sample mixture was transfer from 1 step to the DF column and Centrifuge at 15,000 xg for 30 second. The flow through was discarded and the DF column was placed again in the (2 ml) collection tube. Centrifuge again '15,000 xg' for 3 minute to dry the column matrix.

3- Washing :

[(600 µl) of wash buffer]] (make sure absolute ethanol was add) was add into the center DF column and let for 1 minute. Centrifuge at 15,000 xg for 30 second. The flow through was discarded and the DF column was placed again in the 2 ml collection tube.

4- DNA Elution :

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

[The dried DF] column was transfer to a new 1.5 ml microcentrifuge tube[[Add 20 -50 μ l of elution buffer into the center DF column matrix]]Let stand for 3 minutes to allow elution buffer to be completely absorbed , Centrifuge at 15,000 xg for 2 minutes to elute the purified DNA. (DNA was stored at - 20° C).

Sequencing Some DNA fragments of the expected length obtained after PCR were purified with the LaboPass™ PCR kit (Hokkaido System Science, Sapporo, Japan), eluted in 10 mM Tris-Cl (pH 8.5) and directly sequenced with PCR primers. Sequencing was carried out with Bigdye® Terminators on an ABI 3730 genetic analyzer (Applied Biosystems) according to the manufacturer's protocol. The nucleotide sequences were submitted to 'BLAST' [[sequence similarity searching at the National Center for Biotechnology Information]] (NCBI) database and were aligned with the gene sequences available from the Gene Bank database (Kim *et al.*, 2010).

* Extraction of toxin:

For enterotoxin production, *E. coli* strains were inoculated into 250 ml Casamino Acids-yeast extract (CAYE) broth and incubated at 37°C in a rotatory incubator-shaker at 150 rpm for 18 hr. The cultures were then put in cool centrifuged 40C at 6,000 rpm for 30 min, and the respective supernatants were carefully separated and poured into new vials and stored in room temperature after adding 5 μ g gentamicin in each vial. To detect the purity of the toxins, streaking of supernatant was applied on EMB agar and incubated at 37C for 24 hours (Evans *et al.* (1973); Mohd *et al.*, 2012).

*Detection of heat stable toxicity by Mice assay injected:

Mice were obtained from Biotechnology Research Center college of Veterinary medicine, University of Tehran (Iran). Their age range 2days-12 weeks and they were used to carry out the investigations.

* Procedure :

Detection of (STa): six of two-days-old mice were used, all injected I.P. with ST. To study the effect of toxin, eighteen numbers of 12 weeks old mice (males and females) were divided into three groups namely A, B and C consisting of six mice in each group.

In group A: six mice injected intraperitoneally (I.P.) with 0.25 ml supernatant containing crude enterotoxin ST using syringe. In group B: six mice were administered orally with 0.25ml of crude enterotoxin using separate micropipette and all groups were kept in incubation at 37C for 24 hours to observe toxic effects. The mice of group C were kept as control in the same environment (Giannella, 1976; Mohd *et al.*, 2012).

The abdomen was opened and the entire intestine and liver for all groups were removed for the interpretation of toxin effect according to (Giannella, 1976). Mouse intestinal and liver samples were immediately fixed in 10% formalin for overnight at 4°C.

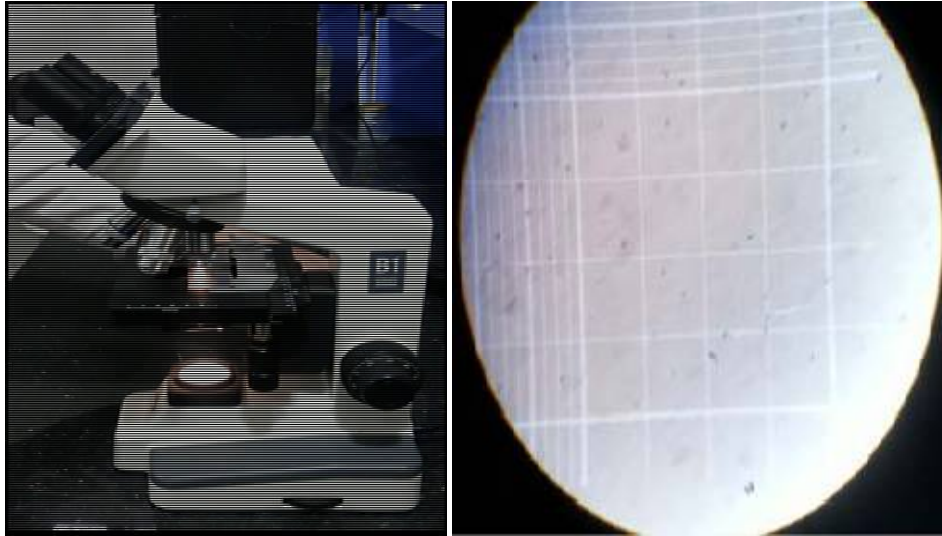
* Histological study:

All routine histopathology examination work was applied in the Medicine College Department of Histopathology University of Basra.

After fixation, the tissue block is embedded in paraffin sections using a regular embedding sectioning and staining of the tissue Martials then cut in a microtome to the desired thickness (approximately 5 microns) and affixed onto the slide(Roebroek *el al.*,2002).

* Cell line viability assay:

All work has been done in the Central Laboratory, cell culture unit, College of Veterinary Medicine, University of Tehran (Iran).



Figure(1): Hemocytometer

*** Protocol for Culture:**

*** Protocol 1 :**

- In this study, HCT 116 (human colorectal cancer cells) were used to investigate the effect of toxin ST on the viability of the cells.
- The HCT 116 cells were cultured in 6-well plate with rose park memorial institute (RPMI), 10% fetal bovine serum (FBS) and 5% CO₂.
- When the confluence of each well reached 90% (approximately 1 million cells in each well), the cells were treated by toxin ST for 24 hours.
- Six different concentration of toxin ST tested on the cells of each plate (10⁻¹ to 10⁻⁶).
- The viability of the cells were examined by Trypan-blue staining.

*** Protocol 2 :**

- Six different concentrations of toxin ST tested on the cells of each plate (1/2 to 1/64).

*** Protocol 3 :**

- The HCT116 cells in one well were treated by 1.5 ml toxin ST Cell lines were obtained from the American Type Culture collection (ATCC). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, fed with fresh medium every three day, and split when sub confluent.

In this study we used different types of cell lines:

1. Colorectal Cancer Stem Cell HCT116 (ATCC) American Type Culture Collection.
2. Vero cell (ATCC) American Type Culture Collection.
3. Normal colon cancer cell line (control).

All the work was done under sterile condition in the laminar airflow hood.

Briefly, cells grown in six-well plates were treated with different concentrations of ST crude enterotoxin and incubated at 37°C for 24 h. To assess cell viability, this done by mixing 0.2ml of cells and 0.2ml of the stain trypan blue loaded onto a hemocytometer. Only the dead cells will take up the dye and appear blue under the microscope while viable cells exclude the dye and appear white, which makes it very easy to distinguish dead cells from viable cells. Neubaur counting chamber hemocytometer was filled with one drop of suspension and excess fluid was wiped off by a filter paper. Cells were counted in the four large squares (Zhu *et al.*, 2011).

□ **[Calculate the number] of cells per ml and total number of cells''**

Cells/ml = number of cell counted/number of squares counted x 10⁴ x dilution factor

Total cells = cells/ml x vol. of original suspension

The percentage of viable cells is calculated as:

% viability = (number of viable cells counted/ total number of cells counted) x 100

*** Statistics analysis:**

The results were evaluated by using the chi square test to determine the statistical significance of the data, and significance was assumed at $P < 0.05$.

III. RESULTS AND DISCUSSION

*** Bacteriology study :**

*** Isolation and identification of *E. coli* isolates :**

In the present study, as it is displayed in **Table (4)**, (115/375) 30.67 % of the stool samples were positive for *E.coli* and (13/115) 11.30% were positive for ETEC.

Table(4):distribution of patients infected with *E.coli* and ETEC in both sexes

Gender	ETEC (%)	Other than ETEC (%)	Total (%)
Female	5 (38.46)	44(43.14)	167 (44.53)
Male	8 (61.54)	58 (56.86)	208 (55.47)
Total (%)	13 (11.3)	102 (88.7)	115 (30.67)
Total(%)			375 (100)

With respect by isolating of *E. coli* (30.67 %) this percentage almost semi approach to (Quiroga *et al.*, 2000; Bublitz *et al.*, 2014) who recorded incidence of *E. coli* in diarrheal patients 43% and 33% respectively, but it wasn't consistent with others like (Biyikli *et al.*, 2004) who recorded higher percent than our study while (Daniel *et al.*, 2005) recorded lower than as the incidence of *E.coli* 63% and 7.3% respectively.

This study focused on the isolation and identification of ETEC infection as there is a lack of studies in Iraq to isolate these important pathogens. The results indicated that 13 out of 115 (11.3%) *E.coli* was ETEC. This result agrees with (Chapman and Mitchelmore, 1988; Qadri *et al.*, 2005). The researchers logged incidence of ETEC 12% and 11.3% respectively, while it disagrees with other researches, who recorded lower frequently compared with our results, since Bueris *et al.*(2007) recorded 3.7% while Nweze (2010) recorded 4.2%.

However, the situation in Iraq " calls for more concern" The possible explanation for high frequency of ETEC in Iraq is poor sanitation, education and personal hygiene were plays a major role in preventing the spread of ETEC infection. The lack of clean water for example play very important role in spreading of many diarrheal organisms, many researchers (Bryce *et al.*, 2005; Qadri *et al.*, 2005) showed that inadequate sanitation is the world's largest cause of illness and it has been estimated that more than two million people, most of them children, die every year from water-borne infections (Bryce *et al.*, 2005).

*** Diagnosis of ETEC isolates :**

Colony morphology on eosin methylene blue agar (EMB) and MacConkey agar (MCA) were as green metallic sheen, bright pink or red respectively. Gram negative rod shaped isolates on Gram staining during microscopic examination. In all 115 *E. coli* isolates obtained from the diarrheal fecal samples were reused for further study.

*** Biochemical characterization of the isolates :**

E.coli isolated and identified by using conventional biochemical tests and API20E system. A series of biochemical tests selective for *E. coli* were performed with the suspected Gram-negative rod shaped isolates (115 culturally positive samples) and all the suspected isolates produced acid and gas due to the fermentation process in sugar fermentation test. All isolates also showed "Indole positive" Methyl red positive" Voges'Proskauer negative and Citrate negative" .

*** molecular genetic study :**

***DNA extraction :**

The DNA from 115 isolates was extracted and the agarose gel electrophoresis was carried out to check extracted DNA, the bands of total genomic DNA were observed on the gel as shown in **figure (2)**.

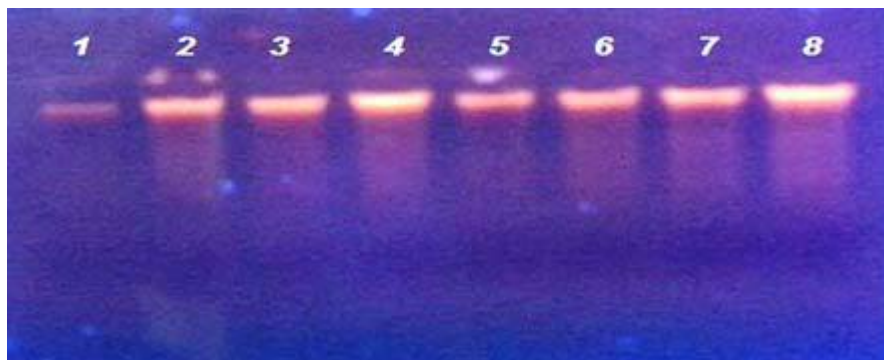


Figure (2): Agarose (0.8%) gel electrophoresis for DNA Bands, (1-8 Lane) = Positive Samples (presence of DNA).

*** Confirmation of ETEC by PCR:**

All the isolates that were identified as *E.coli* by conventional biochemical methods were confirmed by amplification of enterotoxin (*ST* and *LT*) gene.

As seen in **Table 5-6**, the results revealed that among 375 diarrhoeal samples, 13 (3.47)% were positive for *LT* and *ST* gene, 2 isolates positive for *LT*, 6 isolates positive for *ST* and 5 isolates were positive for both gene. The PCR results are presented and illustrated in the **Figure (3, 4)**

Table(5): Isolation of ETEC from the samples of the diarrheal patients

Total No. of sample tested	No.(%) of ETEC positive
375	13(3.47)

Table(6): Percentages of toxins types of the diarrheal patients

Lt	St	Lt/St
2(15.39)	6(46.15)	5(38.46)

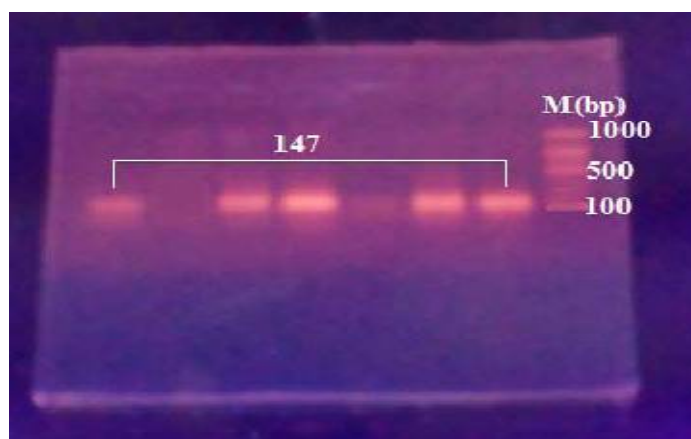


Figure (3): photograph of gel electrophoresis patterns show PCR amplified products of 5 *ST* gene from *E. coli* isolates.

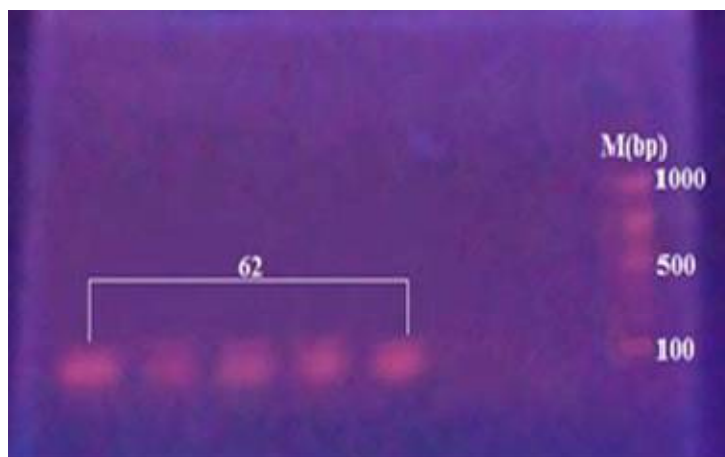


Figure (4): photograph of gel electrophoresis patterns show PCR amplified products of 5 LT gene from *E. coli* isolates.

PCR is a highly sensitive and specific technique for the detection of target DNA in various clinical specimens. In stool samples it can help to differentiate diarrheagenic *E. coli* strains from those of the normal flora (Brandal *et al.*, 2007).

Among 13 ETEC clinical isolates evaluated, 2(15.39%) were positive for LT gene, 6(46.15%) for ST gene, and 5(38.46%) for both ST and LT. This enterotoxin pattern was also reported in the Indonesia and Chile that ST-producing ETEC predominated (Canto *et al.*, 2011; Steinsland, *et al.*, 2011). This is in contrast with other studies from Colombian researchers who recorded that LT, is the most frequently toxin found in the ETEC isolates, be detected in 67.5% of strains, whether alone or in combination with ST (Gonzales *et al.*, 2013). This enterotoxin pattern was also reported in Bangladesh and Peru, where LT-producing ETEC was seen in 52% and 72% of the cases, respectively (Rivera, *et al.*, 2010).

*** Gene sequences :**

The PCR-products of ST gene was sequenced using the same primers as described for the amplification process. Sequences were aligned in the Gene Bank database using the BLASTN program at the National Center for Biotechnology Information (NCBI). The results showed that the isolates which have been detected by sequence were identical in the gene bank in (97%) (**Figure 5**). The strains are: *Escherichia coli* genome assembly FHI92, scaffold scaffold-19 7e-75 LM997169.1 and *Escherichia coli* genome assembly FHI71, scaffold scaffold-24 6e-155 LM996834.1

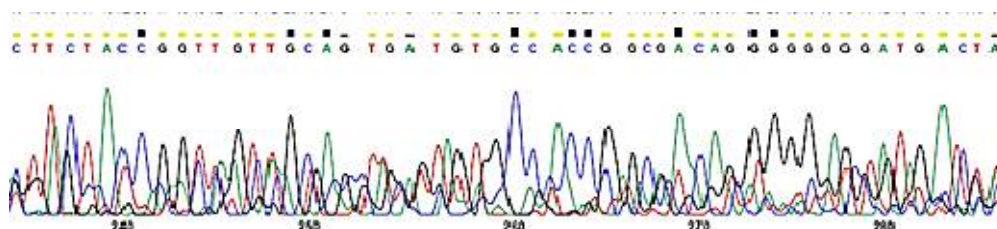


Figure (5): partial of the nucleotide sequence encoding ST gene.

*** Determination the effect of ST< toxicity by Mice assay injected:**

For the six infant mice, all mice which have been injected (I.P.) with ST crude enterotoxin died after 24 hours of injection. This result confirms that in this study the ETEC isolates produce STa toxin. On the other hand, all the control mice(12 weeks) have remained survive during the experiment while in Group A, four mice from six which have been injected intraperitoneally (I.P.) with 0.25 ml supernatant containing ST crude

enterotoxin died after 24 hours of injection of this dose, while all mice which have been administered ST crude enterotoxin orally still survived.

In subsequent images will notice the obvious differences between the controls mice without injected with toxin in compared with infected mice (**figure-6-**)

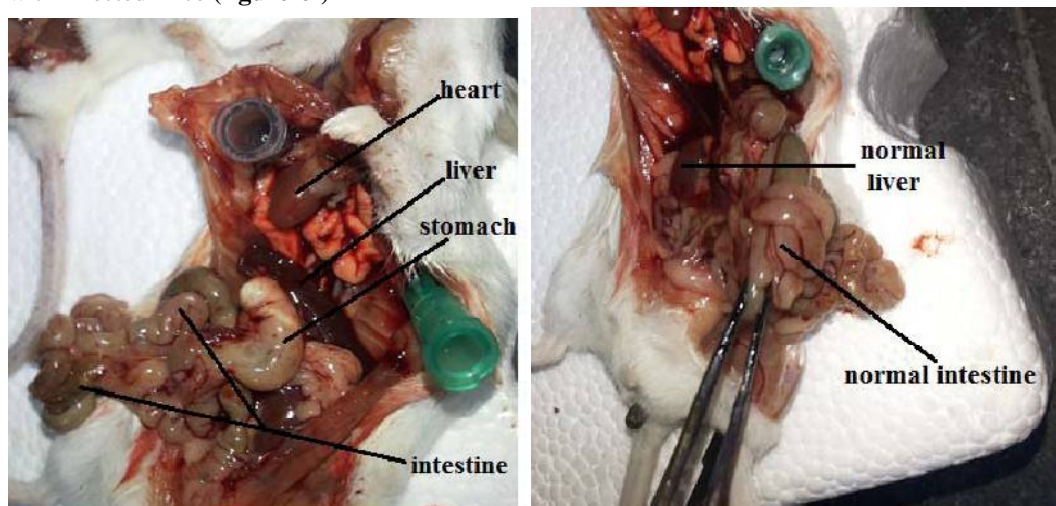


Figure (6): Normal mouse autopsy (control)

* *Light microscopic examination of the:*

* **Intestine:**

In normal large intestine the mucus of normal intestine consist of normal columnar epithelial cells (enterocyte) and lamino properio forming finger like projection between the enterocyte there are mucous goblet cells. The lamino properia consist of loose connective tissue and blood vessels. While the infected intestine consists of sinus hyperplasia of lymphoid tissue in the wall Serosa congested blood vessels and acute inflammatory cells infiltration (Clemente, 2011). (**Figure 7**).

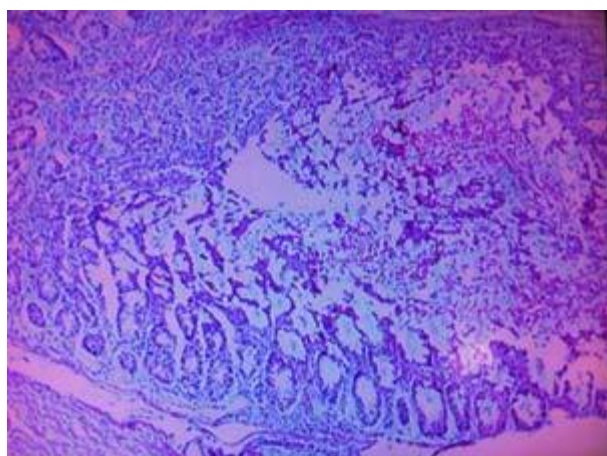


Figure (7): Section from infected intestine shows destruction of most villi , proliferation with crypts and infiltration with inflammatory cells (H&E, 4X).

The results showed that the ST toxin with lower molecular weight (MW=2500) have potent and faster action to cause damage in several organs when injected I.P. after 24 hours in comparison with orally administered since there are no noticeable effects. This is in agreement with (Routhiau and Moreau, 1995). Who stated that bacterial factors such as

enterotoxins can affect the induction of oral tolerance. Oral tolerance is defined as the systemic unresponsiveness to a previously ingested antigen (Ag) when encountered on the parenteral route the gut flora could protect the individual from the toxin's abrogating effect, they believed the gut flora to be an important contributor to the functioning of intestinal immunity (Russo *et al.*, 2013).

When treating intestinal diseases, maintaining its integrity must be taken into account, small quantities of orally administered proteins escape enzymatic digestion in the intestine and can be detected in the circulation antigenically intact (Russo *et al.*, 2013).

*** Cell line tests:**

In this study, HCT 116 (human colorectal cancer cells) were used to investigate the effect of ST toxin on the viability of the cells. The HCT 116 cells were cultured in 6-well plate with RPMI, 10% FBS and 5% CO₂. When the confluence of each well reached 90% (approximately 1 million cells in each well), the cells were treated by ST toxin for 24 hours incubated in 37°C. After incubation, the viability was determined using Trypan-blue staining. The cells calculated for both viable and dead cells .

***Protocol 1:** Six different concentrations of toxin ST tested on the cells of each plate (10⁻¹ to 10⁻⁶).

No considerable changes were observed in viability the cells following the treatment. So protocol two have been done.

*** Protocol 2:** Six different concentrations of toxin ST tested on the cells of each plate (1/2 to 1/64).

□ The viability of cells in 1/2, 1/4 and 1/8 concentrations decreased, but in other concentrations had no significant changes (**Figure 8**).



Figure (8): HCT116 cell line results by Protocol 2

The viability of cells in 1/2 concentration show slightly decreased, but in other concentrations had no significant changes.

*** Protocol 3:** The HCT116 cells in one well were treated by toxin ST directly (1 ml ST in concentration of 0.04 mg/100 ml with 1.5 media).

□ The HCT 116 cells were cultured in 2-well plate with RPMI, 10% FBS and 5% CO₂.

□ When the confluence of each well reached 90% (approximately 1 million cells in each well), the cells were treated by ST toxin for 24 hours . **As shown in Figure (9)**.

(Viability: 94%)

(Viability: 38%)

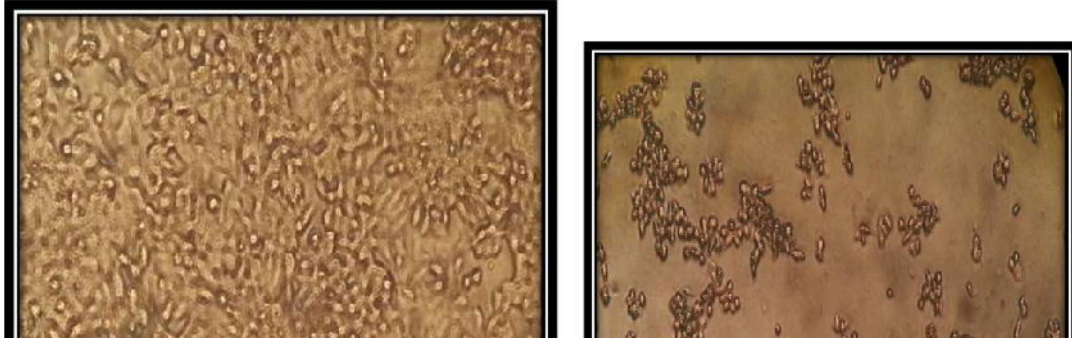


Figure (9): HCT116 cell line results by Protocol 3. A significant decreasing was

observed in the viability of treated cells in comparison with control cells. The results indicated that bacterial enterotoxins ST could inhibit the growth of colon cancer cells *i.e.* inhibition DNA synthesis in human colon cancer cells and regulating cell proliferation. These results similar to those obtained by many authors (Carrithers, 2003; Morgan, 2003 and Pitari *et al.*,2005) who pointed out enterotoxin ST elaborated from ETEC may prevent the hyperproliferative and neoplastic development of intestinal epithelial cells that are associated with initiation and progression of colorectal cancer.

Endogenous [GC-C ligands] guanylin and uroguanylin. {may activate this pathway and promote the transition from proliferation to differentiation of enterocytes along the crypt-villus axis in normal intestine} (Pitari *et al.*,2005). In addition {these observations offer a possible mechanistic insight into the resistance to colorectal cancer observed in geographic areas in which ETEC is endemic} [This putative role for GC-C as a tumor suppressor may contribute to the inverse epidemiological association between colorectal cancer and ETEC infections reflecting, in part longitudinal exposure in under-developed countries to ST-producing bacteria](Ferlay *et al.*,2001).

Drawing from these observations (Pitari *et al.*, 2003) introduced an interesting hypothesis that specific peptides ST elaborated from ETEC may prevent the hyper proliferative and neoplastic development of intestinal epithelial cells that are associated with initiation and progression of colorectal cancer .

On the other hand in the current study, there wasn't record any effect of ST toxin when we used it in the same different concentrations on Vero cells and in the normal colon cell. We provide an attempt to provide anti-cancer agent ST for the treatment of colorectal cancer with less or no cytotoxic effect on normal cells and with more cytotoxic effect on cancer cells Waldman *et al.*(1998) showed that in vero cells, cells lack this receptor/enzyme molecule and do not exhibit ST-induced accumulation of cGMP were unresponsive to the antiproliferative effects of ST [ST/GC-C interaction induced the accumulation of cGMP] which mediated suppression of proliferation by activating CNG channels.

In this study as seen in **figures (10, 11)** the ST toxin test on Vero cell and also on normal colon cell with the same concentration that we mentioned above and it was very clear that the two types of cells were not affected by the toxin notes the continued division and proliferation of cells. These results confirm and prove that the specificity of ST toxin is effective only when there is a special receptors which is what happens in the case of colon cancer.



Figure (10): Vero cell test Normal intestinal cell test treated with ST toxin, No significant effect in the viability in different concentrations .

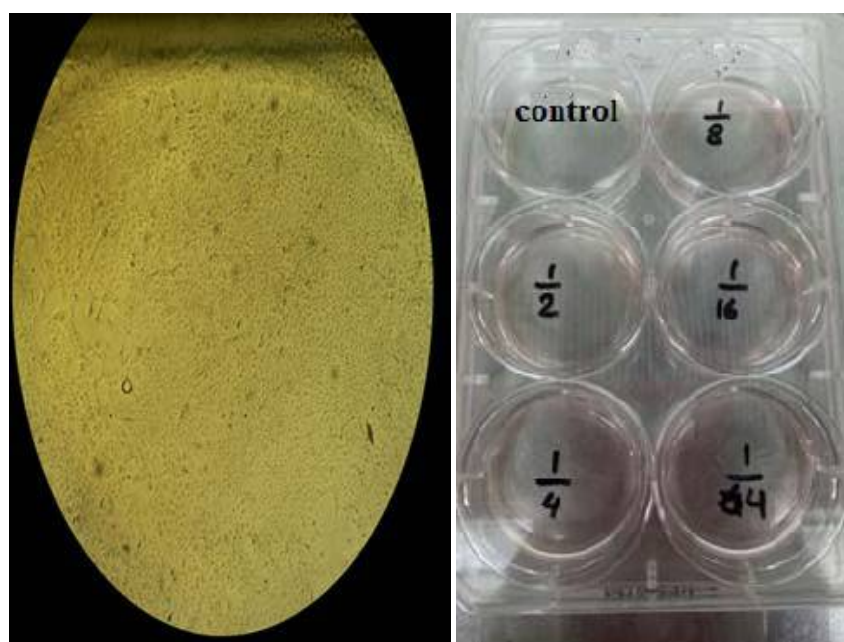


Figure (11): Normal colon cell test treated with ST toxin, No significant effect in the viability in different concentrations .

REFERENCES

1. Asadi, K. M., Bouzari, S., Oloomi, M., Aslani, M.M. and Jafari,A.(2010). Phenotypic and Genotypic characterization of enteropathogenic *Escherichia coli* (EPEC) strains in Tehran, Iran. Iranian J. Microbiol. 2(1):3-7.
2. Biyikli, N., Harika, A., Ozek, E. , Akman, I. and Bilgen, H. (2004). Neonatal
3. Urinary tract infection : Analysis of the patient and recurrences . Black Well
4. Synergy. Pediatrics International.

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

6. Brandal, L.T., Lindstedt, B.A., Lena, A., Stavnes, T.L., Lassen, J. and Kapperud, G. (2007). Octaplex PCR and fluorescence-based capillary electrophoresis for identification of human diarrheagenic *Escherichia coli* and *Shigella* spp. J. Microbiol. Methods; 68: 331-42.
7. Bryce, J., Boschi-Pinto, C., Shibuya, K. and Black, R. E. (2005). WHO estimates of the causes of death in children. Lancet. 365:1147-1152.
8. Bublitz, D.C., Wright, P.C., Bodager, J.R., Rasambainarivo, F.T., Bliska, J.B. and
9. Gillespie, T.R. (2014). Epidemiology of Pathogenic Enterobacteria in Humans, Livestock, and Peridomestic Rodents in Rural Madagascar. PLoS ONE 9(7): e101456.
10. Bueris, V., Sircili, M.P., Taddei, C. R., Santos, I.F., Franzolin, I. M., Martinez, M.,
11. Ferrer, S.R., Barreto, L. and Trabulsi, L.R. (2007). Detection of diarrheagenic *Escherichia coli* from children with and without diarrhea in Salvador, Bahia, Brazil. Mem. Inst. Oswaldo Cruz vol.102 no.7.
12. Canto, F.D., Valenzuela, P. and Cantero, L. (2011). "Distribution of classical and nonclassical virulence genes in enterotoxigenic *Escherichia coli* isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. J. Clin. Microb. vol. 49, no. 9, pp. 3198-3203.
13. Carrithers, S. L. (2003). Diarrhea or colorectal cancer: Can bacterial toxin serve as a treatment for colon cancer? Proc. Natl. Acad. Scie. USA; 100(6): 3018-3029.
14. Chapman, P.A. and Mitchelmore, D.L. (1988). A two-year survey of the incidence of heat-labile enterotoxin-producing *Escherichia coli* and other enteric pathogens in travellers returning to the Sheffield area. Epidemiol Infect. v.101 (2).
15. Clemente, C.D. (2011). Anatomy a Regional Atlas of the Human Body. Philadelphia: Lippincott Williams & Wilkins. p.243.
16. Cohen, M. B., Hawkins, J. A. and Witte, D. P. (1998). Lab Invest 78:101-108.
17. Crawford, J. M. and Kumar, V. (2003). Basic Pathology. 7th (ed.), Kumar, V.; Cortan, R. S. and Robbins, S. L. (eds). Saunders Company, Philadelphia and London. Pp: 543-590.
18. Daniel, R., Santos, D., Santana, J.S., Barreto, J.R., Andrade, M. and Silva, L.R. (2005). Epidemiological and microbiological aspects of acute bacterial diarrhea in children from Salvador, Bahia, Brazil. Braz J Infect Dis vol.9 no.1 Salvador.
19. Evans, D. J., Evans, D. G. and Gorbach, S. L. (1973). Production of vascular permeability factor by enterotoxigenic *Escherichia coli* isolated from man. Infect. Immun. 8:725-730.
20. Ferlay, J., Bray, F., Pisani, P. and Parkin, D. M. (2001). Cancer Incidence, Mortality and Prevalence Worldwide. IARC press, Lyon, France.
21. Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007). Bailey and Scott's Diagnostic Microbiology .12th ed. Mosby company.
22. Ghilardi, A. C. R., Gomes, T.A.T. and Trabulsi, L. R. (2001). Production of
23. Cytotoxic Distending toxin and other virulence characteristics of *Escherichia coli* strains of serogroup O86. Mem. Inst. Oswaldo Cruz, Rio de Janeiro., 96(5):703-708.
24. Gianella, R. A. (1976) . Suckling mouse model for detection of heat stable *Escherichia coli* enterotoxin: characteristics of the model. Infection and Immunity 14(1): 95-99.
25. Gonzales, L., Sanchez, S. and Zambrana, S. (2013) . " Molecular
26. characterization of enterotoxigenic *Escherichia coli* isolates recovered from children with diarrhea during a 4-year period (2007 to 2010) in Bolivia. J. Clin. Microb. vol. 51, no. 4, pp. 1219-1225.
27. Istituto Superiore di Sanità (ISS) . (2013). Detection of Enterotoxigenic *Escherichia coli* in food by Real Time PCR amplification of the *hly*, *stx*, and *stx* genes, encoding the heat-labile and heat-stable enterotoxins. EU Reference Laboratory for *E. coli*. Department of Veterinary Public Health and Food Safety Unit of Foodborne Zoonoses.
28. Kim, K.H., Cho, J., Cheung, C.Y., Lim, J.M., Cho, S., Cho, D.H., Kang, C.S. and
29. Kim, D.H. (2010). Development of Multiplex PCR Assays to Identify *Escherichia coli* Pathogenic Genes in Food. Food Sci. Biotechnol. 19(5): 1205-1210.
30. Mohd, F., Hossain, A., Hossain, T., Hossain, M., Bhuyian, E. and Rahman, M. (2012). Characterization of *Escherichia coli* isolated from migratory water fowls in Hakaluki Haor, Bangladesh. Global Journal of Medicine and Public Health.
31. Morgan, K. (2003). Bacterial toxin may fend off colon cancer. Scie. News; 163(7): 100-101.
32. Nweze, E.I. (2010). Aetiology of Diarrhoea and Virulence Properties of Diarrhoeagenic *Escherichia coli* among Patients and Healthy Subjects in Southeast Nigeria. J. Health Popul Nutr.; 28(3): 245-252.
33. Pachauri, R., Godara, A. and Katari, A. K. (2013). Plasmid profiling and detection of LT and ST (enterotoxin) genes in *E. coli* isolates from diarrhoeic calves, kids and lambs. ELBA Bioflux Volume 5, Issue 1.
34. Pitari, G. M., Baksh, R. I., Harris, D. M. and Waldman, S. A. (2005).
35. Interruption of homologous desensitization in cyclic guanosine 3', 5'-monophosphate signaling restores colon cancer cytostasis by bacterial enterotoxins. Cancer, Res.; 65: 11129-11135.
36. Pitari, G. M., Zingman, L. V., Hodgson, D. M. and Alekseev, A. E. (2003). Bacterial enterotoxins are associated with resistance to colon cancer. Proc. Natl. Acad. Scie. USA; 100(5): 2695-2699.
37. Pitari, G. M., Zingman, L. V., Hodgson, D. M., Alekseev, A.E., Kazerounian, S., Bienengraeber, M., Hajnóczy, G., Terzic, A. and Waldman, S. A. (2003). Proc. Natl. Acad. Scie. USA. 100:2695-2699.
38. Qadri F, Svennerholm AM, Faruque AS, Sack RB, (2005). Enterotoxigenic
39. *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin. Microbiol. Rev., 18, 465-483.
40. Quiroga, M., Oviedo, P., Chinen, I., Pegels, E., Husulak, E., Binztein, E., Rivas, M., Schiavoni, L. and Vergara, M. (2000). Asymptomatic Infections By Diarrheagenic *Escherichia coli* In Children From Misiones, Argentina, During The First Twenty Months Of Their Lives. Rev. Inst. Med. trop. S. Paulo vol.42 no.1.
41. Rajkhowa, S., Hussain, I. and Rajkhowa, C. (2009). Detection of heat-stable and heat-labile enterotoxin genes of *Escherichia coli* in diarrheic fecal samples of mithun (*Bos frontalis*) calves by polymerase chain reaction. J. App. Micro. V:106, issue 2. P:455-458.
42. Rao, M. R., Savarino, S. J., Naficy, A. B., Wieba, T. F. and Abo-Elyazeed, R. S. (2003). High disease burden of diarrhea due to enterotoxigenic *Escherichia coli* among Egyptian infants and young children. J. Clin. Microbiol.; 41: 4862-4864.
43. Rivera, F. P., Ochoa, T. J. and Maves, R. C. (2010). "Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from peruvian children. J. Clin. Microbiol. vol. 48, no. 9, pp. 3198-3203.
44. Roebroek, A. J. M., Xiaosheng, W. u. and Bram, R. J. (2002). Knockin Approaches Methods in Molecular Biology. Vol 209, 2002, pp 187-200.
45. Routhiau, V.G. and Moreau, M.C. (1995) . Gut Flora Allows Recovery of Oral

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

46. Tolerance to Ovalbumin in Mice after Transient Breakdown Mediated by Cholera Toxin or *Escherichia coli* Heat-Labile Enterotoxin. *Ped. Research* .39: 625–629
47. Russo, L.M., Melton-Celsa, A.R., Smith, M.J. and O'Brien, A.D. (2013). Oral Intoxication of Mice with Shiga Toxin Type 2a (STx2a) and protection by Anti-Stx2a Monoclonal Antibody 11E10. *American Society for Microbiology*.
48. Sánchez J, Holmgren J (2005) . Virulence factors , pathogenesis and vaccine protection in cholera and ETEC diarrhoea. *Curr. Opin. Immunol.*, 17: 388-398.
49. Steinsland, H., Valentiner-Branth, P., Grewal, ., H. M. S. , Gastra, W. , Mølbak, K. and Sommerfelt, H. (2011). "Development and evaluation of genotypic assays for the detection and characterization of enterotoxigenic *Escherichia coli*," *Diagnostic Microbiology & Infectious Disease*, vol. 45, no. 2, pp. 97–105.
50. Tsubokura, M.; Matsumoto, A.; Otsuki, K.; Animas, S.B. and Sanekata, T.
51. (1995) . Drug resistance and conjugative R plasmids in strains isolated from migratory waterfowl. *J. Wild Dis.*, 31, 352-357.
52. Waldman, S.A., Barber, M., Pearlman, J., Park, J., George, R. and Parkinson, S.
53. J. (1998). *Cancer Epidemiol Biomarkers Prev.* 7:505–514.
54. Weikel, C. S., Tiemens, K. M., Moseley, L., Huq, M. and Guerrant, R. L. (1986-). Species specificity and lack of production of STb enterotoxin by *Escherichia coli* strains isolated from humans with diarrheal illness. *Infect. Immun.* 52, 323-325.
55. Woodward, M. J., Carroll, P. J. and Wray, C. (1992). Detection of enterotoxin and vero-cytotoxin genes in *Escherichia coli* from diarrhoeal disease in animals using polymerase chain reaction. *Veterinary Microbiology.* 31:251-261.