Egyptian Journal of Medical Microbiology Volume 26 / No.4 / October 2017 9-17

ORIGINAL ARTICLE

Isolation and Identification of Human Pathogenic Bacteria and Related Toxicity Potential to Different Cell Line

Mamdouh S. El-Gamal[1], Aly F. Mohamed[2,*], Mona M. Rashed[3] and Ehab Mabrouk[4]
[1]Faculty of Science Botany and Microbiology Dept. Al-Azhar University; [2]Applied Research sector, VACSERA, Egypt; [3] Damanhour Medical National Institute; [4] Suez Canal Authority

Key words:
Human Pathogenic Bacteria, *E.coli*, Virulence Factors of *E.coli*

Corresponding Author:
Aly F. Mohamed
fahmy.aly@gmail.com
Tel: +201222477069

ABSTRACT

Objectives*: The present study was undertaken to investigate three different virulence mechanisms (adherence, invasion and cytotoxicity) of isolated Escherichia coli from urine and stool specimens and the relation between these different mechanisms and the ability of isolated Escherichia coli to induce apoptosis in Vero cells.* ***Methodology:*** *Different clinical samples have been collected from some Egyptian hospitals* ***(****Suez canal authority hospital, Benha university hospital and Ismailia university hospital****).*** *Bacterial strains were isolated and identified by morphological and biochemical analysis using biolog plates for selecting Escherichia coli strains from clinical samples which resulted in 30 Escherichia coli strains. The In-vitro antimicrobial sensitivity of the E.coli isolates were tested against 11 antimicrobial drugs used in human treatment.* ***Results:*** *Five E.coli isolates were selected from five groups of E.coli isolates which were classified according to antibiotic sensitivity similarity. These five selected Escherichia coli isolates were screened for adherence after 1h , invasion after 3h , 5h and cytotoxicity against Vero cells. All the tested Escherichia coli isolates were able to adhere to Vero cells by variable degrees. Concerning invasion, the tested Escherichia coli isolates were found to have the highest levels of invasion reaching at 3 h and the invasion deacresed after 5h of this assay. on screening for cytotoxicity by the MTT assay, the tested Escherichia coli isolates with highly adherence and invasion showed highly cytotoxic effects to Vero cells after 6 h indicating a relation between adherence and cytotoxicity. using Annexin V/PI staining by flow cytometry, highly cytotoxic tested Escherichia coli isolates showed high percentage of apoptotic Vero cells . Upon screening for activation of caspase 3, highly cytotoxic strains showed highly activation in caspase 3 which plays a central central role in driving the apoptotic pathways indicating a relation between cytotoxic effect and induction apoptosis in Vero cells.* ***Conclusions:*** *These results suggest that the tested Escherichia coli strains exhibit different capacity to adhere to Vero cells and invasion is a post adherence effect and it's preferable time is 3 h because after this peroid Vero cells were ruptured. The cytotoxicity of the isolated Escherichia coli is associated with higher adherence and invasion to Vero cells. This demonstrates that Escherichia coli strains induce apoptosis of Vero cells at different degrees . Induction of apoptosis in Vero cells is associated with higher adherence ,invasion and cytotoxicity Escherichia coli of to Vero cells. The induction of apoptosis is correlated with the virulence of Escherichia coli.*

INTRODUCTION

Infectious diseases are important leading causes of the death worldwide , Bacterial infection remains one of the most critical threats to human health, especially in third world countries[1]. Virulence factors ared used as bacterial tools to facilitate disease in plants, animals, and humans[2]. Bacterial pathogens use many diverse strategies to cause disease in hosts. In the human hosts these strategies include; attachment via adhesions (ex; fimbriae, capsules, pili, and biofilms) which bind to host cells and facilitate colonization so the process of bacterial adherence to host cells is an important step in the initiation of bacterial infection[3]. In some bacterial species, invasion may follow the adherence step. Different bacterial pathogens have evolved different strategies to gain access to the intracellular compartment. Once intracellular, invasive bacteria can survive, multiply and spread. Invasion is aided by the production of extracellular substances called invasins which are protein enzymes that act locally to facilitate growth and spread of the pathogen[4]. Studying the virulence of bacterial pathogens will help to control disease and develop new strategies to prevent bacterial infection [5].

Escherichia coli is a common inhabitant of the human and animal gut, but can also be found in water,

soil and vegetation. It is the leading pathogen causing urinary tract infections[6] and is among the most common pathogens causing blood stream infections[7], wounds, otitis media and other complications in humans [8]. *E. coli* is also the most common cause of food and water-borne human diarrhea worldwide and in developing countries, causing many deaths in children under the age of five years [9]. Antimicrobial resistance in *E. coli* has been reported worldwide and increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries [10]. A rise in bacterial resistance to antibiotics complicates treatment of infections [10]. Pathogens can modulate apoptosis by utilizing virulence factors that can interact with key components of the cell death pathway of the host or interfere with the regulation of transcription factors monitoring cell survival [11]. Modulation of host cell apoptosis is one way for bacteria to eliminate key immune cells or evade host defenses that act to limit infection [11]. Pathogenic strains of *E.coli* can cause apoptosis during their pathogensis [12].

METHODOLOGY

Collection of clinical specimens

Clinical specimens were collected from (November 2012-March 2013), from urine and stool from Inpatients and Out patients of three hospital, Suez Canal Authority Hospital, Benha University Hospital and Ismailia University Hospital. A total of 100 samples comprising 65 samples from urine specimen and35samples from stool specimen were collected. Urine samples were collected from adult men and women with history of chronic urinary tract infection, fecal samples from patients with history of acute diarrhea [13].

Isolation of Escherichia coli from the collected clinical specimens

Culture of the samples for isolation E. coli

All collected samples were inoculated in nutrient broth and incubated at 37°C for 24 hours. Loopfuls from inoculated broth were cultured on MacConkey agar plates (Oxiod). After overnight incubation at 37°C, rose pink colonies were cultured on selective culture in Eosin methylene blue agar (Oxiod) by streak plate method to observe the colony morphology.

Colonies with the characteristic metallic sheen of *E. coli* were repeatedly subcultured onto EMB agar until the pure culture with homogenous colonies were obtained[14].

Identification of *E.coli isolates*

E. coli isolates were subjected to identification on the basis of their cultural and morphological characters on MacConkey and EMB agar media [15].

Identification of bacterial strains using biolog plates:

Biolog MicroPlates were originally developed for the rapid identification of bacterial isolates by sole-carbon source utilization, through the inoculation of 95 individual carbon sources plus a water control on a 96 well plate. The plates are read between 24 and 72 h following inoculation with a pre-grown isolate. Metabolism of the substrate in particular wells results in formazan production, producing a color change in the tetrazolium dye. Individual species may be identified by the specific pattern of color change on the plate, providing an identifiable metabolic fingerprint. Each microorganism has a unique capacity to oxidize some of the various carbon sources. When these carbon sources are oxidized by the microorganism, a purple dye develops visible patterns of positive (purple) and negative (clear) wells which provide a metabolic signature of the organism. The system's computer examines the pattern signature with its database to determine bacterial species identification [16] .

Antimicrobial susceptibility test

In- vitro antimicrobial susceptibility tests were done by Kirby Bauer disk diffusion method. Muller–Hinton agar (Oxoid, Basingstoke, UK) was prepared in a uniform thickness (4 mm) for testing of *E. coli* isolates. The thirty strains of *E. coli* were tested against 11 antimicrobial agents (Bioanalysis – Turky), which represent the commonly used antimicrobials in humans[17].

The antimicrobial agents tested were: ampicillin (AM/10µg), Amikacin (AK/30µg), Amoxycillin-Clavulanic acid (AMC/20µg/10µg), trimethoprim-sulphamethoxazole (SXT/1.25µg/23.75µg), Cefotaxime (CTX/30µg), Gentamycin (CN/10µg), Aztreonam (ATM/30µg), Ofloxacin (ofx/5µg), Doxycycline (DO/30µg), Imipenem (IPM/10µg), Meropenem (MEM/10µg).

The diameters of the zones of inhibition were interpreted by referring to the table which represents the NCCLS subcommittee's recommendation [18].

Identification using molecular method by sequencing for sample 2

The polymerase chain reaction (PCR) method based on 16S rRNA gene for identification *E. coli* was used. Genomic DNA was extracted and purified by using Qiagen kit (Qiagen company) Cultures of *E. coli* were streaked on tryptic soy- agar medium and incubated at 37 ^{o}C for 24 h. A single colony of this sample2 pathogen was grown in (LB) broth medium in Erlenmeyer flask and incubated at 37 ^{O}C for 24 h. Culture was harvested by centrifugation at 4 _C for 10 min, DNA was extracted from pellets according Qiagen kit instructions Amplification and sequencing of 16S rRNA Full length 16S rRNA (1500 bp) were amplified from isolates (*E. coli*) by PCR using universal forward primer P1 and universal reverse primer P6. P1: 5-AGA GTT TGA TCC TGG TCA GAA CGC T-3), P6: 5-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3) (19). Optimum conditions (denaturation 94–1 min, annealing 63–45 s and extension 72–2 min, 35 cycles). Amplified 16S rRNA was purified from 0.8% melting point

agarose gel. Bands obtained from PCR product were eluted and purify by (Qiagen elution kit) PCR instructions, DNA band desired was excised from ethidium bromide stained agarose gel with a razor blade, transferred to Ependorf tube.

DNA were sequenced directly using specific primer with concentration 20 pmol in Vacsera . The sequence alignment was prepared with DNA STAR software program and phylogentic tree was created [20].

Adherence and invasion assays

Colonies were cultured in test tubes containing 10 ml aliquots of TSB and incubated for 18-20 h at 37oC under static conditions. This culture was kept at 4oC and used for inoculum preparation which was prepared by transferring 50 µl of this culture into 10 ml TSB and incubation at 37oC for 18-20 h under static conditions[5].

Cell line and growth conditions

African green monkey kidney epithelial cells (Vero Cell Line, ATCC No. CCL-81) were used in this study. Vero cells were maintained in Eagle's minimum essential medium with Earl's balanced salts (MEM Earl's; Sigma) supplemented with 2% Fetal bovine serum (FBS; Gibco),100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5 % CO2 and sub cultured every 3-4 days. For experiments, Vero cells suspended in MEM Earl's supplemented with 10 % FBS were seeded in 96-well tissue culture plates and kept at 37°C and 5 % CO2 for 24 h to form a confluent monolayer (5 x 104 cells/well) [5].

Assay Protocol

Firstly preparation of bacterial cells and cell free supernatants, bacteria were harvested by centrifugation at 5000 rpm for 15 min. The supernatants were separated and sterilized by filtration through 0.22-µm-pore-size membrane filters (Millex, Millipore Corporation, Bedford, USA). The bacterial pellets were washed once with PBS, then resuspended in MEM Earl's to an absorbance corresponding to 1×10^7 cfu/ml.
Bacterial adherence and invasion was determined and carried out as follows [21]:

After washing the monolayer, aliquots of 100 µl of the bacterial suspensions (1×107 cfu/ml) were added to the wells of a microtitre plate (8 wells for each isolate). Following 1 h of incubation at 37°C in 5% CO2, the inocula were removed and the Vero cells were washed 3 times with PBS to remove non associated bacteria. Vero cells were then treated with lysis solution (0.025% trypsin and 1% tween 20 in PBS) for 30 min at 37°C and the total number of associated bacteria (adherent and invaded) was assessed by the colony counting method.

Gentamicin survival assay was used to quantify invasion. This was determined using the same method described above except that after 1 h incubation, the infected monolayer was treated with gentamicin solution 300 µg/ml for 1 h to kill the extracellular bacteria just before the addition of the lysis solution. Adherent bacteria were calculated as the difference between the total number of associated bacteria and the number of invaded bacteria.

Invaded bacteria after 3h and 5h were determined by the same method.

Count colonies on the plate and calculate the number of cfu of adhered bacteria after 1 h and invaded bacteria after 3 and 5hours.

Cytotoxicity assay

Protein determination

The biuret and Lowry procedures are methods for protein determination.

Reagents and Equipment

- Biuret ReagentFolin and Ciocalteu's Phenol Reagent
- Microplate reader wavelength 550-750
- 0.85% Sodium Chloride Solution

Procedure

Test tubes for blank and test samples were labeled.

To the Blank tube, add 0.2 ml of 0.85% Sodium Chloride Solution.

Add 0.2 ml of a test sample solution to the appropriately labeled test tube.

To each test tube add 2.2 ml of the Biuret Reagent. Mix well and allow to stand at room temperature for 10 minutes.

Add 0.1 ml of the Folin and Ciocalteau's Phenol Reagent to each tube. Mix each tube well immediately after addition. Allow to stand at room temperature for 30 minutes.

Transfer the contents of the test tubes to microplate and read absorbance using the Blank as reference. Complete readings within 30 minutes.

Determine the protein concentration (mg/ml) of each tested sample from the Standard curve.

MTT assay

Five thousand cells per well were seeded in a 96-well plate (B.D. Bioscience Falcon, Bedford, MA, USA). Cells were allowed to adhere for 24 h at 5% CO2 and 37OC. Various concentrations of total protein were added to the cells. Incubation continued for different 24 h. Control cells were untreated cells. After incubation, the media and superntant were removed and 3-(4,5 dimethylthiazole-2yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium (MTT) at 100 µL per well was added for 1 h at 37AC, followed by 100 µL of lysis buffer (20% SDS, 30%phosphate-buffered saline (PBS) and 50% dimethylformamide). Absorbance was determined by spectrophotometry as 570nm wavelength with Biotek E 1X 800 USA. The concentration of total proteins which is lethal to 50% of the cells (IC50) is calculated. the cell viability was determined by the followed equation: Cell viability % = Mean O.D. / Control O.D. * 100[22].

Apoptotic assay using Flow cytometry

Reagents

FITC Annexin V, Use 5 µl per test

Propidium Iodide(PI), Use 5 µl per test

Annexin V Binding Buffer, 0.1M Hepes/NaOH(PH 7.4),1.4 M NaCL, 25mM $CaCL_2$.

Staining methods

Cells were washed twice with cold PBS and then resuspend cells in Binding Buffer at a concentration of 1x 10^6 cells/ml

100 µl of the solution (1x 10^5 cells) were transferred to a 5 ml culture tube.

5 µl of FITC Annexin V and 5 µl PI were added.

The cells were vortexed and incubated for 15 min at Room Temperature (25 ^OC) in the dark.

400 µl of Binding Buffer were added to each tube and analyze by flow cytometry within 1 hr

Caspase-3 Colorimetric assay

Cell Culture Preparation

Cells were placed in 6-well plate using 2 mL of medium.

The cells were cultured in a CO2 incubator for at least 16 hours

The cell culture medium was removed from the culture wells. Gently remove as much of the medium as possible.

1ml of Cell Lysis Buffer was added to each culture well

Gently shake, rock or tap the plate for 10 minutes to facilitate cell lysis and sample homogenization.

Assay Protocol

100 µL Caspase-3 Reaction Buffer were added to each well in the reaction plate.

100 µL of cell lysate were added to each well in the reaction plate. Mixing between the Caspase-3 Reaction Buffer and lysate is spontaneous.

The absorbance of each sample was measured at 405 nm (Initial Reading). After exactly 30 min, measure the absorbance again (Final Reading).

The absorbance increase is directly proportional to the amount of active caspase-3 present in each culture sample

RESULTS

Thirty strains of *Escherichia coli* were isolated from 100 clinical sources (65 urine &35 stool samples)

Identification using biolog plates

In the present study , all Gram negative bacilli strains with metallic sheen colonies were suspended in "IF" inoculating fluid then 100µl of this suspension were transferred per well of plate. a thirty strains of *Escherichia coli* were isolated by biolog plates from urine and stool specimens. 9 strains were isolated from stool samples and 21 strains from urine samples.

Antimicrobial susceptibility test

The isolated strains were classified into 5 groups according to antibiotic sensitivity similarity figure (1)

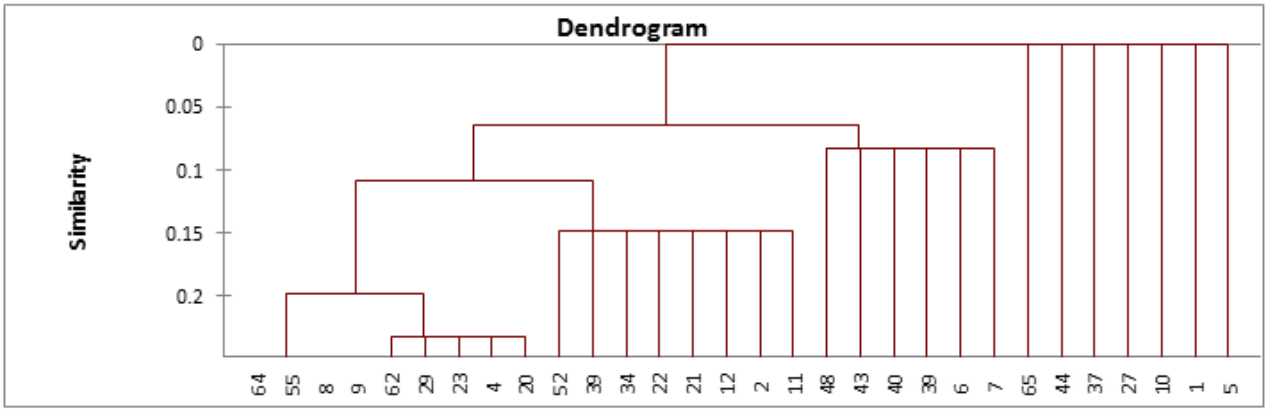

Fig. 1: Dendrogram indicate antibiotic Sensitivity Similarity for *Escherichia coli* isolates.

Group 1 include (1 , 5 , 10 , 27 , 37 , 44 , 65)
Group 2 include (2 , 11 , 12 , 21 , 22 , 34 , 36 , 52)
Group 3 include (4 , 20 , 23 , 29 , 62)
Group 4 include (6 , 7 , 39 , 40 , 43 , 48)
Group 5 include (8 , 9 , 55 ,64)

According to this figure we selected strains 5,2,23,39,64 for adherence, invasion and cytotoxicity assays.

Identification using molecular method by sequencing for sample 2

PCR method based on 16S rRNA gene was used as fast and accurate method for Identification and confirmation of *E. coli* strain sample 2 which gave the highest pathogenicity.

16S rRNA sequence –

AGTCGACGGTAACAGGAAGGAGCTTGCTGCTTT
GCTGACGAGTGGCGGACGGGTGAGTAATGTCT
GGGAAACTGCCTGATGGAGGGGGATAACTACT
GGAAACGGTAGCTAATACCGCATAACGTCGCA
AGACCAAAGAGGGGGACCTTCGGGCCTCTTGC
CATCGGATGTGCCCAGATGGGATTAGCTAGTAG
GTGGGGTAACGGCTCACCTAGGCGACGATCCCT
AGCTGGTCTGAGAGGATGACCAGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCCATGCCGCGTGTATGAAGAA
GGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGA
GGAAGGGAGTAAAGTTAATACCTTTGCTCATTG
ACGTTACCCGCAGAAGAAGCACCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGAGGGTGC
AAGCGTTAATCGGAATTACTGG

The 16S rRNA sequences for *E. coli* were deposited in the gene bank sequence database under accessions AB599716.1.The relevant sequences were downloaded and phylogenic analysis has been carried out. Figure (2) showed phylogenic tree of *E. coli*.

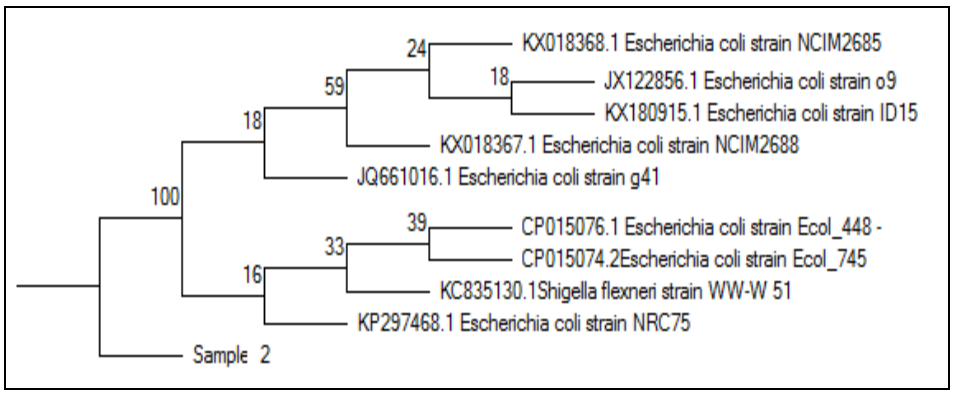

Fig. 2: Phylogenic tree of *Escherichia coli of sample 2*.

Adherence and invasion of the bacterial isolates to Vero cells

The ability of the five collected bacterial isolates to adhere to an invade the Vero cells was investigated and the results were expressed in histograms of chart (1) and chart (2).The five isolates(2, 5, 39, 23, 64) caused detachment of the Vero monolayer within 1 h of the assay . on other hand, these isolates invaded the Vero cells within 3h and 5 h of the assay.

In the mean time the adhesion potential of bacterial isolates was tested and data recorded revealed that adhesion potential was arranged in the order of sample 2>5>39>23>64 recording a significantly elevated adhered bacterial count ($P<0.05$) despite the variation in the start CFU.

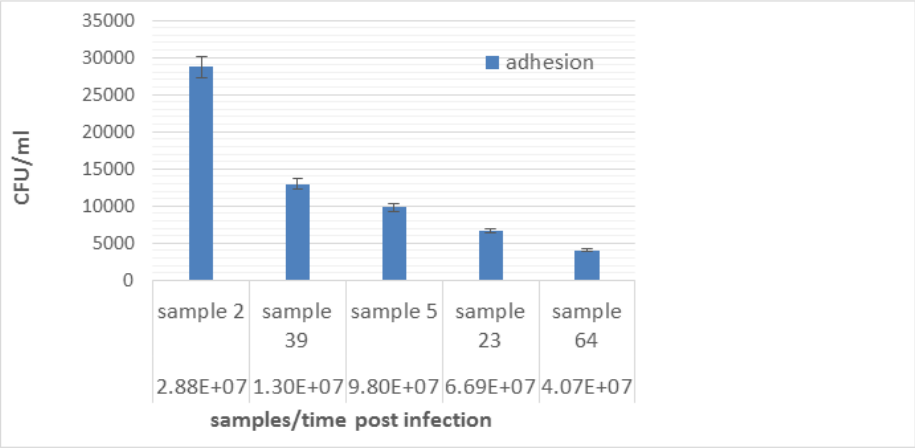

Chart 1: Adherence of the tested *E.coli* isolates after 1 h to Vero cells. The histograms represent the adherent bacteria count t o Vero cells after 1 h and the error bars indicate the standard deviations of the data.

Regarding the invasion potentials of test isolates there was a great variation in invasion potentials despite the variation in the start count . Also., invasion potentials showed a reversible reactivity relative to time as the CFU invaded then cells showed a clear reduction in the invasive colony forming units relative to time (chart. 2).

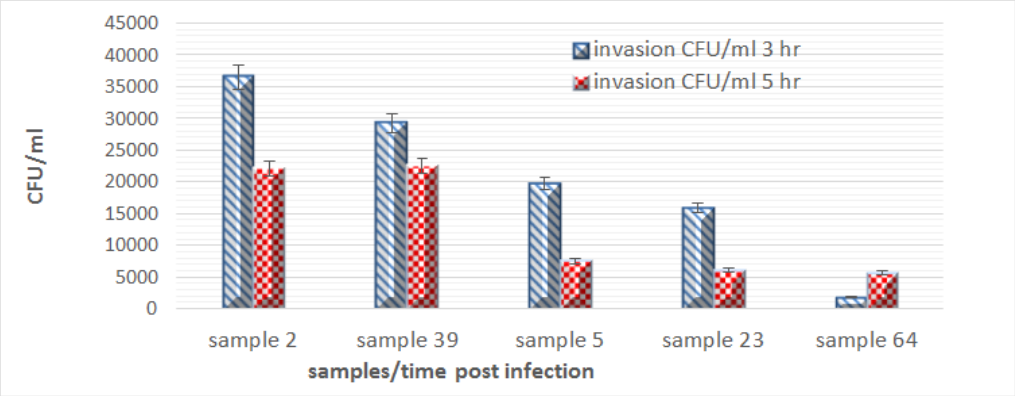

Chart 2: Invasion of the tested *E.coli* isolates after 3 h and 5h to Vero cells. The histograms represent the invaded bacteria count t o Vero cells after 3 h and 5 h and the error bars indicate the standard deviations of the data.

Cytotoxicity studies of tested isolates on Vero cell line

Cytotoxicity of collected isolates was investigated using the MTT assay, after 6 h incubation of metabolites of bacterial isolates with Vero cells. A relationship was made between concentration of proteins content in metabolites of bacterial isolates with viability of Vero cells and The cytotoxicity can be evaluated by half maximal inhibitory concentration (IC_{50}) .according to IC_{50}, sample 2 showed the lowest concentration 3.958 µg% to induce IC_{50} followed by sample 39 recording 4.671µg%, sample5 recording 13.93 µg%, sample 23 recording 39.17 µg% and sample 64 recording 66.74 µg%. as in chart (3).

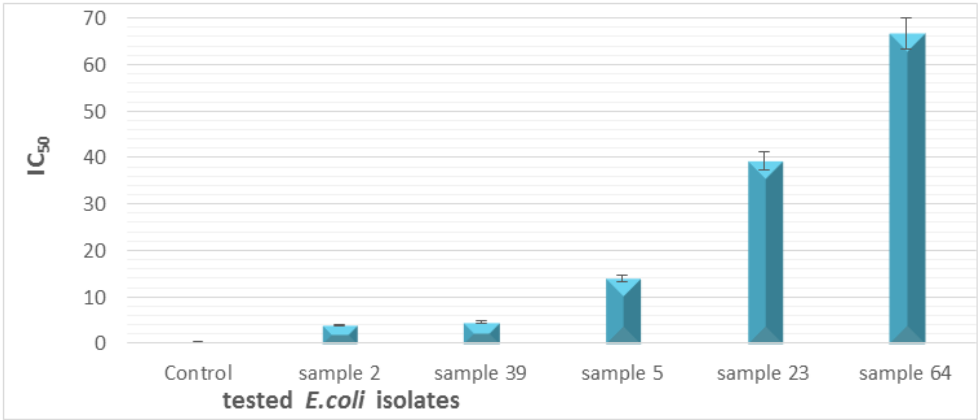

Chart 3: The cytotoxicity of total proteins of the tested isolates**.** The histogram represent half maximal inhibitory concentration (IC_{50}) of the tested E.coli isolates and the error bars indicate the standard deviations of the data.

Apoptotic assay using flow cytometry

In the (control) samples, the majority of cells (97.3%) were viable and non-apoptotic (Annexin V–PI–). In contrast, when cells were treated with metabolites of isolated E.coli samples (64, 23, 5, 39, 2) , (94.9 %, 85.1%, 82.6%, 52.7%, 33.5% , respectively) of Annexin V–PI– cells were observed. There was an increase in early apoptotic cell populations (Annexin V+PI–) from untreated (control) to treated cells by metabolites Of isolated E.coli (64,23,5, 39, 2) (2.14% to 5.08%, 14.9%, 17.4%, 47.3%, 66.4% Annexin V+PI– , respectively). A slight increase in the Annexin V+PI+ population was also observed which indicates late apoptotic or dead cells as seen in chart (4).

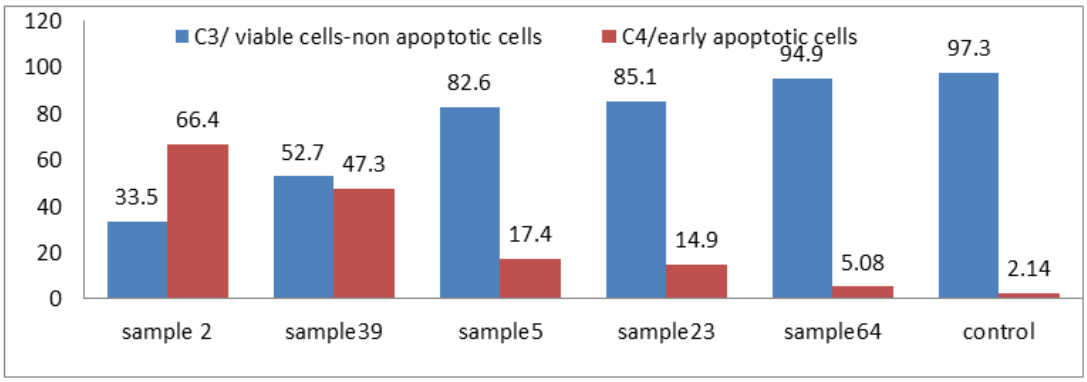

Chart 4: The histograms represent the Percentage of viable Vero cells and the precentage of early apoptotic cells

Caspase-3 Colorimetric assay

Regarding the induction apoptosis via monitoring the biopotential of caspase 3 . Data recorded revealed that there was a elevated caspase 3 level post cellular treatment with bacterial metabolites indicating induction apoptosis of bio-metabolites the activity was arranged according to the sample isolates in the order of 2>39>5>23. Also sample 64 showed no effect on the caspase 3 level and its values was near that of control.

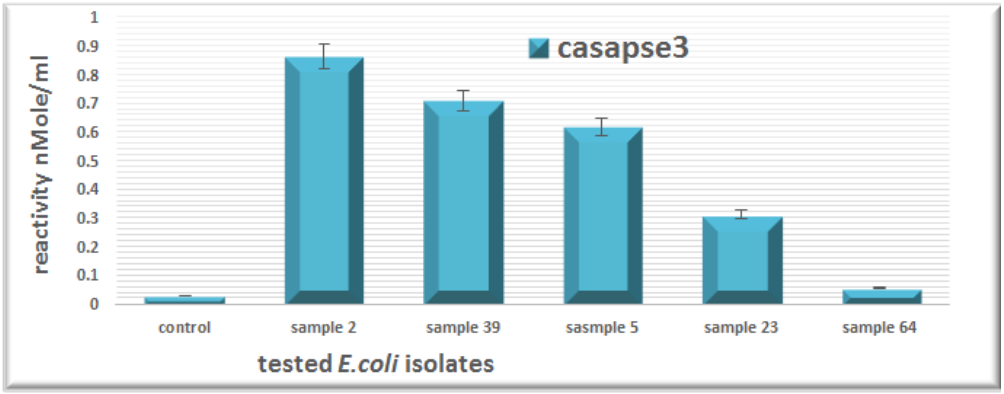

Chart 5: Activation of caspase 3 in Vero cells by tested *E.coli* isolates

DISCUSSION

The isolation rate of *E.coli* in the present study was 30% and it was commonly isolated from urine samples (70%). These finding are in conformity with reports by other researchers [23]. Antimicrobial resistance in E.coli has increased worldwide and its susceptibility patterns show substantial geographic variation as well as differences in population and environment [24].

In this study, the overall resistance of E.coli to antimicrobials was high. the result is consistent with findings of previous studies [25].

The Bacteria have wide ranging & deleterious effects on the hosts [26]. In order to infect hosts, bacteria need different virulence systems. Processes like adherence, invasion and evasion of host defense are crucial for the bacteria during infection [27]. To overcome infections caused by bacterial pathogens, it is necessary to clarify the pathogenicity mechanisms that are used by these pathogens, and the relationship between these different virulence mechanisms. The results of the present study revealed that all the tested isolates were able to adhere to the Vero cell line but by variable degrees. However, no general relation between the origin of isolation and the ability of the tested isolates to interact with Vero cells could be established. In addition.

In some bacterial species, invasion may follow the adherence step where Bacterial adherence is the establishment of the bacterial pathogen at the appropriate portal of entry. The process of bacterial adherence to host cells is an important step in the initiation of bacterial infection [28]. Once intracellular, invasive bacteria can survive, multiply and spread [28] Chart (2) invasion of *E.coli* after 3 hr was increased than adherence after 1 hr and the same samples with

high adherence were the highest invasion as sample 2 and sample 39 followed by sample 5 and sample 23 while sample 64 also showed the lowest invasion. invasion after 5 hr , invasion potentials showed a reversible reactivity relative to time which may be due to the Vero cells rupture.

Invasion is aided by the production of extracellular substances called invasins which are protein enzymes that act locally to facilitate growth and spread of the pathogen [4].

Some bacterial species can establish locally and cause infections that remain extracellular by secreting toxins with local or systemic effects [28]. These toxins may damage or kill host cells by different mechanisms . Cytotoxicity of the collected isolates was also investigated using the MTT assay. MTT assay is a non radioactive colorimetric assay system and is used for measurement of cytotoxicity caused by bacterial pathogens [29]. It detects interference with total protein concentrations. After 24 h of incubation with Vero cells, IC_{50} of sample 2 was the highest followed by sample 39,5,23 and 64. Sample 2 was the most cytotoxic strains to Vero cells and followed 39, 5 ,23 and 64.

In our results, the statistical data showed a significant positive correlation between adherence and invasion of the tested isolates. This indicates that invasion is a post adherence event and that bacterial adherence to the host cells is a prerequisite for invasion to take place. These findings are in agreement with those obtained by they reported that the type I pilus adhesion FimH in some *E. coli* mediates not only adhesion but also invasion of epithelial cells[30].

The statistical data for adherence and cytotoxicity of the tested isolates showed Highly cytotoxic isolates were highly adherent to Vero cells indicating a relation between adherence and cytotoxicity of these isolates. This may be because β-hemolysin is cell bound and so greater contact with host cells is accompanied with a higher cytotoxic effect. It has been reported previously that cytotoxicity caused by bacteria associated hemolysin requires some degree of contact or close proximity between the bacteria and host cells [31].

High cytotoxicity was observed after 3 h only of invasion assay and this cytotoxicity gradually decreased as incubation time increased since secreted toxin were reported to cause rapid cytotoxic effects on cultured mammalian cells. Cytotoxic activity of *E. coli* isolates (producing β-hemolysin) on epithelial cells was reported after 90 min of incubation only [32].

The present study showed that *E.coli* strains induce apoptosis of Vero cell by different degrees and induction of apoptosis is directly proportional to the toxicity of strains . In previous studies, cell death is usually classified into two broad categories: apoptosis and primary necrosis. The term necrosis may be misleading because it corresponds to the late stage of death associated with membrane disruption, whether cells are dying of apoptosis or oncosis [33]. Apoptotic cells exhibit an early loss of phospholipid asymmetry, leading to the exposure of PS residues on the outer layer of the plasma membrane [34]. Because annexin V binds to negatively charged phospholipids such as PS, FITC-conjugated annexin V is classically used to identify apoptotic cells by flow cytometry [35]. During apoptosis, the cells become reactive to annexin V prior to the loss of both plasma membrane integrity and the ability to exclude PI. Combined staining with fluorescein-conjugated annexin V and propidium iodide (PI) distinguishes between early (Annexin V+PI) and late apoptotic cells Annexin V+PI+ [35,36]. In one study revealed that PS exposure on the outside layer of the plasma membrane is not specific to apoptotic cells; it also occurs in oncotic cells, thus significantly interfering with the detection of apoptotic cells in the annexin V/PI assay. Many authors use the annexin V/PI assay for apoptosis detection and quantitation but usually do not confirm the type of cell death by morphological examination. We have shown that the presence of oncotic cells within an apoptotic population could significantly interfere with, then quantification of apoptosis by the annexin V/PI assay. Both oncosis and apoptosis can coexist in response to certain stimuli [37].

In our study other events involved in apoptosis, such as the activation of caspase 3 was done. This study will better characterize the two types of cell death and will help to identify cell death occuring by apoptosis or oncosis. We examined the activation caspase-3 where it plays a central role in driving the apoptotic pathways and it is required for certain distinctive biochemical and morphological changes during apoptosis, these changes almost invariably involve chromatin condensation and its margination at the nuclear periphery, extensive double-stranded DNA fragmentation and cellular shrinkage and blebbing .

REFERENCES

1. Yestrepsky BDY; YXU, ME.; Breen, MEXL; Rajeswaran WG; Ryu, JG; Sorenson, RJ; Tsume, Y; Wilson, MW; Zhang, W ; Sun, H. and Larsen, SD. Journal of public Bioorganic& Medical chemistry.21,18880-1897, 2013.
2. Turgeon, Z; Jorgensen, R; Visschedyk, D; Edwards, PR.; Legree, S.; McGregor, C.; Fieldhouse, RJ.; Manggroo, D.; Schapira, M. and Merrill, AR. Antimicrobial agents and chemotherapy.55,983-991,2011.
3. Rasko, DA and Sperandio, V. Nature Reviews Drug Discovery.9,117-128,2010.
4. Pizzaro-Cerda, J. and Cossart, P. Cell 124(4): 715-727,2006.
5. Ghadir S. El-Housseiny, Mohammad M. Aboulwafa and Nadia A. Hassouna. American science. 6 (10), 269-283, 2010.

6. Wagenlehner FM, Naber KG, Weidner W. (2008) Med Monatsschr Pharm. 31: 385-90,2008.
7. Biedenbach DJ, Moet GJ, Jones RN. Diagn Microbiol Infect Dis. 2004; 50: 59–69,2004.
8. Khan NA, Saba N, Abdus S, Ali AQ Pak J Biol Sci. (1):111-113, 2002.
9. Turner SM, Scott-Tucker A, Cooper LM, Henderson IR FEMS Microbial Letters. 263(1): 10-20,2006.
10. El Kholy A, Baseem H, Hall G, Procop GW, Longworth DL J Antimicrob Chemother. 51: 625–630,2003.
11. Gao LY, Kwaik YA. Trends Microbiol 8, 306-313,2000.
12. Yajing chen and Arturo Zychlinsky(2001): Apoptosis induced by bacterial pathogens. Skirball institute and Department of Microbiology, New york university Medical centre, 540 First Avenue , New York, NY 10016, U.S.A.
13. Miller, JM. A guide to specimen management in clinical microbiology. 3Th ed. book. American society for microbiology, united states of America,2005.
14. Zinnah, MA; Bari, MR; Islam, MT; Hossain, MT; Rahman, MT; Haque, MH.; Babu, SAM; Ruma RP. and Islam, MA. Bangl. j. vet. med. 5(1&2):25-32, 2007.
15. Quinn, P.J.; Carter, M.E.; Markey, B. and Carter, G.R. Clinical veterinary microbiology. 6th ed., Mosby, Edinburgh, London, New York, Philadelphia, St. Louis, Sydney, Toronto,2004.
16. Fahad Abdullah M. Al-Dhabaan, Ali Hassan Bakhali Journal of Biological sciences 24, I4, 2017, 901-906,2017.
17. Bauer, AW, Kirby, WMM sherris, JC and Turk, M. Am J. Clin pathol 45:493-496,1996.
18. NCCLS "National Committee for Clinical Laboratory Standards", Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved Standard M31-A2. Villanova, PA, USA,2002.
19. Jill E. ClarridgeMicrobiol.Rev.17(4) 840-862,2004.
20. Hall TA Nucl. Acids. Symp. Ser. 41: 95-98,1999.
21. Plotkowski, MC, Saliba, AM, Pereira, SHM., Cervante, MP and Bajoletlaudinat, O. Infection and Immunity 62(12): 5456- 5463,1994.
22. Zhang B, Hirahashi J, Cullere X, Mayadas TN. J Biol Chem 278, 28443-28454,2003.
23. G. Yismaw, S. Abay, D. Asrat, S. Yifru, A. Kassu, Ethiopian medical journal, 48 ,293-300, 2010.
24. Von Baum H, Reinhard M. Inter J Med Microbiol. 295: 503– 511,2000.
25. 25.Orrett FA, Shurl SM. Prevalence of resistance to Escherichia coli. University of Texas Medical Branch, Galveston, TX, USA, *2*Universidad de Chile, Santiago, Chile,2001.
26. Heinzelmann, M., Scott, M. and Lam, T. The American Journal of Surgery 183: 179- 190,2002.
27. Kaper, J.B., Nataro, J.P., Mobley, H.L., Nat. Rev. Microbiol. 2 (2), 123–140,2004
28. Sansonetti, P. Host–pathogen interactions : these duct ion of molecular cross talk. Gut50,Suppl.3S2–S8,2002.
29. Kubica, M., Guzik, K., Koziel1, J., Zarebski, M., Richter, W., Gajkowska, B., Golda, A., Maciag-Gudowska, A., Brix, K., Shaw, L., Foster, T. and Potempa, J. 1, e1409, 2008.
30. Martinez, J.J., Mulvey, M.A., Schilling, J.D., Pinkner, J.S. and Hultgren, S. J. Embo Journal19 (12): 2803-2812,2000.
31. 31.Gadeberg, O. V. and Orskov, I. Infection and Immunity 45(1): 255-260, 2004.
32. Sobieszczanska, B. M., Osek, J., Gryko, RL. and Dobrowolska, M. Journal of Medical Microbiology 55(3): 325-330,2006.
33. Majno G, Joris I. Am J Pathol;146:3–16,2005.
34. Fadok VA, Voelker DR, Campbell PC, Cohen JJ, Bratton DL, Henson PT J Immunol; 148:2207–2216, 2002.
35. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. J Immunol Methods;184:39–51,2005.
36. Van Engeland M, Ramaekers FCS, Schutte B, Reutelingsperger PM Cytometry 1996;24:131–139,2006.
37. Herve´ Lecoeur, Marie-Christine Pre´vost, and Marie-Lise Gougeon . 44,1,65-72,2001.