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The influence of nutrient culture media on *Escherichia coli* adhesion and biofilm formation ability

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Abstract

Adhesion of Escherichia coli strains to inert and cellular substrates and formation of biofilms are complex processes that are influenced by many factors. This work highlighted the variation of bacterial adhesion in different culture conditions in E. coli strains isolated from different infections sites. The experimental infection of THP-1 and HeLa cells showed that the degree of adhesion, the adhesion pattern and the virulence ability of the tested strains are influenced by the composition of the used medium. A nutrients rich medium increases the level of adhesion and strains ability to produce slime. In addition, the ability to form biofilms is biased by the nutrient media: in minimal medium the production of biofilms is very low, almost non-existent, but in a rich nutrient medium ability of bacteria to produce biofilms increases exponentially. Changes of the culture medium composition lead to major differences in the degree of adhesion and hence the degree of formation of biofilms. Lack of standardized methods contributes to different results regarding the contribution of different metabolic and virulence features on the ability of adhesion and biofilm formation, and on the progress of the whole infectious process.

Keywords: *Escherichia coli, human monocytes THP-1, Ekaryotic cells*

1. Introduction

Escherichia coli has the ability to colonize and persist for different periods of time in many environmental and animal hosts niches, requiring the adaptation and survival of these bacteria in constantly changing environments, achieved through coupling individual cell responses.

E. coli, along with other commensal bacteria from the intestinal microbial of mammals, most often creates beneficial symbiotic relationship with the host, providing nutrients for growth regulation and key signals and protective immunity against foreign pathogens (F. YAN & D.B. POLK [1]). However, certain *E. coli* strains can become pathogenic and cause gastrointestinal or extra-intestinal diseases. Based on the epidemiology and host symptoms, pathogenic *E. coli* pathogenic strains are divided in enteropathogenic and extraintestinal pathovars (J.B. KAPER & al. [2]), exhibiting specific combinations of virulence factors (C.F. MARRS & al. [3])]. *E. coli* pathotypes affect host cell processes and functions such as signal

transduction, protein synthesis, mitochondrial function, cytoskeletal functions, ion secretion, cell division, transcription and apoptosis (V.K. VISWANATHAN & al. [4]; M.G. GAREAU & al., [5]; J.L. ROXAS & al. [6]; V. YASHUNSKY & al. [7]; J.L. ROXAS & al. [8]).

The cultivation and growth of *E. coli* strains using various static and dynamic models for the biofilms study has led to the identification of a large number of adhesins, and complex linkages between regulatory systems involved in the biofilms formation. Some studies of commensal and pathogenic *E. coli* isolates revealed the operation modalities of signaling molecules, receptors and the communication within the biofilm involved in maturation and maintenance of biofilm architecture.

The aim of this work was to study the influence of nutrient media on the adhesion ability and biofilm formation by *E. coli* strains, in order to bring new data on the involved mechanisms, useful to discover more effective methods to inhibit unwanted biofilm formation.

2. Materials and methods

2.1. Bacterial strains: In this study there were used *E. coli* 32 strains (16 of them being extended spectrum beta-lactamase positive ESBL+), from the CC Iliescu Clinical Institute of Cardiovascular Diseases. The strains identification was performed using theautomated VITEK 2 Compact Ssystem (GN card for identification).

2.2. Ekaryotic cells: The HeLa cell line (ATCC CCL-2) and human monocytes THP-1 cell line (TIB 202) were used. The cells were maintained in DMEM:F12 (HeLa) and RPMI (THP1) supplemented with 10% fetal bovine serum, at 37°C, and 5% CO_2.

2.3. Media for *E. coli* cultivation: simple broth culture medium (3% meat extract, 10% peptone, 5% NaCl solution, and distilled water); simple broth with casiminic acid (3% meat extract, casiminic acids 10%, 5% NaCl solution, in distilled water), M9 medium (M9 salt (6% Na2HPO4-7H2O, 3% KH2PO4, 2.5% NaCl, 5% NH4 Cl solution, in distilled water), 2 mM MgSO4, 20% glucose, 0.1 mM CaCl2, distilled water ad 1000 ml) and M9 medium with casiminic acid (M9 medium containing 20% casiminic acids).

2.4. Adherence to HeLa cells: testing of bacterial adhesion capacity to HeLa cells grown in monolayer was done using the modified Cravioto method (A. CRAVIOTO & al. [9]; A.M. HOLBAN & al. [10]). Initial bacterial inocula, with a density of 0.5 Mc Farland units (corresponding to 108 cells/ml), were obtained from fresh 24 hours bacterial cultures. Decimal dilutions of initial bacterial inocula were performed in a) simple meat broth; b) simple broth with casiminic acid; c) M9 medium and d) M9 medium with casiminic acids. The tubes were then incubated for 24 hours at 37°C. After this step was accomplished, a second decimal passage were done in the same media type used in the first passage, the tubes being then placed incubated for 24 hours at 37°C.

TheHeLa cells were grown in monolayer in the DDMEM:F12 medium with 10% fetal bovine serum (FBS), in plastic plates with 6 wells. The plates were then incubated for 24 hours at 37°C. HeLa cells monolayers were washed with phosphate buffered saline (PBS), and then 1 ml of bacterial suspension from the second passage was added to each well. The plates were incubated for 2 hours at 37°C and thereafter were washed with PBS to remove non-adherent bacterial cells. Monolayers were fixed with 1 ml of cold methanol for 5 minutes, stained with 1% Giemsa solution for 30 minutes, and then washed with tap water and dried. Microscopic examination was made using immersion objective in light microscopy. Microscopic analysis allowed on the one hand to establish the adherence pattern, and on the other hand, to perform a semi-quantitative analysis of the level of bacterial adhesion to the cellular substratum, through the determination of the adhesion index, which is the ratio between the

number of eukaryotic cells presenting adhered bacteria and the total number of eukaryotic cells, expressed as a percentage (H. ZEPEDA-LOPEZ & al. [11]).

2.5. Analysis of bacterial adhesion to THP-1 cells grown in suspension: the bacterial culture obtained in: a) simple meat broth; b) simple broth with casiminic acid; c) M9 medium and d) M9 medium with casiminic acids were centrifuged for 10 minutes at 10,000 rpm, washed with PBS solution, vortexed and placed for 20 minutes at 60°C. After cooling, the samples were diluted in PBS until 0.5 Mc Farland densities, and the amount of 1 mL from each sample were stained with FITC for an hour in the dark. The bacterial cells were washed for 5 times in PBS by centrifugation 10 minutes at 10000 rpm.

The THP-1 cells were grown in the flow cytometer sterile tubes at a concentration of 2 x 105 cells/ mL. 1 ml bacterial suspension was added to THP1 cell and they were maintained at 37°C for 6 h. Samples were successively introduced into flow cytometer for analysis to determine the intensity of adhesion process.

2.6. The slime production test: bacterial suspensionswere prepared as described above, by 2 passages in medium with and without casiminic acid and incubation for 24 hours at 37°C. Bacterial suspensions were then aspirated, the tubes were washed with PBS, and stained by adding 1% safranin solution for 30 m inutes. After staining, the tubes were washed with distilled water, dried and the ring was read from each tube to determine the ability of adherence to the inert substratum, due to the secretion of a polysaccharide called slime (G. O'TOOLE & al. [12]). The test involves observing the formation of a colored ring adherent to glass at the air-liquid interface. Depending on the thickness of the ring, the ability of bacterial strains to produce slime was quantified as: strong (+++), moderate (++), low (+), absent (0).

2.7. Determination of biofilm formation using microtiter plate assay: in order to determine the biofilms formation ability of bthe tested strains, bacterial suspensions prepared as described above, grown in medium with and without casiminic acid, were subjected to a qualitative and quantitative test. 200 µl of bacterial strain suspensiongrown in the appropriate medium were seeded to each well and the plates were incubated for 24, 48 and 72 hours at 37°C. To the appropriate time, the test plate was washed with PBS, followed by a fixing step for 5 minutes with 5% methanol, and then drying, followed by staining of each well with 200 µ1 crystal violet solution (l%) for 30 minutes. After washing, drying, and addition of 33% solution of acetic acid, the plates were read at the spectrophotometer at a wavelength of 492 nm (J.H. MERRITT & al. [13]).

3. Results and discussion

3.1. Optic microscopy assessment of the bacterial adhesion to HeLa cells monolayers

The evolution of pathogenic *E. coli* strains results in the occurrence of pathotypes able to colonize the gastrointestinal tract, the urinary tract, the meninges etc. This illustrates how the genetic elements may affect the adaptation of one strain to different host environments. From the physiological point of view, this bacterial species is adaptable, responding to signals from the environment such as pH, temperature, its chemical composition and osmolarity. Adherence is an essential step in bacterial biofilm formation, since the survival of all cells in a biofilm structure depends on interactions between bacterial cells and substrate. Anchoring to a fixed surface in a microenvironment of increased amounts of nutrients ensure the survival of the bacterial cells.

In order to determine the adhesion patterns and indexes of *E. coli* strains, experimental infection of the HeLa monolayer was carried out. The main adherence patterns were represented by localized adhesion (bacterial cells form microcolonies on the surface of the host cell), aggregative adherence (cell adhered to the surface of the host cell membrane and between

them) and diffuse adherence (diffuse cell adhered to the surface the host cell membrane) (Figure 1). Patterns of adherence allow correlation of strains with certain pathotypes (J.P. NATARO & J.B. KAPER [14]). Localized adherence is characteristic enteropathogenic *E. coli* strains that cause type A / E damage on cell substrate and aggregative adherence is typical for EAEC strains of *E. coli* and diffuse adherence is characteristic of DAEC and ETEC (S. BOUZARI & al. [15]; L.R. TRABULSI & al. [16]; I.C.A. SCALETSKY & al. [17]).

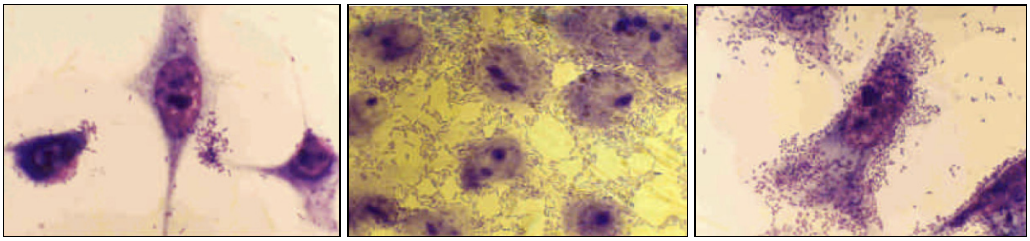

Fig. 1. Adherence patterns of *E. coli* strains to HeLa cell line; **A**) localized adhesion; **B**) aggregative adhesion; **C**) diffuse adherence; Giemsa staining; Magnification: x 2500.

The adhesion index and pattern of the analyzed *E. coli* strains varied with the composition of the used culture medium. For example, the bacterial strains grown in nutrient media (simple broth and broth with casiminic acids), showed a localized adherence pattern in 43.75% of bacterial strains, an aggregative one in 31.25% and a diffuse adherence in 25% of the tested strains. It was also noted that the same pattern of adhesion was evidenced both in simple meat broth and broth containing casiminic acids.

In adition, when the bacterial strains were grown in nutrient media (simple meat broth and broth with casiminic acids), no major differences in the adhesion index of tested strains was observed, 56.25% of the tested strains exhibiting a high adhesion index (50-100%). Increased adherence index demonstrates the potential of these strains to adhere and colonize human tissues and initiate an infectious process (N. WAKIMOTO & al. [18]; L. MESHRAM & al. [19]; M. MARTINEZ-MEDINA & al. [20]).

When the strains were grown in a poor nutrient medium (M9 medium) and in a nutrient medium M9 medium supplemented with casiminic acids, major differences are observed both in the adhesion index and the patterns of adhesion. The bacterial strains maintained in poor nutrient medium, showed a lower adherence index in 93.75% of cases, but in the nutrient media M9 supplemented with casiminic acids the bacterial adhesion was increased (Figure 2).

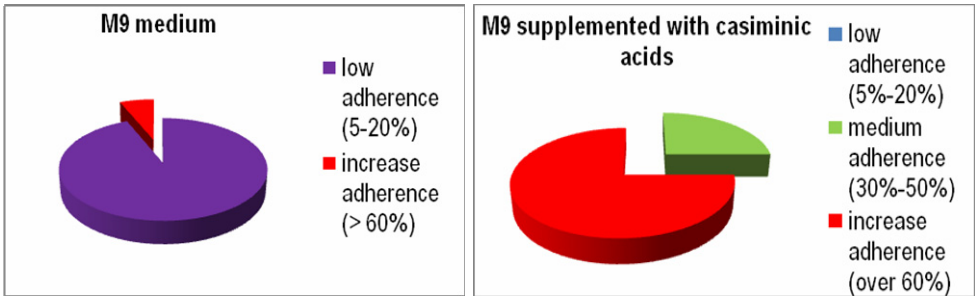

Fig. 2. The adherence index of *E. coli* growing in M9 medium (left) or M9 supplemented with casiminic acid (right).

Casiminic acids are composed of amino acids and peptides obtained by acid hydrolysis of casein, a source of nutrients and thus favoring the growth and adherence of bacteria, processes that occur with low efficiency in poor nutrient media; these nutrients are known to stimulate the biosynthesis of extraparietal protein structures, like pilli and fimbriae, which explains the intense adherence when bacterial strains were cultivated in medium supplemented with casiminic acids.

On the other hand, adherence pattern was modified from a difuse (in the cases of bacteria growing in M9 medium) to a difuse-localizedone (in the case of M9 supplemented with casiminic acids).

Our results showed that bacterial adhesion to a cellular substrate is highly dependent on the nutrients concentration in the medium.

3.2 Flowcitometry assessment of the bacterial adhesion to THP1 cells grown in suspension

After the contact of the THP-1 cells with FITC marked bacterial cells, the adhesion process was evaluated by flowcytometry. The obtained results were analysed using dot-plots (figure 3). This allows to determineareas of interest corresponding to cell subpopulations, facilitating the calculation of the percentageof adheredbacterial cells to THP1 cells, depending on the intensityof analyzed signals.

Bacterial strains grown in M9 minimal medium showed lower values (between 0.01%-0.62) which demonstrate that there has been a lower adhesion process. Instead, bacterial strains grown in M9 medium containing casiminic acids showed values in the range 7.05 - 97.1%, which shows an increased capacity of adhesion to eokaryotic cells.

The adherence percentages obtained using flowcytometry method were smaller than optical microscopy. This issue is probably due to the fact that the method uses killed and then fluorescently labeled bacteria. Instead, live bacterial cells, used in microscopic method, multiply exponentially and thus the number of bacteria that can adhere increases. Flow cytometry allows the end point measurement of bacterial adhesion much faster and in a much more accurate than optical microscopy.

3.3. Evaluation of capacity to adhesion to inert substrate

E. coli developed specific mechanisms by which the various components of the surface, in particular adhesins that are necessary to the steps of biofilm formation are produced (M.W.VAN DER WOUDE [21]).

The ability of the slime production by *E. coli* strains, depending on the thickness of the ring formed in the air-liquid interface is shown in Figure 4.

It was demonstrated that the protein composition of culture media influence the biofilm development. For example, the increase in intracellular levels of cyclic guanosine tetraphosphate, an intracellular signaling molecule that plays a role in the cellular response due to the absence of amino acid in the medium, promotes the expression of genes, including those for type I fimbriae, which leads to adaptation of cells in a slow growth and survival in stressful environments (D.W. JACKSON & al. [22]). In our study, strains growing on a nutrient-poor medium were not producing slime, while 25% of strains growing on nutrient-rich media (including *E. coli* reference strain ATCC 1842) showed a strong ability to produce slime, 37.5% strains a moderate one, and 37.5% strains had a low capacity to produce slime.

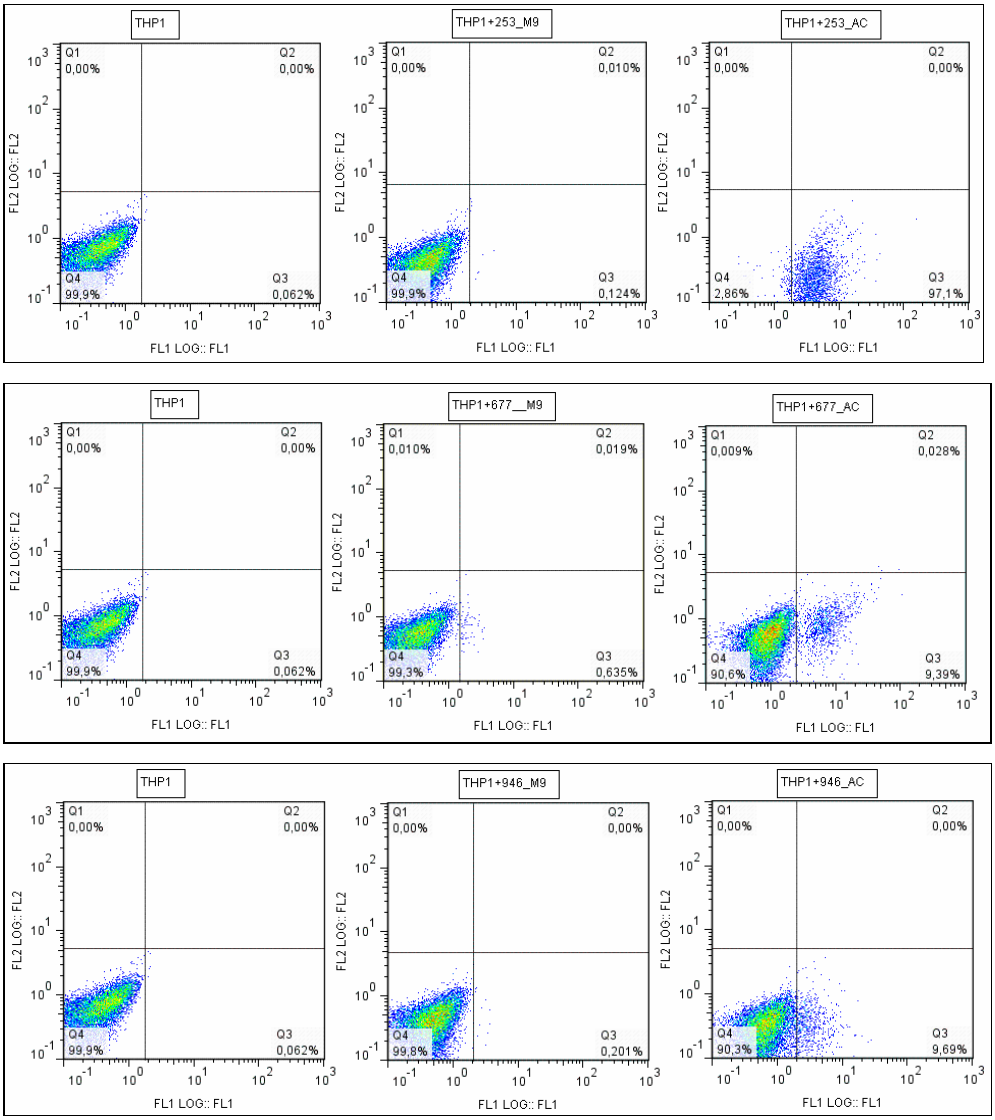

Fig. 3. Biparametric dot-plots representative for bacterial adhesion.

Production of slime is considered as a virulence factor of *E. coli* strains responsible for infections development, persistence and colonization of tissues in susceptible hosts, and different production levels invarious mediacan be an argument for diverse manifestations of these infections produced in different patients.

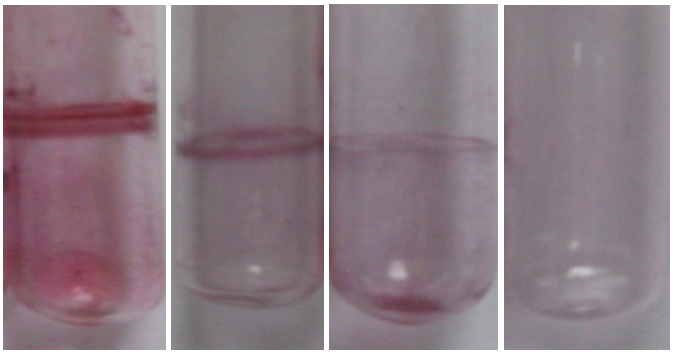

Fig. 4. The capacity of *E. coli* strains to produce slime;
A) strong; **B**) Moderate; **C**) low; **D**) absent

Strains grown in M9 medium supplemented with casiminic acids have a greater ability to adhere to inert substratum and form biofilms (0.375 ± 0.084) compared to those grown in M9 medium (0.0476 ± 0.0056) (Figure 5). These statistically significant increases of absorbance ($p < 0.0001$) mark biofilm development. A slight increase in absorbance values at 48 hours indicates the maturation of biofilm produced by strains grown in nutrient medium. These results confirm the study by Pratt and Kolter (1998), which states that the ability to form biofilms is influenced by cultivation medium: in a minimal medium biofilms producing is very weak, almost non-existent, but in a highly nutritive medium bacterial capacity to produce biofilms grows (L.A. PRATT & R. KOLTER [23]).

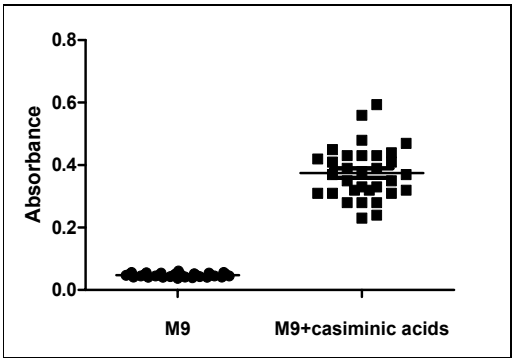

Fig. 5. Graphic representation of the absorbance values obtained for biofilm formation using *E. coli* strains grown on poor nutrients medium M9 and nutritional medium (casiminici acid in M9 medium).

4. Conclusions

Adhesion of *E. coli* strains to inert and cellular substrates and formation of biofilms are complex processes that are influenced by many factors. This work highlighted the variation of bacterial adhesion in different culture conditions in *E. coli* strains isolated from different infections sites. The experimental infection of THP-1 and HeLa cells showed that

the degree of adhesion, the adhesion pattern and the virulence ability of the tested strains are influenced by the composition of the used medium; a nutrients rich medium increases the level of adhesion. Also, strains cultured in a rich nutrient medium have a strong ability to produce slime; this method is simple, fast, and can be used as a screening test to assess the risk of infection consecutive bacterial adherence to medical devices. In addition, the ability to form biofilms is biased by the nutrient media: in minimal medium the production of biofilms is very low, almost non-existent, but in a rich nutrient medium ability of bacteria to produce biofilms increases exponentially. Changes of the culture medium composition lead to major differences in the degree of adhesion and hence the degree of formation of biofilms. Lack of standardized methods contributes to different results regarding the contribution of different metabolic and virulence features on the ability of adhesion and biofilm formation, and on the progress of the whole infectious process.

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References

1. F. YAN, D.B. POLK, Commensal bacteria in the gut: learning who our friends are. Curr Opin Gastroenterol, 20, 6, 565-571 (2004).
2. J.B. KAPER, J.P. NATARO, H.L.T. MOBLEY, Pathogenic *Escherichia coli*. Nature Microbiology, 2, 2, 123-140 (2004).
3. C.F. MARRS, L. ZHANG, B. FOXMAN, *Escherichia coli* mediated urinary tract infections: Are there distinct uropathogenic *E. coli* (UPEC) pathotypes? FEMS Microbiology Lett, 252, 2, 183-190 (2005).
4. V.K. VISWANATHAN, A. WEFLEN, A. KOUTSOURIS, J.L. ROXAS, G. HECHT, Enteropathogenic *E. coli* – induced barrier function alteration is not a consequence of host cell apoptosis. Am J Physiol Gastrointest Liver Physiol, 294, 5, G1165-70 (2008).
5. M.G. GAREAU, N.K. HO, D. BRENNER, A.J. SOUSA, L. LEBOURHIS, T.W. MAK, S.E. GIRARDIN, D.J. PHILPOTT, P.M. SHERMAN, Enterohaemorrhagic, but not enteropathogenic, *Escherichia coli* infection of epithelial cells disrupts signalling responses to tumour necrosis factor alpha, Microbiology, 157, 2963-2973 (2011).
6. J.L. ROXAS, K. RYAN, G. VEDANTAM, V.K. VISWANATHAN, Enteropathogenic *Escherichia coli* dynamically regulates EGFR signaling in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol. 307, 3, G374-80 (2014).
7. V. YASHUNSKY, L. KHARILKER, E. ZLOTKIN-RIVKIN, D. RUND, N. MELAMED-BOOK, E.E. ZAHAVI, E. PERLSON, S. MERCONE, M. GOLOSOVSKY, D. DAVIDOV, B. AROETI, Real-Time Sensing of Enteropathogenic *E. coli* – Induced Effects on Epithelial Host Cell Height, Cell-Substrate Interactions, and Endocytic Processes by Infrared Surface Plasmon Spectroscopy. PLoS ONE, 8, 10, e78431 (2013).
8. J.L. ROXAS, J.S. WILBUR, X. ZHANG, G. MARTINEZ, G. VEDANTAM, V.K. VISWANATHAN, The enteropathogenic *Escherichia coli* – secreted protein EspZ inhibits host cell apoptosis. Infect Immun, 80, 11, 3850-7 (2012).
9. CRAVIOTO, R.J. GROSS, S.M. SCOTLAND, B. ROWE, An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes, Curr Microbiol, 3, 2, 95-99 (1979).

10. A.M. HOLBAN, M.C. CHIFIRIUC, A.C. COTAR, C. BLEOTU, A.M. GRUMEZESCU, O. BANU, V. LAZAR, Virulence markers in Pseudomonas aeruginosa isolates from hospitalacquired infections occurred in patients with underlying cardiovascular disease, Rom Biotechn Lett., 18, 6, 7243-7254 (2013).
11. H. ZEPEDA-LOPEZ, G. GONZALEZ-LUGO. *Escherichia coli* adherence to HEp-2 Cells with Prefixed Cells, J Clin Microbiol, 33, 5, 1414-1417 (1995).
12. G. O'TOOLE, H.B. KAPLAN, R. KOLTER, Biofilm formation as microbial development. Ann Rev Microbiol 54, 49-79 (2000).
13. J.H. MERRITT, D.E. KADOURI, G.A. O'TOOLE, Growing and analyzing static biofilms. Curr Protocol Microbiol, 22, 1B.1.1-1B.1.18 (2011).
14. J.P. NATARO, J.B. KAPER, Diarrheagenic *Escherichia coli*. Clin Microbiol Rev, 11, 142-201 (1998).
15. S. BOUZARI, M.N. JAFARI, F. SHOKOUHI, M. PARSI, A. JAFARI, Virulence-related sequences and adherence patterns in strains of enteropathogenic *Escherichia coli*. FEMS Microbiol Letts., 185, 89-93 (2000).
16. L.R. TRABULSI, R. KELLER, T.A.T. GOMES, Typical and atypical enteropathogenic *Escherichia coli*. Emerg Infect Dis, 8, 508-513 (2002).
17. I.C.A. SCALETSKY, K.R.S. ARANDA, T.B. SOUZA, N.P. SILVA, Adherence factors in atypical enteropathogenic *Escherichia coli* strains expressing the localised adherence-like pattern in Hep-2 cells. J Clin Microbiol, 48, 302-306 (2010).
18. N. WAKIMOTO, J. NISHI, J. SHEIKH, J.P. NATARO, J. SARANTUYA, M. IWASHITA, K. MANAGO, K. TOKUDA, M. YOSHINAGA, Y. KAWANO, Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*, Am J Trop Med Hyg, 71, 5, 687-690 (2004).
19. L. MESHRAM, R.K. PATIDAR, M. KHARE, S. BAGDE, K.N. SAHARE, V. SINGH, Comparative analysis between biofilm formation of commensal and pathogenic *Escherichia coli* isolates, Asiatic J Biotechn Res, 03, 10,1441-1446 (2012).
20. M. MARTINEZ-MEDINA, L.J, GARCIA-GIL, *Escherichia coli* in chronic inflammatory bowel diseases: An update on adherent invasive *Escherichia coli* pathogenicity, World J Gastrointest Pathophysiol, 5, 3, 213-227 (2014).
21. M.W. VAN DER WOUDE, Re-examining the role and random nature of phase variation. FEMS Microbiol Letters, 254, 2, 190-197 (2006).
22. D.W. JACKSON, J.W. SIMECKA, T. ROMEO, Catabolite repression of *Escherichia coli* biofilm formation. J Bacteriol, 184, 12, 3406-3410 (2002).
23. L.A. PRATT, R. KOLTER, Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 30, 285-293 (1998).