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Virulence profiles of pathogenic bacterial strains isolated from different sources

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ABSTRACT

The main goal of present study was to investigate the biofilm formation, adherence to inert and epithelial cells and invasion ability of 20 pathogenic strains isolated from meat, cerebrospinal fluid, blood, wound secretions and faeces. *Escherichia (E.) coli* ATCC 10532 was included in the present study as a reference strain. Two methods were performed to identify slime production and biofilm formation, the Congo red agar qualitative method and a more confident quantitative one, , i.e. the microtiter plate assay. Congo red agar method indicated the *Staphylococcus* (*S.) aureus* 53 (100) strain followed by *E. coli* 7 as the strongest biofilm production strains. The microtiter plate assay identified *Bacillus* (*B.) cereus* 53(100) to be a very good biofilm former strain, followed by *E. coli* 15 159, 16, *E. coli* ATCC 10536, *Salmonella* (*S.) arizonae* 18, *S. pullorum* 19 and *S. aureus* 54. The qualitative assay for bacterial adherence to eukaryotic cells, performed using the HCT-8 cell line, demonstrated that all strains adhered to the epithelial cell line excepting *E. coli* 8, *S. arizonae* and *B. cereus53*(100). Various adherence patterns including some mixed diffuse or aggregative localized patterns were evidenced. Quantitative assay of total bacterial adherence and invasion ability were performed only for 17 gentamycin-sulphate sensitive strains, showing great invasion ability of *S. arizonae* 19, no invasion capacities of all *E. coli* and *L. monocytogenes* strains and interspecific variability of *Salmonellae*. Motility assay revealed that excluding *E. coli* EPEC O126B16 and *FQ2b* strains, all of the tested strains expressed with different levels the swarming behavior.

Keywords: *biofilm formation, adherence, invasion, HCT-8, microtiter assay, Congo red, pathogens, slime production, motility.*

1. INTRODUCTION

In order to support colonization, invasion, and pathogenesis, most pathogens have developed different effective virulence factors depending on the type of infection and the nature of the host response. One of the main virulence factors is biofilm formation [1].

Development of a biofilm involve an initial adhesion to the surface and a further maturation phase. Bacterial adherence to the surface represents the first step of biofilm formation and is initially mediated by Van der Waals forces, electrostatic and hydrophobic interactions. The maturation phase requires intercellular aggregation that generate the typical „mushroom-like" 3-dimensional structure of mature biofilms [2].

Bacterial strains in natural habitats, especially from aquatic ecosystems, are commonly organized in biofilm consortia.

Communities of microorganisms within biofilms are firmly attached to biotic or abiotic surfaces and embedded in an extracellular self-produced matrix composed of polymeric substances which gives them several advantages compared to those living as planktonic cells. The ability of microorganisms to form biofilms plays a major role in pathogenesis as it promotes bacterial survival under rough environmental conditions, being shielded in a polysaccharide matrix, as well as confers nutrient availability, metabolical cooperation, acquisition of new genetic traits by horizontal genetic transfer and enhanced resistance to desiccation, peeling, host defence factors and antimicrobial agents [1,3,4,5].

Additionally, cells inside biofilm are able to communicate by a system of population density detection named quorum sensing, which proved to be involved in cell attachment and detachment from biofilm [6]. According to Casadevall and Pirofski [7], quorum sensing regulation is a three steps process: first, bacteria produce small signaling molecules which then accumulate as a function of cell density and when finally a threshold concentration is reached, bacterial response starts.

Regulatory responses to quorum-sensing molecules may affect the microbial physiology and virulence.

It is hard to correlate biofilm formation experimented *in vitro* with the virulence showed *in vivo*, where the interactions between biofilm formation and other factors can be more complex. Thus, it should be noted that for various pathogens such as *Listeria (L.) monocytogenes* and *Staphylococcus (S.) aureus*, the biofilm tendency established *in vitro* may not reflect the *in vivo* situation [7].

The classical method that can detect biofilm formation on inert surfaces is the microtiter plate assay. Although it hardly represents the *in vivo* characteristics of biofilm-associated infections, this method is very useful in large screens for biofilm-related factors [2]. Biofilms must be treated as an important public

health problem because of their role in certain antibiotic resistance infectious diseases and device-related infections [6].

The first steps of bacterial infection after oral contamination are the adherence and invasion of the host gastrointestinal epithelium [8]. Epithelial cell lines serve as models to simulate the interaction between enteric pathogens and gastrointestinal host cells *in vivo*. Three main adherence patterns of pathogenic strains to different cell lines have been described: diffuse, localized, and aggregative. So far, the most studied phenotype is the localized adherence (LA) of enteropathogenic *Escherichia (E.) coli* (EPEC) to HEp-2 (human epidermoid carcinoma) cells. In this adherence pattern, bacterial-eukaryotic cell association triggers the accumulation of actin filaments followed by pedestals formation that provide the capacity of bacteria to produce attaching/effacing lesions [9].

Even if virulence mechanisms of EPEC are not well known yet, it's clear that bacterial adhesion is crucial in several infections, so many studies developed to evidence the adherence process of EPEC. It has been reported by transmission and scanning electron microscopy a localized adherence to HEp-2 and HeLa cells and a localized destruction of microvilli. Researches showed a high bacterial adherence to intestinal cells in infants and animals with diarrhea induced by EPEC [10].

Diarrheal illness is a public health problem responsible for over 2 million deaths each year, especially among infants, most common caused by EPEC [4]. The development of diarrhea may be considered a host defense mechanism intended to get rid the host from enteric pathogens such as EPEC. However, this kind of defense mechanism is double-edged since it may allow the spread of bacteria from host to host [8].

Despite it is not an invasive pathogen, in few cases EPEC is able to penetrate epithelial cells. EPEC pathogenesis assumes three main stages consisting of localized adherence to epithelial cells, signal transduction and intimate adherence to epithelial cell surface. Most of EPEC adhered cells induce attaching and effacing lesions, but a subpopulation is internalized by epithelial cells [11]. However, in tissue culture, the infection with A/E bacteria kills eukaryotic infected cells, after a prolonged contact, which can become round and detach.

Most infections can be contracted through contaminated food or water. Although direct person-to-person transmission is possible, due to the harsh gastrointestinal conditions, a number of minimum 10^6-10^9 microorganisms are necessary to develop the symptomatic illness. The resisting bacteria have the opportunity to show their invasive capacity by internalizing and eventually passing through the intestinal epithelium. Because most of invasive pathogens are able to invade even cultured epithelial cells in vitro, the invasion process can be studied experimentally.

After host cell invasion, the microorganism is captured in a phagosomal vacuole that becomes acidified as a defensive response of the host. Some invasive pathogens, such *L. monocytogenes*, manage to escape the vacuole and reach the host cytosol. Once inside the cell, bacteria remain capable of intracellular replication.

The most widely used assay for internalization of bacteria is the antibiotic protection assay, based on the assumptions that the addition in medium of antibiotics that can't penetrate the eukaryotic cell membrane will kill all bacteria but those internalized. Both adhesion and internalization can be measured by determining the total number of CFU before and after the antibiotic treatment [8].

2. EXPERIMENTAL SECTION

2.1. Bacterial isolates. A number of 20 pathogenic strains purchased from NIRDMI Cantacuzino Zoonosis Laboratory Collection, belonging to *Bacillus*, *Escherichia*, *Klebsiella*, *Listeria*, *Salmonella* and *Staphylococcus* genera were maintained on BHI (Brain Heart Infusion broth) with 20% glycerol at -70°C. Beside these isolates, *E. coli* ATCC 10532 (*American Type Culture Collection*) was included as a reference strain.

2.2. Detection of biofilm formation by the Congo red agar method. Qualitative detection of biofilm formation was performed according to the protocol previously described by Saising *et al*., [12]. Briefly, the overnight cultures grown in liquid BHI broth at 37°C were inoculated on a specific medium containing 37 g/L brain heart infusion broth (Difco), 50 g/L sucrose, 0.8 g/L Congo red and 10 g/L agar (Merck). Inoculated plates were then incubated for 24 hours at 37°C. Biofilm producing strains developed as black colonies while non-biofilm producing strains presented red colonies.

2.3. Detection of biofilm formation by the microtiter plate method. Detection and quantification of biofilms were performed using 96-well polystyrene microtiter plate, using crystal violet staining, according to the method of Cendra *et al*., [13], with slightly modifications. Isolated strains were inoculated in solid LB broth and grown overnight at 37°C. The colonies were resuspended in phosphate buffered saline (PBS) with a density corresponding to 0.5 Mc Farland, then 100 µL was inoculated in four replicate in 96-well microplate. Controls consisted of uninoculated wells containing only sterile medium. Plate was incubated without mixing for 48 h at 37°C to allow biofilm formation. Following incubation, non-adherent bacteria were removed by aspiration and plate was washed two times with 200 µl/well of PBS. Thereafter, plate was air dried, and stained for 15 minutes with 180 µl crystal violet 0.1% solubilized in ethanol 95%. Excess stain was decanted off and plate was washed with PBS and dried. The remaining crystal violet, corresponding to atached cells, was solubilized in 200µl acetic acid 33% and resuspended. Finally, the amount of crystal violet was determined by measuring Optical Density at 620 nm. Optical density of each four replicates was averaged.

2.4. Adhesion assay. Quantitativ bacterial adhesion assay was performed according to the method of Scaletsky *et al*., [10], in 6 well plates using the HCT-8 epithelial cell line. HCT-8 cell line was cultivated in RPMI 1640 (Gibco) supplied with 10% FBS medium in anaerobic conditions at 37°C and 5% CO_2. After tripsinization, cells were seeded in 6-well plates and incubated nearly 3 days until the cellular confluence becomes 90%. RPMI medium containing planktonic cells is decanted off and 1.5ml of

0.5% McFarland bacterial suspension in PBS from tested strains grown overnight at 37°C in LB was added in each of the 6 wells. Plates were then incubated at 37°C in aerobic conditions for 3h., washed twice with PBS and fixed 5 minutes with methanol. After drying, the plates were Gram stained and examined under a light microscope at imersion objective.

2.5. Total adherence and invasion assay. For this experiment we choose 17 pathogens, that were senzitive to gentamicin-sulphate. All 20 pathogens were previously tested for 400µg/ml and 800µg/ml gentamicin-sulphate resistance by counting CFU and only 3 had been resistant. (*E.coli* 159, Fqa2 and Fq2b). The rest of them were senzitive to both concentrations of gentamicin-sulphate. Total adherence and invasion of HCT-8 cells was tested according to the method of McKee and O'Brien [9], with few changes. In order to determine the total adherence, corresponding to adherent and intracellular bacteria, the HCT-8 cells were lysed after 2h infection period and the bacterial cells were quantified. For assesing the invasion, coresponding only to the internalized bacteria, the infected cell monolayers were treated with 400 µg/ml gentamicin-sulphate before lysis, (remaining only the number of bacteria surviving the gentamicin kill assay). Total adherence and invasion assays were performed in a 24-well plate containing 90% confluent HCT-8 cell line. RPMI medium was removed and the wells washed 2 times with PBS, remaining only the adhered monolayer. Bacterial cultures grown 24h at 37°C in LB were used to obtain a 0.5 McFarland PBS suspension which was added to confluent epithelial cell monolayers for 2 h, then the bacterium–HCT-8 mixture was washed gently two times with sterile PBS, pH 7.4. Half of the wells were mild mixing 15 minutes with 100µl of 0.1% Triton X-100 (Sigma) and processed to determine the total number of associated bacteria with the cells without gentamicin treatment by decimal serial diluting up to 10^4 and plating to LB broth. The other half was incubated for 2 additional hours with 300 fresh RPMI medium supplemented with 400 µg/ml gentamicin-sulphate. The infected monolayer was washed 2 times with sterile PBS and also treated 15 minutes with 0.1% Triton X-100. Finally, the solution was serially diluted and plated on LB agar broth. Colonies resulted from total adherence and invasion were counted next day to determine the number of CFU/ml.

2.6. Motility assay. Motility tests were performed only for Gram negative pathogenic strains. 24h grown overnight cultures were plated on semisolid Muller Hinton medium (0.4% agar) with sterile tooth picks, right in the middle of the plate. After 48 hours of incubation at 37°C, motility was observed as a diffusion area around the inoculation point. Results were expressed by measuring the diameter of the diffusion areas.

3. RESULTS SECTION

3.1. Morphological and biochemical characteristics of pathogenic strains. All strains included in the study were already taxonomical identified excepting those isolated from enterocolitis newborns faeces (FQ2b and FQa2). Different pathogenic strains, belonging to *Bacillus*, *Escherichia*, *Klebsiella*, *Listeria*, *Salmonella* and *Staphylococcus* genera, were isolated from meat, cerebrospinal fluid, blood, plague secretions and faeces. Pathogenic strains were inoculated on selective media to evidence the hemolysins, lecithinases, lipases and DN-ases synthesis capacity, as virulence factors. The results were summarized and are presented in the Table 1.

Table 1. Isolation source and biochemical characters of pathogenic strains.

Bacterial strains	Isolation source	Hemolysin synthesis	Lecithinase synthesis	Lipase synthesis	DN-ase synthesis
B. cereus 54(100)		+ (β-hemolysis)	+	±	+
E. coli 159	Plague secretion	-	-	-	-
E. coli 7	Beef sausages in sheep gut	-	-	-	-
E. coli 8	Minced meat (mixture)	-	-	-	-
E. coli 15	Minced meat	-	-	-	-
E. coli 16	Minced meat	-	-	-	-
E. coli 52	Minced meat	-	-	-	-
E. coli EPEC O126B16	Feces	-	-	-	-
K. pneumoniae 2	Feces	-	-	-	-
L. monocytogenes 322	Cerebrospinal fluid	±	-	-	-
L. *monocytogenes* 325	Cristim product	±	+	-	-
L. *monocytogenes* 331 a	blood(septicemia + meningitis)	±	+	-	-
L. *monocytogenes* 331 b	cerebrospinal fluid (meningitis)	±	+	-	-
L. monocytogenes 333	cerebrospinal fluid	±	-	-	-
S. arizonae 18	Sausages	-	-	-	-
S. pullorum 19	Minced meat (mixture)	-	-	-	-
Salmonella sp. 14	Minced meat (mixture)	-	-	-	+
S. aureus 54		+	+	+	-
FQ2b	Enterocolitis newborns faeces	-	-	-	-
FQa2	Enterocolitis newborns faeces	-	-	-	+

3.2. Detection of biofilm formation by the Congo red agar method and microtiter plate method. Microtiter plate method is a confident quantitative assay for biofilm identification and which can detect the process of biofilm formation, while the Congo red agar method is a qualitative one that can only detect slime production, an esential step which suggests biofilm formation [12]. Congo red stain binds to polysaccharides generally synthesized by biofilm producing strains, turning the colony color in black (Figure 1). Slime-producing strains develop black colonies, while non-producing strains develop red colonies.

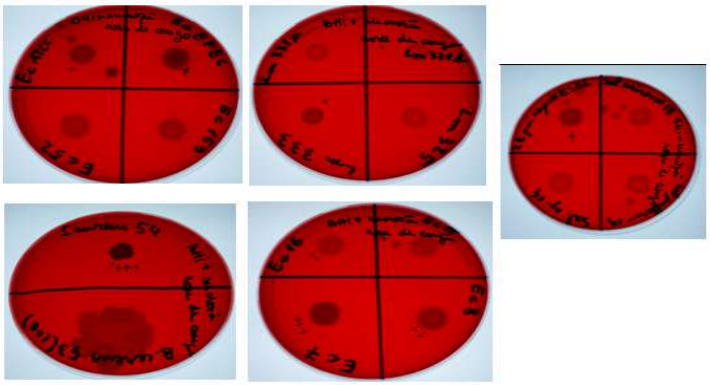

Figure 1. Congo red agar plates after 24 hours of incubation at 37°C. Biofilm formatting strains (black colonies) are marked with "+" in a scale of 1 to 4, depending on the color intensity of colonies.

According to Congo red agar (CRA) method, *S. aureus* 54 was associated with strong biofilm production, followed by *E. coli* 7 as a moderate one, while *E. coli* EPEC, *L. monocitogenes* 333, *E. coli* 16, *E. coli* 8, *E. coli* ATCC 10536 and *K. pneumoniae* 2 ESBL showed week biofilm production.

The microtiter plate assay is a more accurate method than the Congo red agar method, which takes less time and is easyer to perform but the results are less concudent, depending on subjective chromatic evaluation of the colonies.

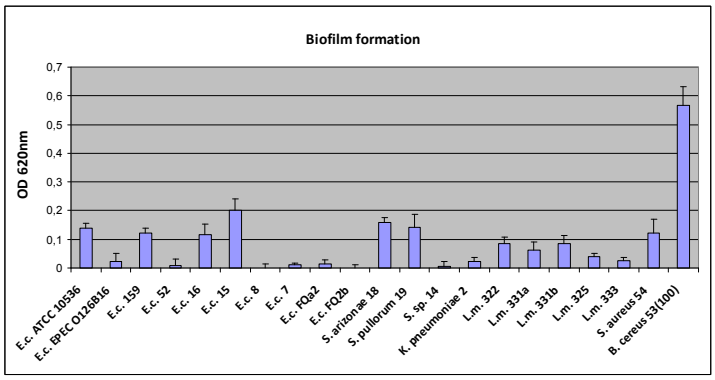

Figure 2. Quantification of biofilms formed on a 96-well polystyrene plate, using the microtiter plate method with crystal violet staining. Each value represents the 620nm absorbance mean of four replicates and the error bars represent standard deviations.

Examining biofilm formation among pathogens, we observed that *Bacillus cereus* 53(100) strain formed a substantially more biofilm than did the other tested strains, showing an absorbance of 0.661667. *E. coli* 15 is also a good biofilm producing strain, because it has the second absorbance value after *B. cereus*, reaching 0.2955. Strains *E. coli* 159, *E. coli* 16, *S. arizonae* 18, *S. pullorum* 19 and *S. aureus* 54 showed week biofilm capacity, presenting similar OD_{620nm} values to *E. coli* ATCC 10536 strain. Besides *L. monocytogenes* strains, which had low levels of biofilm formation, the rest of the strains did not produce any biofilm mass (Figure 2).

Slime-forming ability, evaluated by the Congo red agar method, did not correlate with the biofilm-forming ability on microtiter plates. The possible cause for low correlations between methods could be that slime production, although a favoring condition, is not necessarily associated with biofilm formation, or the broth conditions did not allow slime production by some of the biofilm forming strains revealed from crystal violet method. However, sensitivity, specificity and accuracy of CRA assay were very low in a comparative study of different biofilm detection methods performed by Hassan *et al.,* in 2011. Moreover, Knobloch *et al.,* did not recommend this method for investigation of *S. aureus* biofilm formation in their study [14,15].

3.3. Adhesion assay. From 21 strains tested for HCT-8 epithelium cells adhesion capacity, only *E. coli* 8, *S. arizonae* 18 and *B. cereus* 53(100) didn't adhere, so we obtained an adherence rate of 85.7%. Three patterns of adherence were distinguish: localized adherence (LA), aggregative adherence (AA) and diffuse adherence (DA). The most frequent pattern was LA (42.8%), followed by AA (28.6%) and DA (14.3%) (Figure 3).

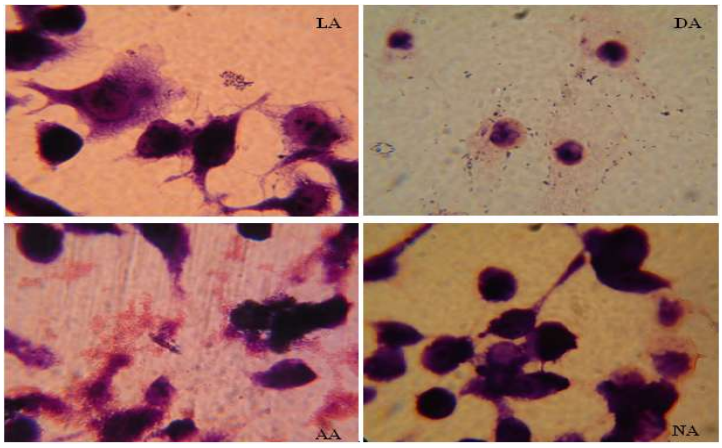

Figure 3. Light photomicrograph of Gram-stained HCT-8 cells showing the three types of pathogen adherence patterns: localized adherence (LA), aggregative adherence (AA), diffuse adherence (DA) and no adherence (NA). Note the clusters of microorganisms in the LA pattern. In the DA pattern, microorganisms adhere to all of the cell surface. In the aggregative pattern, they attach to both bacterial and epithelial cells.

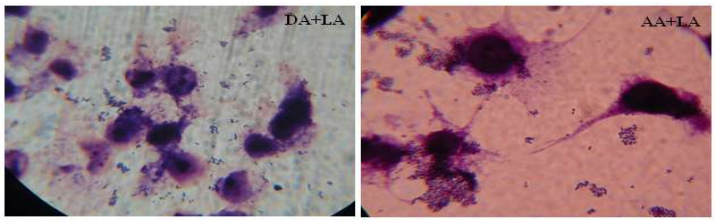

Figure 4. Mixtures of DA and AA with clusters of LA patterns.

In case of *B. cereus* 53(100), *E. coli* 8 and *E. coli* 15 bacterial cells did not adhere or adhered only to plastic surface. Probably these strains have the ability to detach or kill epithelial cells by the synthesized virulence factors.

3.4. Adherence and invasion assay. The total adherence and invasion were calculated by counting the colonies, from a particular dilution that allows the reading, then multiplying the number of colonies with the dilution factor (Figure 5).

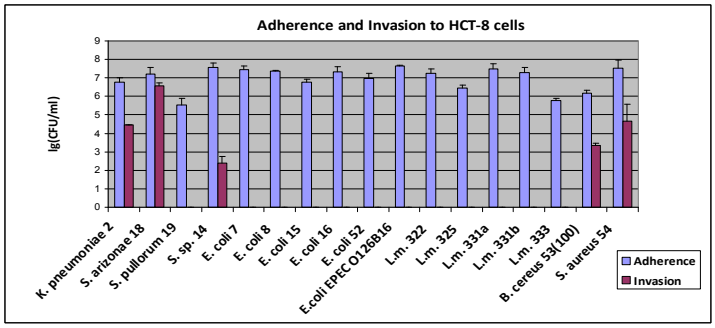

Figure 5. Quantification of the total bacterial adherence and invasion ability to HCT-8 cell line in four pathogen strains sensitive to gentamycin-sulphate, by the gentamycin protection method. The resulting levels are expressed in lgCFU/ml and represent the mean of three replicates.

In this case, the grafic indicates for all tested strains, that showed simillar levels of adherence, with the lg(CFU/ml) values ranging between 5.54 (*S. pullorum* 19) and 7.56 (*S. sp*. 14). Interestingly, strains belonging to *Salmonella* genus exhibited both maximum and minimum adherence rates. Concerning invasion capacity of the pathogens, *S. arizonae* 18 is the most invasive strain, followed by *S. aureus* 54, *K. pneumoniae* 2, *B. cereus* 53(100) and *Salmonella. sp*. 14. Again, except for the strains having no invasion ability, the maximum (6.59) and minimum (2.4) value of invasion belonged to *Salmonella sp.* The fact *Salmonella* strains presented high, weak and no invasion capacities, suggests a great interspecific variability, that is supposed to be a result of species adaptation to a wide range of environmental conditions. A contradictory result is that all of *L. monocytogenes* tested strains showed to be non-invasive, although from most scientific reports it is known the high invasive capacity of this species. The discordance could be explained by the possibility that our strains may lack some of bacterial surface proteins, so the cell-to-cell jonction with epithelial cells could not occur [16]. *E. coli* EPEC O126B16 appeared to be a non-invasive strain, wich is in accordance with the scientific literature, because *E. coli* EPEC is generally considered a non-invasive extracellular pathogen. Researches demonstrate that pathogenesis of EPEC is mainly associated with the attaching/effacing lesions characterized by microvilli destruction, tight adherence to intestinal epithelium, pedestal formation and cytoskeletal alterations as a result of actin filaments agregation at bacterial adesion sites [17]. However, sporadic reports revealed the existence of atypical EPEC strains that can invade non-phagocytic epithelial cells through intracellular actin pedestal formation [18].

The antibiotic protection method is feasable and reproducible if the cell membrane is not damaged during invasion, allowing antibiotic to kill the internalized bacteria. Also, the inability to distinguish between the rate of internalized bacteria from that of intracellular multiplication or between adherent bacteria from those that invade the cells, but are released after triggering the apoptotic process, represents another limit of this method [8].

3.5. Motility assay. Motility was performed only on Gram negative strains, because it is an important virulent factor due to certain motion organelles (flagella, pilli, fimbriae), giving them certain advantages in the competition for nutrients, colonization of the intestinal epithelium and avoiding antimicrobial compounds.

The result was quantified by measuring the diameter of the diffusion zones that appear only at swarming strains. Non swarming strains were developed only in the center of the plate, at the inoculation site (Figure 6).

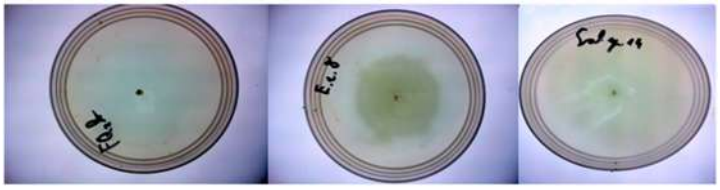

Figure 6. Exemplification of the motility test results: lack, moderate and high motility is observed from left to right. All images were captured after 24 h at 37°C.

Besides *E. coli* EPEC O126B16 and *FQ2b* strains, that failed to exhibit motility, all of the strains presented moderate (*E. coli 8*, *E. coli 15*, *E. coli 16)* and high swarming behavior. Motility levels varies among the strains (Figure 7).

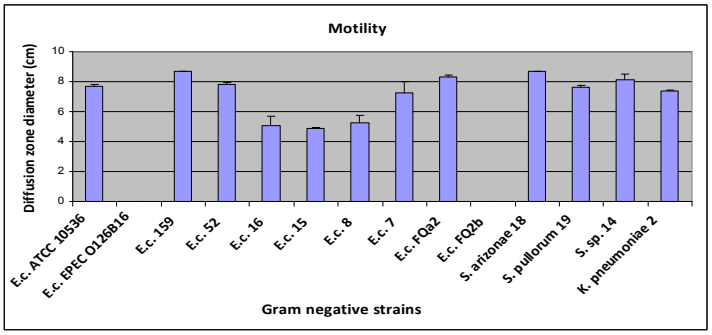

Figure 7. Quantification of Gram negative pathogenic strains swarming capacity, based on diffusion zone diameter.

4. CONCLUSIONS

In this study, a number of 21 pathogenic strains were tested regarding biofilm formation, adherence and invasion ability, using different quantitative and qualitative methods such microtiter plate, Congo red agar, Gram stained cell adhesion and antibiotic protection assay. Examining biofilm formation among pathogens by microtiter method, *B. cereus* 53(100) strain demonstrated a very good level, followed by *E. coli* 15, *E. coli* 159, *E. coli* 16, *E. coli* ATCC 10536, *S. arizonae* 18, *S. pullorum* 19 and *S. aureus* 54 with moderate and week biofilm formation capacity.

Unfortunately, these results didn't correlate with those obtained by Congo red agar method which finds *S. aureus* the strongest biofilm production strain followed by *E. coli* 7 as a moderate one. However, Congo red assay is a low accurate method that can detect indirect biofilm formation by slime production capacity.

A 85.7% adherence rate was obtained after adhesion assay to HCT-8 epithelial cells. Generally, *E. coli* and *L. monocytogenes* strains were the most adherent strains. The three main adherence patterns were recorded, but the most frequent was the localized adherence. The *E. coli* strains showed especially an aggregative pattern, but other strains like all *L. monocytogenes* and *E. coli* EPEC O126B16 strains displayed a mixture of AA or DA with LA patterns.

Quantification of the total bacterial adherence and invasion ability showed that all of the tested strains had simillar levels of adherence, and regarding invasion capacity, *S. arizonae* 18 is the most invasive, followed by *S. aureus* 54, *K. pneumoniae* 2, *B. cereus* 53(100) and *Salmonella sp*. 14 strains. According to other scientific reports, our strain didn't exhibit any invasive characteristics. All *L. monocytogenes* and *E. coli* tested strains showed to be non-invasive as well, but this is in contradiction with literature data, because both species are generally known to possess invasive properties.

Motility test revealed that excepting *E. coli* EPEC O126B16 and *FQ2b*, all Gram negative strains have swarming abilities, which mean they have an advantage in reaching the intestinal epithelium through the intestinal mucus layer.

5. REFERENCES

[1] Mohandas V., Ballal M., Distribution of *Candida* species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in Southern India, *Journal of Global Infectious Diseases*, 3, 1, 4–8, **2011.**

[2] Otto M., Staphylococcal biofilms, *Current Topics in Microbiology and Immunology,* 322, 207–228, **2008**.

[3] Ramli N.S.K., Guan C.E., Nathan S., Vadivelu J., The effect of environmental conditions on biofilm formation of *Burkholderia pseudomallei* clinical isolates, *Plos one* , 7, 9, 44104, **2012**.

[4] Weiss-Muszkat M., Shakh D., Zhou Y., Pinto R., Belausov E., Chapman M.R., Sela S., Biofilm formation by and multicellular behavior of *Escherichia coli* O55:H7, an atypical enteropathogenic strain, *Applied and Environmental Microbiology,* 76, 5, 1545–1554, **2009**.

[5] Burmolle M., Webb J.S., Rao D., Hansen L.H., Sorensen S.J., Kjelleberg S., Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms, *Applied and Environmental Microbiology*, 72, 6, 3916–3923, **2006**.

[6] Rodney M.D., Biofilms: microbial life on surfaces, *Emerging Infectious Diseases*, 8, 9, 881–890, **2002**.

[7] Casadevall A., Pirofski L., Virulence factors and their mechanisms of action: the view from a damage–response framework, *Journal of Water and Health*, 7, 51, 52-518, **2009**.

[8] Allen-Vercoe E., Apicella M.A., Bitar D.M., Cornelis G.R., Courtney H.S., Denecker G., DeVinney R., Edwards J.L., Fives-Taylor P., Freitag N.E., Harvey H.A., Kwaik Y.A., Lamont R.J., Lippmann J.E., McCormick B., Meyer D.H., Molmeret M., Podbielski A., Sasakawa C., Wong K.K.I., Yilmaz O., Bacterial invasion of host cells*, Cambridge University Press*, 1-315, **2004**.

[9] McKee M.L., O'Brien A.D., Investigation of enterohemorrhagic *Escherichia coli* O157:H7 adherence characteristics and invasion potential reveals a new attachment pattern shared by intestinal *E. coli, Infection and Immunity,* 63, 5, 2070-2074, **1995**.

[10] Scaletsky I.C., Milani S.R., Trabulsi L.R., Travassos L.R., Isolation and characterization of the localized adherence factor of enteropathogenic *Escherichia coli*, *Infection and Immunity*, 56, 11, 2979-2983, **1988**.

[11] Budeau J., Glasser A.L., Masseret E., Joly B., Darfeuille-Michaud A., Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease, *Infection and Immunity*, 67, 9, 4499-4509, **1999**.

[12] Saising J., Singdam S., Ongsakull M., Voravuthikunchai S.P., Lipase, protease, and biofilm as the major virulence factors in staphylococci isolated from acne lesions, *BioScience Trends*, 6, 4, 160-164, **2012**.

[13] Cendra MdM., Juarez A., Torrents E., Biofilm modifies expression of ribonucleotide reductase genes in *Escherichia coli*, *Plos one*, 7, 9, 46350, **2012**.

[14] Hassan A., Usman J., Kaleem F., Omair M., Khalid A., Iqbal M., Evaluation of different detection methods of biofilm formation in the clinical isolates, *Brazilian Journal of Infectious Diseases*, 15, 4, 305-311, **2011**.

[15] Knobloch J.K., Horstkotte M.A., Rohde H., Mack D., Evaluation of different detection methods of biofilm formation in *Staphylococcus aureus*, *Microbiology and Immunology*, 191, 2, 101-106, **2002**.

[16] da Silva C.V, Cruz L., da Silva Araújo N., Angeloni M.B, Fonseca B.B., Gomes A.O., Carvalho F.R., Ribeiro Goncalves A.L., Barbosa B.F., A glance at *Listeria* and *Salmonella* cell invasion: Different strategies to promote host actin polymerization, *International Journal of Medical Microbiology*, 302, 19–32, **2012**.

[17] Alikhani M.Y., Mirsalehian A., Aslani M.M., *HeLa* cell adherence patterns of enteropathogenic *Escherichia coli* strains isolated from faecal samples. *Pakistan Journal of Biological Sciences,* 9, 8, 1465-1469, **2006**.

[18] Bulgin R., Arbeloa A., Goulding D., Dougan G., Crepin V.F., Raymond B., Frankel G., The *T3SS* effector *EspT* defines a new category of invasive enteropathogenic *E. coli* (EPEC) which form intracellular actin pedestals, *Plos Pathogens*, 5, 12, 1000683, **2009**.

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