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Ability of *Cronobacter sakazakii* to adhesion and invasion AMGM5 cell line

Hayder A. Al-mandalawii[1], Luma Abdulhady Zwain[2], Estabraq A. Mahmoud[2]

1 Directorate of education of Rasafa /ministry of education/Iraq
2 Department of Biology /College of Education Pure Science Ibn Al-Haitham/University of Baghdad/Iraq.

Abstract:

Background: Cronobacter sakazakii is gram-negative bacteria , rod–shaped , facultative anaerobic the growth temperature range is 6–45 ° C . It is have many virulence factor such OmpA , flagella and extracellular glycoproteins help to adhesion and invasion of the host and found in macrophage to use invasion other organs in body .
Aim of the study: Study ability of bacterial to adhesion on non-living surfaces (polystyrene) and living surfaces (brain cancer cell line) and its invasion , its cytotoxicity.
Patients and methods
Fifteenth of C. sakazakii were obtained from previous studies (5 infant formula , 5 spinal fluid and 5 bloods) . Study of movement ability of bacteria , adhesion to polystyrene , adhesion to AMGM5 , invasion of AMGM5 and its cytotoxicity .
Results: In this study , the number of C. sakazakii colony adherent to polystyrene plate more than 300 CFU for four isolated , while adhesion to (AMGM5) in dilution (1 : 10 and 1 : 100) were more than 300 CFU to four isolated , the number of invasion colonies to AMGM5 were CSF5 (285 CFU) , A1C (220 CFU) , B1 (<300 CFU) , C1 (235 CFU) . Cytotoxicity of C . sakazakii was 45. 81 % when using A1C isolate.
Conclusion: C . sakazakii have the ability to movement , adhesion to polystyrene , adhesion and invasion to Cerebral glioblastoma multiforme and cytotoxic .

Keyword: *Cronobacter sakazakii, AMGM5 cell line.*

Introduction:

Cronobacter sakazakii is one of the Enterobacteriaceae family, and it is gram- negative bacteria , rod–shaped , peritrichously flagellated and non-spore forming, it is facultative anaerobic as it can grow without oxygen or grow with a small of oxygen ,the growth temperature range is 6–45 C with Optimum temperature of 37–43 C (1) . C. *sakazakii* is ability to survive on long time in environment as powder infant material which leads transmission of bacteria to the immune compromised infant (2) . C. *sakazakii* caused necrotizing enterocolitis in infants , dangerous neurological diseases, meningitis and septicemia (3) . C. *sakazakii* growth in the intracellular of macrophages and its use to invasion the other organs in body (4) . Biofilm formation and its adhesion to plastic surfaces and capsular production helps protect it from macrophages and preserve them in dry environments (5) . It is have many virulence factor such as zpx gene responsible for the lysis of collagen , and its role in the crossing of bacteria to the blood barriers of brain (6) . OmpA help to break blood-brain barrier and invasion central nervous system causing meningitis (7) many studies refer to C . *sakazakii* have plasmid (PESA3) encode to Cpa ,its role in protecting bacteria from complement as well as plasmogen activation and inhibition 2- alpha antiplasmin (8) . The attack of bacteria to the host's cells is unknown, but C. *sakazakii* have outer membrane proteins , its help to affect the host's cells (9) . Penetration of the blood-brain barrier through the small blood vessels leads to meningitis, causing C. *sakazakii* The death rate is high, depending on the immune system of the host body (10). The role of flagella in chemotaxis , adhesion and invasion of the host (11) . Extracellular glycoprotein lead to adhesion intestinal epithelial and endothelial cells (12) .

Corresponding Address:
Hayder A. Al-mandalawii
Directorate of education of Rasafa/ ministry of education/Iraq
Email: hayderbiology1987@gmail.com

Materials and methods:

Identification of isolates

Fifteenth of C. sakazakii were obtained from previous studies . Isolates were (infant formula , spinal fluid and bloods) . All isolates were cultured on MacConky agar and Tryptone soy agar (TSA) . All isolates of identification based on microscopic , biochemical test and confirmed by 16SrRNA .

Motility test

Motility test according to (13) bacteria were grown in the motility media (TSB + 0.3 agar) by transferring a single isolate to the center of the plate and incubated at 37 c for 4 and 8 hours , and its diameter was measured in millimeter .

Adhesion to polystyrene

Adhesion to Polystyrene microtiter plate were carried out according to modified (13) , The C. sakazakii was inoculated on MacConky agar and incubated at 37˚C for 24 hours . Isolated was inoculated in to normal saline and compared with 0.5 Macfarlane standards then place 100 µl of the saline in Polystyrene plate with 100 µl of media and incubate at 37 ° C for 2 hour, media were removed and then washed at least 3 times with phosphate-buffered saline (PBS) then a series of dilution (1 : 10 , 1 : 100) and on MacConky agar was incubated at 37 ° C for 24 hours and according to the number of colonies per dish .

Adhesion to AMGM5 cell line (14)

It was prepared according to (13) , modified and it is according to the Iraqi Center for Cancer Research and Medical Genetics. Transfer 200 µl of AMGM5 cell line to 96 well microtiter plate . Incubate at 37 ° C for (24 – 72) hours until the cells are attached to the wells, then remove the medium and add 100 µl of RPMI-1640 (without antibiotic) and 100 µl of C. sakazakii culture , incubated at 37 ° C for 2 hours, and media removed , the microtiter plate were washed at least 3 times with PBS , add 200 µl of trypsin-versene , Then a series of dilutions (1:10 ,1:100) was performed and 100 µl of all dilution was diffuser on the surface of MacConky agar , incubated at 37 ° C for 24 hours and according to the number of colonies per dish .

Invasion to AMGM5 cell line

The same steps were used for adhesion to (AMGM5) cell line except for the treatment of cells with RPMI-1640 culture medium containing antibiotics (pencillin / streptomycin) .

Results:

Fifteenth isolates were identified depending on the macroscopic , microscopic characteristics and biochemical test . Identification of all C. sakazakii isolates were definite with 16SrRNA , study of ability of all isolates on motility . Results showed that all isolates have the ability to move after 4 hours of incubation and it increased after 8 hours , as isolates (A1b , A1c , B2 , B5 ,CSF4 and CSF5) had strong movement after 8 hours of incubation , while the other isolates had slow and medium movements (Table -1) .

(15) pointed out that movement of bacteria towards the cell is a provision for adhesion and invasion , as flagella play a fundamental role in colonizing bacteria and he noticed when mutations in the flagella genes that led to a decrease adhesion to Coca-2, cell line . Enterobacteriaceae bacteria can invasion epithelial cells if they are able to move (15).

Table (1) Ability test of *C. sakazakii* on the motility

source	isolation	Diameter mm after 4 hours	Diameter mm after 8 hours
Dialak	A1a	0.7	1.6
Dialak	A1b	1.3	2.9
Dialak	A1c	2	3
Novolac Allernova	C1	0.7	1.4
Novolac AD	C2	0.7	1.6
Blood	B1	0.2	0.3
Blood	B2	1	1.3
Blood	B3	0.7	1.3
Blood	B4	0.7	1.5
Blood	B5	1.4	2.5
Cerebrospinal fluid	CSF1	0.4	0.9
Cerebrospinal fluid	CSF2	0.6	0.7
Cerebrospinal fluid	CSF3	0.4	1
Cerebrospinal fluid	CSF4	1	3
Cerebrospinal fluid	CSF5	2	3.5

Number : (0.1 – 0.5) weak / (0.6 – 1) medium / (1.1 – 3.5) strong .

The ability of these isolates on adhesion were studied in two way the first adhesion on polystyrene microtiter plate , the result obtained after account the number of colonies. the results of the study showed that the number of adherent *C. sakazakii* to Polystyrene microtiter plate more than 300 CFU to four isolated (Table - 2) .

Table (2) Number of *C. sakazakii* adhesion to Polystyrene plates .

Isolation Sample	The number of colony adherent cells dilution 10 : 1	The number of colony adherent cells dilution 100 : 1
CSF5	> 300	>300
A1C	>300	>300
B1	>300	>300
C1	> 300	>300

The second method adhesion by use (AMGM5) cell line , the results of our current study we observed that the number of *C . Sakazakii* colony adhesion in (AMGM5) cell line to dilution (1 : 10 and 1 : 100) were more than 300 CFU to 4 isolated (Table - 3) .

Table (3) Number of C . *sakazakii* attached to the AMGM5 cell line .

Cronobacter *sakazakii* isolation	The number of colony adherent to AMGM5 cells dilution 1 : 10	The number of colony adherent to AMGM5 cells dilution 1 : 100
CSF5	300 >	300 >
A1C	300 >	300 >
B1	300 >	300 >
C1	300>	300>

Finally invasion AMGM5 cell line the results of our current study we observed that the number of C . sakazakii colony invasion in (AMGM5) cell line were CSF5 (285cfu) , A1C (220cfu) , B1 (<300 cfu) , C1 (235cfu) (Table – 4) .

Table (4) Number of C . *sakazakii* invasion to the AMGM5 cell line .

Cronobacter *sakazakii* isolation	The number of colony invasion AMGM5 cells
CSF5	285
A1C	220
B1	300 >
C1	235

Discussion:

(15) pointed out that movement of bacteria towards the cell is a provision for adhesion and invasion , as flagella play a fundamental role in colonizing bacteria and he noticed when mutations in the flagella genes that led to a decrease adhesion to Coca-2, cell line . Enterobacteriaceae bacteria can invasion epithelial cells if they are able to move (16) . It was also observed through the results that bacteria C.sakazakii has the ability to adhere to the non- living surface of polystyrene and the surface of the living cell (AMGM5) and invasion , this is due to its have of many factors adhesion . The production of lipopolysaccharides has a role in adhesion to both living and non-living surfaces (17) . The ability of C .sakazakii to adhesion to and invasion cell lines is due to their have of ompA, ompX and inv gen , mutations occur of ompA and deletion of the inv gene ,it was reduced adhesion and invasion, as well as the role of the mcp gene in the bacterial virulence and regulating its movement that helps it adhesion and invasion (13 , 18) Both (17 , 19) indicated the ability of bacteria to adhesion to and invasion intestinal epithelial cells to possess flagellating genes that depend on the type of proteins , especially those that encode genes . The high number of adherent cells is due to the bacterial density and the period of exposure, as the more cells the number increases , the less cells adhere to the cell line (20) . C .sakazakii have of zpx encodes the protein lytic enzyme , and LPS increases the permeability of intestinal epithelial cells, They have a role in adhesion and invasion (6, 21) .

Cytotoxic of C. sakazakii on (AMGM5) cell line

The effect of C. sakazakii on brain tumor line(AMGM5) expressive to percentage of inhibition cells and results are shown in Figure (1) . The effect was within 2 hours of exposure of the cell line to the bacteria , as it gave an inhibition rate of (45.81)% when using A1C isolate , while B1 isolate did not give any toxic effect .

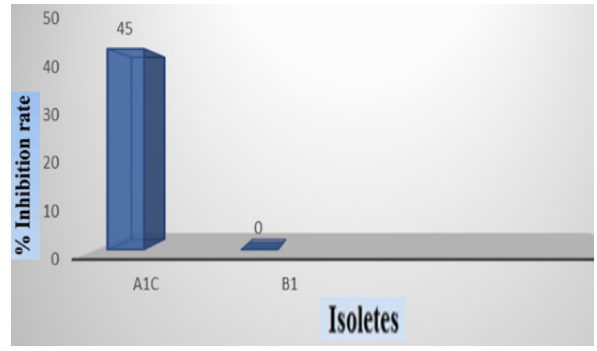

Fig (1) The ability of effect of *C. sakazakii* (A1C) and (B1) bacteria isolates on cell line AMGM5 carcinoma after 2 hours of incubation .

The toxic effect of C. sakazakii was observed in the AMGM5 cell line the bacteria gave a toxic effect that differs depending on the type of cell line and on the source of isolation , if it is environmental isolation has more effect than clinical isolation , which did not give any inhibition , and ability bacteria on adhesion and invasion (AMGM5) . pointed that (21) a decrease in absorbance when using (MTT) method after exposing the Caco-2 and HBMEC cell line with C. sakazakii for 3 hour incubation , indicates the events of cell death and the amount of decrease depends on the type of cell line , and found the Caco-2 line was lower effect than HBMEC .

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