ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

The cytotoxicity behavior of *Acinetobacterbaumanii* supernatant on some of mammalian cell lines in vitro

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Manuscript Info

Manuscript History:

Received: 11 January 2014
Final Accepted: 12 February 2014
Published Online: March 2014

Key words:
Acinitobacterbaumanii, RD and L20B cell lines, Cytotoxicity.

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Abstract

The clinical isolate of *Acinetobacterbaumanii* wasisolated previously amongmany bacterial genusfrom some of surgical tools and hospitals halls in Baghdadand reconfirmed during routine worksof quality control unite in central public health laboratory .There is no lysesshown around their grown colonies on blood agar and skim milk agar respectively. This isolate was appeared sensitive toImipenem, ciprofloxacin, meropenem, gentamicin, amikacin, doxycycline, norfloxacin and enorfloxacin, while resistant a gainstcloxacilin, pencillinG, ceftriaxone, colistin, oxytetracyclin, cephalothin, chloramphenicol, pipracillin, ampicillin, amoxicillin, ceftazidime and co-trimoxazole respectively, However,it was appeared moderate in its behavior against cefotaxime. The bacterial supernatant non-heated and heated (100°C for 60 min) were caused morphological changes in both animal cell lines (RD and L20B) including ((detaching, accumulation, size enlargement, rounding, shrinkage, lyses and death after 96hrs of treatment).the cytotoxicity effect is not related to protease and heamolysinsecretion.Other extracellular heat stable toxins may share in pathogenicity of *A.baumanii* on human cells.
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Introduction

Acienetobacterbaumanii is a gram negative bacteria,stronglyaerobic,catalasepositiveoxidasenegative,non-motile encapsulated coccobacilli (Constantiniuet al.,2004). *A.baumanii* is generally as allow virulent pathogen(Pleg et al.,2008;Gordon et al.,2010),but the full genome sequencing shows that this organism harbors a remarkable number of putative virulence associated genes and elements homologous to the Legionella coxiellatypeIVsecretion apparatus(Smith et al.,2007).Several virulence determinants ,such as biofilm formation(Lee et al.,2008;Gaddy et al.,2009),adherence and ability to invade host cells(Choi et al.,2008), iron acquisition(Zimber et al.,2009) and host cell death(Choi et al.,2005).*A.baumanii* is an important nosocomial pathogen that causes a variety of human infections,particularly in severely ill patient(Pleg et al.,2008).It can be found in soil ,there is evidence that most of the recent infections in military personnel are caused by stains that populated the hospital environment (Towner,2009).*Acientobacter* infections cause of morbidity and mortality in soldiers in intensive care units in Vietnam,Iraq,and Afghanistan(Breslow et al.,2011).*A.baumanii* infections have been shownto manifest as bacteremia,pneumonia,urinary tract infections (UTI),and soft tissue infections (Wisplinghoff et al.,2004).Severalof infections rodent models of *A.baumanii* infections have been reported ,these include pneumonia models using intratracheal routes of infections (Jacobs et al.,2010;Vanfassen et al.,2007), a rat soft tissue model (Luke et al.,2010;Russo et al.,2010),and a rabbit endocarditis model (Rodrigues et al.,2000).Multi drug resistance to clinically available antimicrobial agents in this organism induces serious therapeutic issues(Gordon et al.,2010;Falagas et al.,2005),plasmid-mediated antibiotics and metal resistance has been most widely reported among strains of *A.baumanii* bacterium(Constantiniuet al.,2004),this study amid to identify the activity of hemolytic and

protease,antibiotics sensitivity and testing the heat stable extra cellular cytotoxin that produced from *A.baumanii* by two different mammalian cell lines rhabdomyosarcoma-RD(tumors cells)and LB20 cells.

Materials and Methods

Bacterial Isolates and Maintenance In the beginning, the clinical isolate of*A.baumanii* was obtained from the Central Public HealthLaboratory in Baghdad- Quality control unit; it was maintained on Nutrient agar slants for 5 weeks (pH7.5) (Atlas and Snyder , 2006) , and on Nutrient broth that supplemented with 35 %(vol/vol) glycerolin freezer at -20°C for long time maintenance.

Hemolytic Activity

The hemolytic activity of the*A.baumanii* isolate was determined using blood agar plate that containing 5% (v/v) human blood; the pattern of hemolysis around the colonies were recorded after 24hr incubation at 37°C (Fischbach,2000).

Preparation of Bacterial Filtrate

The bacteria were cultured overnight in 10 mL of LB broth and incubated at 37°C; the culture was harvested by centrifugation at 10000 rpm for 10 min at 4°C, the supernatant were immediately filter sterilized using 0.2 mm pore size syringe filter (AL-Rubai et al., 2011) and kept until using to detect protease production and cytotoxicity effect.

Determination of Protease Production

The ability of bacterial isolates to produce protease were determined by skim milk agar plate containing 10% of skim milk powder and incubated at 37°C for 24 hr; and protease activity was determined by appearance of clear zone around the isolates and wells indicated to protease production and have proteolysis activity (AL-Rubai,2009).

Antibiotic Susceptibility Testing

The antibiogram of*A.baumanii* was determined by Kirby Bauer (single disk) method against Cefotaxime 30µg, ceftriaxone 30µg, ceftazidime 30µg, amoxicillin10 µg, imipenem 10µg , ciprofloxacin 5µg,Cephalothin30µg,Colistin 10 µg, Meropenem 10 µg, Pencillin G 10 µg ,Oxytetracyclin30µg ,Chloramphenicol 30µg,Pipracillin 10 µg,Gentamicin10 µg,Ampicillin 10µg,Amikacin30 µg,Co-trimoxazole 30 µg,Doxycyclin 30 µg,Cloxacillin 5µg ,Norfloxacin 10 µg and Enorfloxacin 5 µg(all were provided from Oxoid); The diameters of inhibition zone for individual antibiotics were measured in millimeters values were interpreted as resistant and sensitive categories according to Clinical and Laboratory Standards Institute (CLSI,2011).

Heat Treatment of Culture Filtrate

The sterilized bacterial filtrate of*A.baumanii* was divided for (500 µl) in Eppendroff tubes and heated in water bath at 100°C for 1hr, the filtrate was immediately placed on ice pieces until applied to monolayer of RD and L20B cells (AL-Rubai et al., 2011).

Cytotoxicity Effect

Human Rhabdomyosarcoma cells (RD) and mouse fibroblast cells (L20B) were used for the cytotoxicity analysis of*A.baumanii* isolate, these cells were obtained from the center public health laboratory in Baghdad (Iraqi National Polio Laboratory); RD human cell line originally was derived from a biopsy specimen obtained from pelvic rhabdomyosarcoma of a 7-year old Caucasian girl (Mcallister et al.,1969).

Results and discussion

The clinical isolates of A.baumanii was obtained from central health laboratory/Baghdad ,No hemolysis pattern and clear zone around colonies that grown on blood agar and skim milk agar plates respectively, the antibiotics susceptibility was measured by millimeter of diameter of inhibition zones table (1).

Table(1):The susceptibility of *A.baumanii*

Antibiotic	Results	Antibiotic	results
Imipenem	S	Colistin	R
Ciprofloxacin	S	Oxytetracyclin	R
Meropenem	S	Cephalothin	R
Gentamicin	S	Chloramphenicol	R
Amikacin	S	Pipracillin	R
Doxycycline	S	Ampicillin	R
Norfloxacin	S	Amoxicillin	R
Enorfloxacin	S	Ceftazidime	R
Cloxacilin	R	Co-trimoxazole	R
Pencillin G	R	Cefotaxime	M
Ceftriaxone	R		

S:sensitive R:resist M:moderate

The Cytotoxic effect of *A.baumanii* filtrate was investigated on both RD and L20B cellslines ; several changes on some of these cells were shown including:, shrinkage,detachment, rounding and finally the pH value was changed started within less than 24 hr after treatment with heated and non- heated of tested supernatant, these changes may attributed to cytotoxicity. The death of cells and lyses for both RD and L20B were observed within 96 hr with complete detachment and destruction of the monolayer of cells comparative with control wells (Fig1). It was sensitive to Imipenem, ciprofloxacin, meropenem, gentamicin, amikacin, doxycycline, norfloxacin and enorfloxacin, while resistant to cloxacilin, pencillinG, ceftriaxone, colistin, oxytetracyclin, cephalothin, chloramphenicol, pipracillin, ampicillin, amoxicillin, ceftazidime and co-trimoxazole, *A.baumanii* in this study was appeared moderate in its behavior against cefotaxime comparatively with standard *E.coli* ATCC25922 strain that according to CLSI,2011 (22) . The pattern of antibiotics susceptibility testing was performed to see the reaction to several antibiotics that gives a clear information that *A.baumanii* has a variable behavior against different antibiotics.. In previous studies for antibiotics susceptibility testing were sensitive to meropeneme,ciprofloxacin , gentamicin, colistin and ampcilin when tested by antibiogram (disc diffusion) method for same cases reports (Neou et al.,2013).our results disagreed with results that observed by Kuo et al(2012) &Jaggi et al., (2012) where founded that the clinical isolates of *A.baumanii* resistant to amikacin, gentamicin, ciprofloxacin, imepinem, meropenem, and sensitive to colistin, pipracilin, co-trimoxazole.It is interesting to find that the results of present study were close to the other researchers, that all clinical isolates of *A. baumanii* were resistant to colistin(Cai et al.,2012). The changes in susceptibility to the antibiotics may referred to enhancing resistance to these agents is caused of concern and periodic monitoring of drug resistance of these pathogens must be performed in different world areas , a mutations and miss diagnosis, so that suitable agent can be selected for empiric therapy. To test the ability of *A.baumanii* to destruction and dismantle of mammalian cells as virulence factor , we used two types of cell lines,; The results were showed that the growth rate of cells was decreased ,detaching ,and changing in cellular morphology from spindle to round shape also,the size was changed to enlargement and shrived cells showed for both RD and L20B cells when it was treated with *A.baumanii* supernatant(heated and non - heated at 100°C) after less than 24 hr of addition on monolayer of these cells in comparative with control cells which appeared complete confluent monolayer of cohesive cells; these changes in shapes were followed by destruction , cytotoxic activity on monolayer of both cells RD and L20B cells and complete damaged after 96 hrcausing death of the cell culture and can be considered as cytotoxicity (Fig 1). there is no locally and global studies refer to ability of *A.baumanii* to cytotoxic effect on RD or L20B cell lines, but many studies were refer to cytotoxic effect and apoptosis of clinical isolates of *A.baumanii* on different cells such as human epithelial HeLa cell, U937 cells and HEp-2 cells (Jin et al., 2011;Lee et al.,2001).

Many studies determined that the bacterial molecules secreted from *A. baumannii* were directly responsible for host cell death(Choi et al.,2008), such as, outer membrane protein A of *A. baumannii* (AbOmpA) was identified as a potential virulence factor to induce host cell death via both mitochondrial and nuclear targeting (Choi et al.,2008;Kuehn and Kesty,2005). AbOmpA is a porin which allows for the passing of small solutes in the outer membrane and its consider as one of the important proteins in culture supernatants (Choi et al.,2008;Kwon et al.,2009).. The variety of pathogenic bacteria have been showed ability to secrete outer membrane vesicles (OMVs) in their growth, OMVs are spherical Nan vesicles are composed of different compounds such as lipopolysaccharides (LPS), lipids,proteins, and DNA or RNA (Beveridge et al.,1999Galka et al.,2008;Lee et al.,2008)., OMVs carry many toxins and other virulence factors, such as,the heat-labile toxins of enter toxigenic*E. coli* (ETEC) (Horstman

and Kuehn2000, Kesty et al., 2004), the Shiga toxin of E. coli O157:H7(Kolling and Matthews1999), the cytolethal distending toxin of *Campylobacter jejun* (Lindmark et al .,2009) and the Cif protein of *Pseudomonas aeruginosa* (Bomberger et al .,2009). after the delivery of virulence factors to host cells, OMVs have an important role in bacterial pathogenicity without the direct interaction between the pathogens and the host cells. The clinical isolate of *A. baumannii* DU202 secreted OMVs into the extracellular during their in vitro growth (Kwon et al., 2009), the secretion of OMVs from *A. baumannii* delivery of virulence factors to host cells via OMVs and subsequent cytotoxicity to host cells and also that a the AbOmpA enriched in the OMVs contributes directly to host cell death(Jin et al.,2011). Many studies referred to that bacterial haemolysin and protease have acytotoxic effect on some mammalian cells(Shrooq et al.,2010;Al-Rubai et al.,2012);the cytotoxic effect in our study was not related with haemolysin and protease production,that lead to believe the cytotoxicity effects of isolate on human cells is attributed to other extracellular heat stable toxins,thus we must not only characterize the different protein toxins, but also address the genetic regulation of transcription. These results demonstrated the presence of widespread, pathogenically characterized, multiple antibiotic resistant, cytotoxic of *A. baumanii*. No relation between the haemolysin; protease and cytotoxic activities,but must be further evaluated in relation to genetic control of other extracellular virulence factors(toxins), molecular mechanisms and network of these virulence factors in exhibiting the pathogenesis of *A.baumanii*.

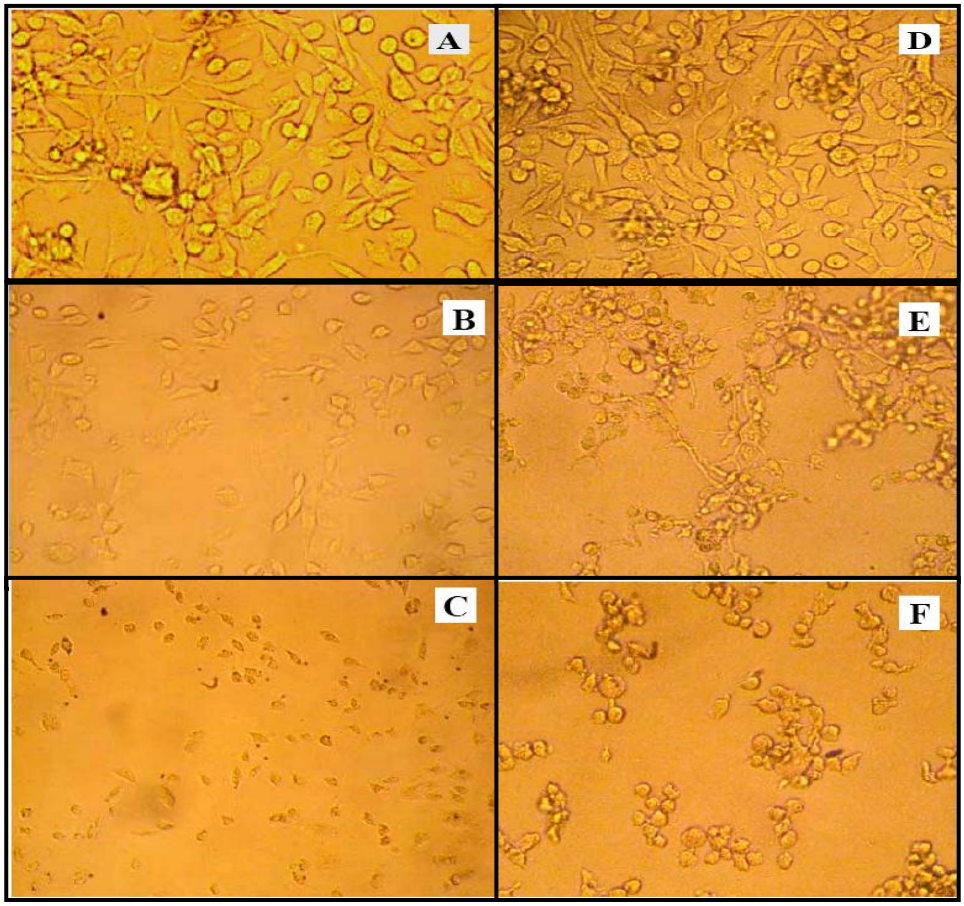

Fig 1: The cytotoxicity effect of *A.baumanii* supernatant on different Cell lines

A: untreated L20B cell **B:** L20B cells after treatment with non-heated supernatant for24hr cells shrinking, detaching and rounded **C:**degredation, lyses and death most treated cells with heated supernatant at 100°Cafter 96 hr.
D: untreated RD cell **E:** RD cells after 24hr of non-heated supernatant, cells shrinking, detaching , accumulation and rounded **F:**death most treated RD cells with heated supernatant at 100°Cafter 96hr.

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