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Major article

Major biologic characteristics of *Acinetobacter baumannii* isolates from hospital environmental and patients' respiratory tract sources

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Background: This prospective study investigated major biologic characteristics of *Acinetobacter baumannii* isolates from hospital environment and respiratory tract samples of patients admitted to adult intensive care units (ICUs) at the Jordan University Hospital.

Methods: *A baumannii* isolates from both sources were examined for antimicrobial susceptibility and for presence of specific metallo-β-lactamase genes (VIM-2, IMP-1) and OXA-type β-lactamase genes (OXA-type) using polymerase chain reaction and biofilm formation and surviving under various temperatures and pH conditions.

Results: The majority of *A baumannii* isolates from environmental and patients sources was multidrug resistant (MDR), except for colistin and tigecycline. All *A baumannii* examined carried a *bla*OXA_{51}-like gene, 58% has a *bla*OXA_{23}-like gene, and 38.8% has a *bla*OXA_{24}-like gene. Representative MDR *A baumannii* isolates from both sources were capable to form biofilm. *A baumannii* environmental isolates were capable to survive for a longer time in tap, normal saline, and distilled water than respiratory tract isolates with pH range of 4.5 to 8 and temperature between 18°C to 37°C.

Conclusions: This study demonstrates that *A baumannii* isolates from the patients' respiratory tract and hospital environment carried much similar multidrug resistance patterns and biologic characteristics. In conclusion, this study shows that all MDR *A baumannii* strains survived well in the hospital environment, especially in water and moist environment and produced biofilm, which might be responsible for high colonization in the respiratory tract of patients in ICU.

Acinetobacter baumannii has emerged in recent years as an important infectious agent in hospitalized patients worldwide and especially in developing countries.[1-4] The ability of *A baumannii* to survive in the environment under unfavorable conditions for prolonged periods of time may have contributed to the endemic and epidemic behavior of this opportunistic pathogen.[5] There are few major factors identified to contribute to the survival of *A baumannii* in the hospital environment including their resistance to antimicrobial drugs, disinfectants, desiccation, and nutritional starvation in the moist envirnment.[6,7] Previous studies demonstrated that adhesion and biofilm phenotypes of clinical *A baumannii* isolates seem to be associated with antibiotic resistance.[5,8-10]

The high capacity of multidrug-resistant (MDR) clinical isolates of *A baumannii* to form biofilms and to adhere to respiratory epithelial cells make them virulent and enhance their potential to colonize human skin and respiratory tract mucosa.[10] Bacterial colonization of the respiratory tract frequently precedes the onset of serious invasive infection. Investigators have reported that biofilm producing bacteria can be up to 1,000-fold more resistant to antibiotic treatment than the same organism grown in planktonic stage.[11-13] Therefore, one major reason for persistence of *A baumannii* in environment seems to be related to its capacity to grow within biofilms, which protect them against adverse environmental factors. This study compared the survival potential, biofilm production, and antimicrobial resistance in *A baumannii* isolates from both hospital environment and respiratory tract of hospitalized patients.

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MATERIALS AND METHODS

Bacterial isolates

Over the period of this investigation (May 2009-February 2010), a total of 74 (49.7%) *A baumannii* isolates was recovered by culturing 149 environmental samples collected from 3 adult intensive care units (ICUs) of the Jordan University Hospital (JUH). The majority of these samples was collected randomly from pillows, bed linens, and ventilation masks used by patients as well as from the floor and sinks using wetted swabs with physiologic saline. At the same period, a total of 64 (45.1%) of *A baumannii* isolates was recovered from 142 patients' respiratory samples of bronchoalveolar lavage, endotracheal aspirate, and sputum, which were consecutively collected from of 93 patients in the first 48 hours after admission to ICUs at JUH. All collected samples were cultured within 1 to 2 hours on blood agar, cysteine-lactose-electrolyte-deficient agar (cysteine-lactose-electrolyte-deficient medium; Oxoid, Cambridge, England), and minimal salt agar supplemented with 1% acetate for detection of colonization with *Acinetobacter* spp. All suspected *Acinetobacter* growing colonies were first identified by conventional bacteriologic techniques and confirmed later as *A baumannii* by Rapid NF plus system, Remel Kit (Lenexa, KS). Only 1 positive culture of *A baumannii* isolate was included for each environmental sample or patient. All cultures were kept frozen at −70°C in brain-heart agar with 15% glycerol (Difco, Sparks, MD) until used. *Pseudomonas aeruginosa* (ATCC 29852) was used for quality control of antimicrobial susceptibility test and biofilm formation. All the used media were obtained from Oxoid. This study has been approved by the Postgraduate Committee of the Faculty of Medicine and the Ethics Committee of JUH, a 550-bed, tertiary care, teaching hospital with 24 ICU beds and 3 adult ICUs.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed for 11 different therapeutically relevant antibiotics by disk diffusion method, and susceptibility of each isolate to the antimicrobial drugs was determined according the guidelines of the Clinical and Laboratory Standard Institute.[15] The tested antibiotics (Mast Group LTD, Merseyside, England) and their concentrations are shown in Table 1. Minimum inhibitory concentrations (MICs) for amikacin, colistin, imipenem, and tigecycline were determined by the E-test method according to the manufacturer's guidelines (AB Biodisk, Solna, Sweden). MDR was defined as resistance to $\geq$ 3 classes of antimicrobial agents, including cephalosporins, aztreonam, carbapenems, aminoglycosides, and fluoroquinolones. The following tests (OXA-51, OXA-23, OXA-24, VIM-2, IMP-1, and OXA-58 genes) were included for each 8 MDR *A baumannii* isolates from hospital environmental and patients' respiratory tract sources as shown in Table 2.

Detection of β-lactamase genes

A polymerase chain reaction multiplex was used to detect metallo-β-lactamases (VIM-2, IMP-1) and OXA type β-lactamase genes (OXA-58, OXA-51, OXA-24, OXA-23) as described by Sung et al.[16] Genomic DNA was extracted according to the manufacturer's instructions of Wizard Genomic DNA purification kit (Promega, Madison, WI).

Determination of survival at different temperature in vitro

A single colony of each fresh overnight culture was added to 10 mL Mueller Hinton broth. The resultant bacterial suspension was plated onto nutrient agar using standardized (0.001 μmL) loop and incubated at 4°C, laboratory room temperature (range, 18°C-24°C), 37°C, 42°C, 45°C, and 48°C for 24 hours, and each test was performed in duplicate on nutrient agar.

Table 1
Antimicrobial resistant patterns of 74 environmental and 64 *A baumannii* patients' respiratory tract isolates

Antimicrobial agent (disk concentration/μg)	No. (%) resistant environmental isolates*	MIC_{90} (mg/L)	No. (%) resistant patients isolates*	MIC_{90} (mg/L)
Amikacin (Am/30)	47 (63)	14	47 (73)	13.2
Aztreonem (Atm/30)	68 (92)	-	63 (98)	ND
Ceftazidime (Caz/30)	72 (97)	-	64 (100)	ND
Ciprofloxacin (Cip/5)	46 (62)	-	58 (91)	ND
Colistin (Col/10)	Null	0.5	Null	1
Gentamycin (Gm/10)	66 (89)	-	60 (94)	ND
Imipenem (Imi/10)	51 (69)	12	41 (64)	12
Meropenom (Mem/10)	72 (97)	-	64 (100)	-
Piperacillin/tazobactum (Ptz/110)	69 (93)	-	63 (98)	-
Tigecycline (Tag/15)	Null	1.5	Null	2

MIC, minimum inhibitory concentration; *ND*, not done.
*Data from Al-Dabaibah et al.[14]

Table 2
Distribution of β-lactamase genes in representative of each 8 *A baumannii* isolates from hospital environment and patients' respiratory tract samples

	Antibiotic resistance pattern of each examined isolates*	β-Lactamase resistance genes		
		OXA-51	OXA-23	OXA-24
No. *A baumanni* isolates from patients†				
1	Atm, Caz, Mem	+	+	−
2	Atm, Caz, Gm, Cip, Ptz	+	+	+
3	Atm, Caz, Cip, Gm, Mem, Ptz	+	+	−
4	Ak, Atm, Caz, Gm, Imi, Mem, Ptz	+	+	+
5	Ak, Atm, Caz, Cip, Gm, Mem, Ptz	+	+	+
6	Ak, Atm, Caz, Gm, Imi, Mem, Ptz	+	+	+
7	Ak, Atm, Caz, Gm, Imi, Mem, Ptz	+	+	+
8	Ak, Atm, Caz, Cip, Gm, Imi, Mem, Ptz	+	+	+
No. *A baumannii* isolates from environment†				
1	Atm, Caz, Mem	+	+	−
2	Caz, Gm, Imi, Mem, Ptz	+	−	+
3	Ak, Caz, Cip, Gm, Imi, Mem, Ptz	+	+	+
4	Ak, Caz, Cip, Gm, Imi, Mem, Ptz	+	+	+
5	Atm, Caz, Gm, Imi, Mem, Ptz	+	−	+
6	Ak, Atm, Caz, Cip, Mem, Ptz	+	+	−
7	Ak, Atm, Caz, Gm, Imi, Mem, Ptz	+	+	+
8	Ak, Atm, Caz, Imi, Gm, Mem, Ptz	+	+	−

NOTE. Antimicrobial agent definitions for column 1 are found in Table 1.
*All tested *A baumannii* isolates were negative for metallo-β-lactamases (VIM-2, IMP-1 genes) and OXA-58 genes.
†There was no significant difference in biofilm formation between patients and environmental isolates.

Determination of survival at different environmental conditions

The effect of pH on *A baumannii* growth was performed as described by Benjamin and Datta[17] with some slight modification. The effect of temperature on the *A baummanni* was determined by suspending a single colony of 8 fresh overnight cultures in 10 mL Mueller Hinton broth. The bacterial suspension was plated onto nutrient agar using standardized (0.001) loop and incubated at 4°C, 18°C, 24°C, and 37°C for 24 hours. The effects of pH range (4.5-8), type of water (distilled, tap, saline), room temperature (range, 18°C-24°C), and days on the viability of *A baumannii* were presented as major if the colony count was less than or equal to 10 colony-forming units/plate and minor if the count was more than 50 colony-forming units/plate. All tests were performed in duplicate.

Biofilm formation

Biofilm formation on polystyrene 96-well plates (Greiner Bio-one, Frickenhausen, Germany) and silica catheters (Nunhai, New Delhi, India) was performed according to the methods of Burton et al.[18] Significant difference in biofilm density was defined as equal or more than 1-log cycle difference in viable count. For test biofilm formation on catheter, a 3-cm-long catheter segment was added to each well that contains the fresh nutrient broth, and the bacteria were inoculated as described above. The adherent bacteria were removed initially by scratching followed by sonication. The dynamics of biofilm formation on 96-well plates and catheters using 2 different inocula of approximately 2×10^9 and approximately 4×10^2 for both clinical and environmental strains were determined after 2, 4, 6, 8, 24, 48, and 72 hours.

Antibiofilm assay

The antibiofilm efficacy of 2 substances (Sterillium; BODE, Bode Chemie, Hamburg, Germany) and 0.2% hypochlorite solution on catheters' biofilms was assessed using viable count method of Kim et al.[19] Each test was repeated to simulate dirty conditions by mixing the test suspension with 0.3% or 3.0% (wt/vol) bovine serum albumin (Sigma-Aldrich Chemie, Taufkirchen, Germany) solution as reported by Kawamura-Sato et al.[20]

RESULTS

The present study demonstrated high and almost similar incidence rates of MDR *A baumannii* (37.5%-100%) among isolates from both environmental and patients' respiratory tract sources as shown in Table 1. All 16 *A baumannii* isolates from both sources were positive to $bla_{\text{OxA-51}}$-like gene and negative to $bla_{\text{OxA-58}}$-like genes (Table 2). The patient and environmental isolates showed mostly similar distribution of the β-lactamase resistance genes (Table 2). All 8 tested *A baumannii* isolates from both sources have grown well at the following temperatures: laboratory room temperature, 37°C, 42°C, and 45°C. None of the tested isolates have shown growth at 4°C or 48°C. Environmental isolates of *A baumannii* survived in distilled, tap, and normal saline water (0.90% NaCl) until the 23rd day; within the pH range of 4.5 to 8; and room temperature range of 18°C to 24°C, whereas the patients isolates survived at higher pH range (5.5-8) until the 23rd day and only in tap and normal saline water under the same conditions (Table 3). All *A baumannii* isolates from environment and patients were capable to form biofilms. There was no significant difference in biofilm formation on 96-well plates and catheters between both groups of isolates. This biofilm formation was removed successfully to 99.99% by applying Sterillium or 0.2% hypochlorite (NaOCl) within 1 minute and with no significant difference among all isolates. The antibiofilm efficacy of hypochlorite was decreased under dirty conditions, where higher concentration of 0.3% was needed to effect 99.99% biofilm reduction.

DISCUSSION

This study revealed that environmental *A baumannii* isolates have mostly similar MDR profiles compared with those isolates colonizing the respiratory tract of hospitalized patients over the same period (Table 1). All recent studies from different countries have shown increasing incidence of MDR clinical isolates of *A baumannii*, including resistance to used carbapenems as demonstrated in this study.[1,2,21,22] In addition, all *A baumannii* isolates in this study were susceptible to colistin and tigecycline. There are only few studies reported limited resistance toward colistin and tigecycline.[23-25] The increased prevalence of carbapenem resistance within *A baumannii* isolates is attributed to the production of carbapenemases, mostly the oxacillinases (OXAs), and, to a lesser extent, the metallo-β-lactamases (MBLs), which are expressed by bla_{OXA} and *MBL* genes, respectively.[4,25] Similar to our findings, many recent studies reported high prevalence of the resistance genes of $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}51}$ in *A baumannii* clinical isolates.[25-28] The high incidence of these genes may explain the wide spread of the MDR *A baumannii* isolates among both the environment and the patients' respiratory tract as demonstrated in this study and other similar studies.[16,28]

Table 3
Survival means of 8 *A baumannii* isolates from environmental and clinical sources in water at different pH and number of days at room temperature, ranging from 18°C to 24°C

Type of water	Survival of environmental isolates at pH range (4.5-8), days	Survival of patients' isolates at pH range (5.5-8), days
Distilled	23	14
Tape	23	23
Saline	23	23

This study demonstrated that all tested *A baumannii* environmental and patients' strains can survive relatively over a long time at wide range of pH and temperatures in various types of water (Table 3). The only difference was the longer survival capacity of environmental over patients' isolates in distilled water (23 vs 14 days, respectively).

A previous study of Jawad et al (1998) reported the capability of clinical *A baumannii* to survive under dry and 31% relative humidity conditions for an average of 3 and 20 days, respectively.[29] A second study of the same research group found no statistically significant difference between the survival times of sporadic strains of *A baumannii* and clinical outbreak strains (27.2 vs 26.5 days, respectively).[30] This biologic feature of *A baumannii* appears to support its survival in the hospital environment and may contribute to nosocomial infection outbreaks among hospitalized patients.[29-31]

Biofilm formation of isolates from patients and environmental sources is in concordance with previous studies that demonstrated the capability of certain strains of this organism to form biofilms on abiotic surfaces such as polystyrene and biotic surfaces of living cells especially human epithelial cells.[5,8,10] Exposing 24-hour growth of *A baumannii* isolates to Sterillium or 0.2% hypochlorite (NaOCl) for 1 minute resulted in the removal of biofilm with no significant difference between patients' and environmental isolates. The antibiofilm efficacy of hypochlorite was decreased under dirty conditions, where higher concentration of 0.3% was needed to have the same effect of biofilm reduction as Sterillium under dirty conditions. Biofilm formation is considered a surviving strategy that protects organisms from the external environment pressure and from the antimicrobial and biocides effects.[13] Biofilm formation of *A baumannii* in the environment makes their control and eradication a challenging process. This study demonstrated the capability of the biocides of hypochlorite and Sterillium to completely eradicate the formed biofilms of *A baumannii* within 1 minute if the necessary concentration is used. The high prevalence of *A baumannii* in the hospital environment with its ability to survive over a long time and develop biofilm demonstrates the importance of implementing continuous and proper disinfection and sanitization measurements in the health care settings. These measurements could be considered major steps in controlling nosocomial infections with *A baumannii*.

In conclusion, the present study shows that *A baumannii* strains isolated from both hospital environment and respiratory tract of hospitalized patients have mostly similar antimicrobial resistance

profiles and biologic characteristics that allow them to survive for prolonged period in the hospital environment.

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