

Emergence of plasmid mediated *aac(6')-Ib-cr* Gene in Fluoroquinolon- resistant *Acinetobacter* spp.

Adnan H. Al-Hamadani¹, Ali M. Al-Mohana² and Ali S. Al-Khazaali

الخلاصة

هدفت الدراسة الحالية الى التحري عن جين *aac(6')-Ib-cr* البلازميدي في بكتريا *Acinetobacter* المقاومة لمضادات الفلوروكوينولون. اذ جمعت 154 عينة من مرضى رافدين وبعض المعدات الطبية في مستشفى الديوانية التعليمي للفترة من تشرين الاول 2011 الى اذار 2012 وتوزعت مصادر هذه العينات من وحدة العناية المركزة (85 عينة)، وحدة الحروق (43 عينة) ومن اخماج المجاري البولية (26 عينة)، تم الحصول على 16 (10.3%) عزلة عائدة لبكتريا *Acinetobacter*. ثم شخصت بكتريا *Acinetobacter* على مستوى النوع بواسطة نظام VITEK-2 حيث اظهرت النتائج ان الغالبية العظمى تعود للنوع *A. baumannii* complex. 15 (93.8%) عزلة وعزلة واحدة (6.2%) تعود للنوع *A. lowffii*. أُخْتِبرت حساسية عزلات *Acinetobacter* الـ 16 تجاه 20 مضاداً حيوياً تابعة لأصناف مختلفة بواسطة نظام VITEK-2 بأشرط GN-AST23. أظهرت النتائج أن جميع العزلات مقاومة الى ما لا يقل عن ثلاثة اصناف من المضادات الحيوية، كما اظهرت العزلات نسباً عالية لمقاومة لكل من نتروفورانتوين، الجيل الاول و الثاني والثالث والرابع لل سيفالوسبورينات و الأمينوكليوسايد. بين اختبار الحساسية للمضادات الحياتية ان 11 (68.7%) عزلة كانت مقاومة لمضادات الفلوروكوينولون السبروفلوكساسين، الليفوفلوكساسين والنورفلوكساسين. استخلص الـ DNA لعزلات بكتيريا الاسيتوباكتر المقاومة لمضادات فلوروكوينولون واستخدم في تفاعل سلسلة انزيم البلمرة للتحري عن جين *aac(6')-Ib-cr* البلازميدي. بينت النتائج وجود 4 عزلات (36.3%) حاوية على هذا الجين.

Summary

This study aimed to detection of plasmid mediated *aac(6')-Ib-cr* gene in fluoroquinolon resistant *Acinetobacter* spp. A total of 154 clinical and inanimated samples were collected from inpatients and medical devices from different units in Al-Dewaniyah Teaching Hospital during a period of five months from November 2011 to March 2012. Sources of specimens were ICU (n= 85), burn unit (n=43) and urine from inpatients suspected with UTI (n=26). Totally, 16(10.3) *Acinetobacter* spp. were obtained. Identification of *Acinetobacter* isolates to species level was done by VITEK 2 compact system, results showed that the vast majority of isolates belong to *A. calcoaceticus* *A. baumannii* complex (15, 93.8%) while only one isolate (6.3%) was belong to species *A. lowffii*. All of the 16 *Acinetobacter* spp. isolates were testwd to susceptibility against 20 antibiotics by VITEK 2 system with GN-AST23 cassettes.

Result showed that all of isolates were resistant to at least three classes of antibiotics.

1-College of Medicine/ Al-Qadisiya University 2-College of Medicine/ Kufa University.

Isolates showed high resistance rates to first-, second-, third- and fourth generation cephalosporin, aminoglycosides and nitrofurantoin. Results revealed that 11 isolates were fluoroquinolon resistant. DNA of the fluoroquinolon resistant isolates was extracted and used in PCR assay. Results showed that 4(32.3%) isolates were harboring *aac(6')-Ib-cr* gene.

Introduction

Acinetobacter is gram-negative, non motile, strictly aerobic bacteria; it is a heterogeneous group of organisms that is ubiquitous, widely distributed in nature ⁽¹⁾. Taxonomy of the genus *Acinetobacter* was achieved in 1986 by Bouvet and Grimont, who distinguished 12 DNA groups (genospecies) based on DNA-DNA hybridization studies ⁽²⁾. Recent application of molecular methods has established the identity at least 33 named and unnamed species of *Acinetobacter* ⁽³⁾. *A. calcoaceticus*–*A. baumannii* complex is used to represent *A. baumannii*, *A. calcoaceticus*, *A.pittii* and *A. nosocomialis* that are difficult to differentiate on the basis of phenotypic characteristics. These members of complex, except *A. calcoaceticus*, are the species of greatest clinical importance ^(4,5).

This bacterium is well adapted to survive and tolerates both wet and dry hospital environment conditions ⁽⁶⁾. Moreover, Sources of *Acinetobacter* transmission was identified in outbreaks in health care setting include medical devices and equipments such as ventilator circuits, spirometers, suction catheters. ⁽⁷⁾.

The incidence of *Acinetobacter* infections has reached a point of concern and poses a threat to hospitalized populations worldwide. It is found to be associated with a wide spectrum of nosocomial infections including: pneumonia, meningitis urinary tract infections, bacterimia and wound infections ⁽⁸⁾.

The challenges of treating multidrug-resistant bacteria continue to be at the forefront of the clinician's practice in caring for hospitalized patients ⁽⁹⁾. Until 1970s, most clinical *Acinetobacter* isolates were susceptible to commonly used antimicrobials, so that infections caused by these organisms could be treated relatively easily ⁽¹⁰⁾. Nowadays, *Acinetobacter* demonstrates increasing resistance to commonly prescribed antimicrobials and multidrug-resistant (mainly *A.baumannii*) has been reported worldwide and is now recognized as one of the most difficult healthcare-associated infections to control and to treat ^(9,11).

Quinolone are synthetic chemotherapeutic agents, which have broad spectrum of antimicrobial activity as well as a unique mechanism of action resulting in inhibition of bacterial DNA gyrase and topoisomerase IV ⁽¹²⁾.

Resistance to quinolones involves mutations in genes (*gyrA*, *gyrB* *parC*, *parE*) that codify the production of some enzyme with role in DNA replication

(DNA gyrase, topoisomerase IV) or porins synthesis from outer-membrane, a nother mechanism is the reduction of intra-bacterial concentration by efflux pumps that eliminate the antibiotic molecules from the bacterial cell ^(13, 14). The most well-described mechanism of resistance to quinolones in *Acinetobacter* spp. are mutations in the genes encoding DNA gyrase A (*gyrA*) and subunit A of topoisomerase IV(*parC*), the most important mutations, Ser83 for *gyrA* and Ser80 for *parC*, have been mapped to a unique location in these genes, the quinolone resistance determining region (QRDR) ⁽¹⁵⁾.

Resistance to quinolones can also be mediated by plasmids that produce the protein, which protects the quinolone targets from inhibition ⁽¹⁶⁾. Three kinds of plasmid-mediated quinolone resistance (PMQR) determinants have been described: Qnr that include QnrA (Martinez-Martinez, 1998) *qnrS* ⁽¹⁷⁾, *qnrB* ⁽¹⁸⁾, *qnrD* ⁽¹⁹⁾ and *qnrC* ⁽²⁰⁾, *aac (6)-Ib-cr* ⁽²¹⁾ and *qepA* ⁽²²⁾. Plasmid mediated quinolon resistance genes have been not confirmed in *Acinetobacter* ⁽²³⁾.The *aac(6')-Ib-cr* gene is a new variant of common aminoglycoside acetyltransferase that acetylates piperazinyl substituent of some fluoroquinolones, including norfloxacin and ciprofloxacin ⁽²¹⁾. The first report of this gene was in 2003 and confers 2- to 4-folds increase in MICs ⁽²⁴⁾. There is little information regarding in the occurrence of PMQR gene in *Acinetobacter*. This study aimed to investigate occurrence of *aac(6')-Ib-cr* gene in *Acinetobacter* by PCR technique

Method

Sample collection

This study was carried out at Al-Diwaniya Teaching Hospital, the largest hospital in Al-Diwaniya province. A total of 154 clinical and inanimated samples were collected from different units during the period from November 2011 to March 2012 from patients admitted to the hospital and medical devices Sources of specimens were ICU (n= 85), burn unit (n=43) and urine from inpatients suspected with UTI (n=26).

Isolation and identification

Samples that obtained from clinical and inanimated samples were cultured onto MacConkey agar and incubated for 18-24 hrs at 37°C. All non-lactose-fermenting isolates were streaked onto CHROMagar™ *Acinetobacter*/MDR medium and incubated for 18-24 at 37 °C, *Acinetobacter* appears as a red colonies after the incubation period. Suspected bacterial isolates that appeared as typical *Acinetobacter* CHROMagar™ *Acinetobacter*/MDR medium were tested by morphologic characteristics and standard biochemical tests according to MacFaddin, (2000) ⁽²⁵⁾. Confirmation of *Acinetobacter* spp. isolates was also conducted by PCR assay to detect the presence of a specific 16s rDNA gene.

Species Identification of *Acinetobacter* Isolates

In present study, VITEK- 2 Compact system was used to identify *Acinetobacter* isolates to species level according to manufactures' instructions.

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Antibiotics Susceptibility Testing:

Isolates tested against 20 antibiotics by VITEK-2 Compact system according to the manufacturer's instructions (Biomerieux/ France) with GN-AST23 cassettes that. VITEK- 2 Compact system was previously programmed to interpret the results according to National Committee for Clinical Laboratory Standard (CLSI, 2006).

PCR Assay

DNA of Fluoroquinolon resistant *Acinetobacter* isolates was extracted using Genomic DNA Extraction Mini Kit (Genied/ USA) according to manufactures' instructions. Amplification of the 16S ribosomal DNA was performed with specific primer to 16 rDNA of *Acinetobacter* spp.⁽²⁶⁾ F: AGAGTTTGATCCTGGCTCAG, R: TACCAGGGTATCTAATCCTGTT under the following cycling conditions: Initial denaturation at 95°C for 3 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

Fluoroquinolon resistant *Acinetobacter* isolates amplified using *aac(6')-Ib-cr* specific primer ⁽²⁷⁾ F:TTGCGATGCTCTATGAGTGGCTA, R:CTCGAATGCCTGGCGTGTTT. Amplification reactions were carried out under the following conditions: initial denaturation at 95°C for 5 m, followed by 30 cycle of denaturation at 95°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 40 s, and a final extension at 72°C for 4 min. A molecular marker (promega/ USA effective size range: 100 to 1500 bp) was used to assess PCR product size.

Results

Isolation

Totally, 16 (10.4%) cultures from the 154 specimens reported in this study were positive for *Acinetobacter* spp. , type and sources of isolates listed in table (1)

Table (1): Distribution of *Acinetobacter* spp. isolates in various specimens in Al-Diwaniya city

Source of specimen	Unit	No. of samples	No. of <i>Acinetobacter</i> spp.	Percentage%
Wound	ICU	38	5	13.2
Ventilators	ICU	19	2	10.2
Suction machine	ICU	28	4	14.3
Burn wound	Burn	43	2	4.7
Urine	wards	26	3	11.5
Total		154	16	10.4

Identification

The suspected colonies appeared as typical *Acinetobacter* species when they were subcultured onto CHROMagar™ *Acinetobacter*/MDR plates of red colonies, figure (1). All isolates showed positive results for catalase test, whereas all isolates were negative to oxidase test and exhibited positive results with Simmons citrate test. Triple sugar iron agar test results were alkaline/acid type of growth. Isolates shows negative results to indol, methyl red, Voges-Precauer and nitrate reduction. All isolates that were identified by the biochemical test were also positive for 16 rDNA gene, figure (2).

Based on the results of the VITEK-2 compact, the species of *Acinetobacter* identified were *A. boumannii* complex (*A. calcoaceticus* *A. baumannii* (*Acb*) complex) (15, 93.8%) and *A. lwoffii* (1, 6.3%).

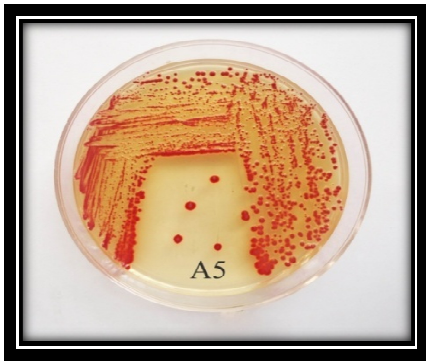


Figure (1): *Acinetobacter* spp. isolate A5 colonies on selective medium (CHROMagar™ *Acinetobacter*/MDR). The isolates appeared red colonies after incubation period for 24 hr at 37°C.

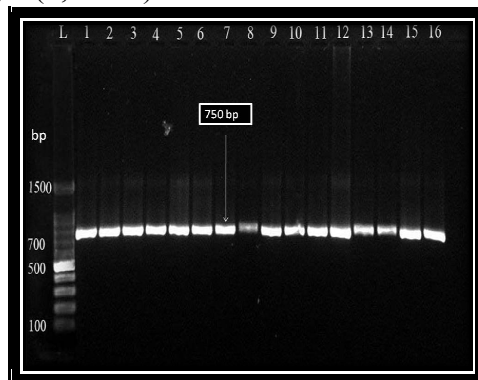


Figure (2): Ethidium bromide-stained agarose gel of PCR products amplified with 16s rDNA primer from *Acinetobacter* spp. extracted DNA (750 bp). L: 100 bp standard size reference marker Lane (1-16): *Acinetobacter* spp. (A1-A16) showing positive result with 16s rDNA gene.

Antimicrobial Susceptibility Testing

All isolates showed resistance to at least three classes of antibiotic and demonstrated full resistance to ampicillin, cephalothin, cephazolin and cephoxitin. The present study also showed that the isolates had very high rates of resistance to the following β -lactam antibiotics tested, cefpodaxime, ceftriaxone, cefuroxime, cefuroxim-exaxtile and ceftazidime and cefepime figure (3).

Results have demonstrated that in general the *Acinetobacter* spp. isolates have notably high rates of resistance to the commonly used fluoroquinolones (ciprofloxacin, norfloxacin and levofloxacin). Out of 16 isolates, 11(68.8%) that belong to *Acinetobacter baumannii* complex were fluoroquinolon resistant.

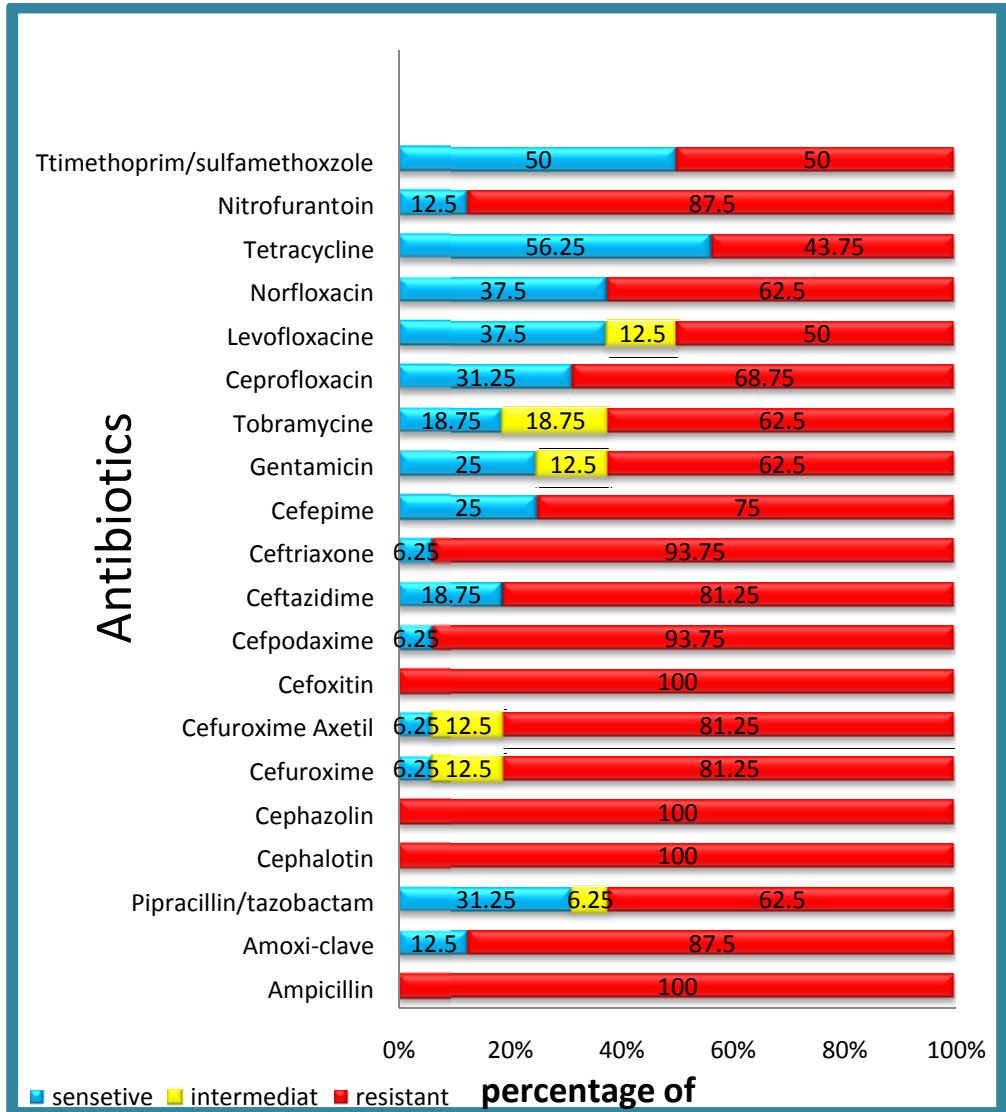


Figure (3): Antibiotic susceptibility profile of 16 *Acinetobacter* isolates recovered from Al-Diwaniya Teaching hospital.

Detection of Plasmid Mediated *aac(6')-Ib-cr* Gene

All of 11 flouroquinolon resistant isolates were investigated to the presence of plasmid mediated *aac(6')-Ib-cr* Gene. A total of 4(36.3%) were positive to presence of this gene (figure 4).

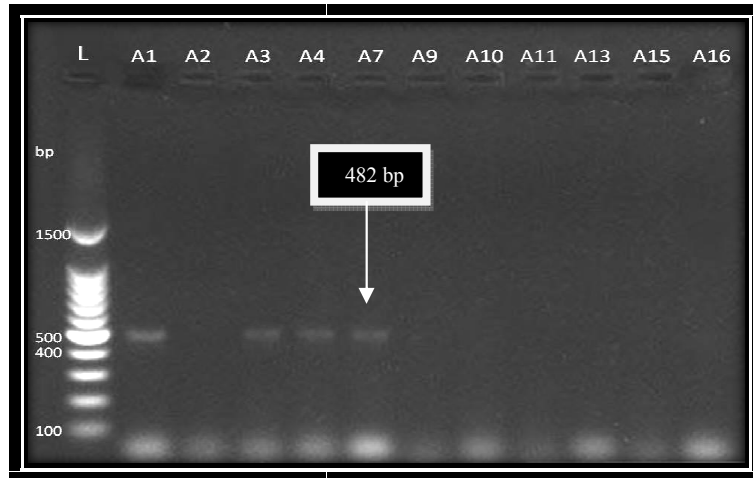


Figure (4). Ethidium bromide-stained agarose gel of PCR Products amplified with *aac(6)-ib-cr* primer from flouroquinolon resistant *Acinetobacter* spp extracted DNA (485 bp). L: 100 bp standard size reference marker Lane (1, 3, 4, 7): Showing positive result with *aac(6)-ib-cr* gene.

Discussion

In current study, only 16(10.3%) *Acinetobacter* isolates were obtained. Compared with local studies, present results disagree with Al-Garaawi⁽²⁸⁾ who found that incidence of *Acinetobacter* in Al-Diwaniya city was 3.7%. This study is relatively in agreement with a study carried out in Baghdad by Mosafer⁽²⁹⁾ who recovered 21 *Acinetobacter* isolates from 296 clinical samples. It is noted that incidence of *Acinetobacter* spp. differs from region to another even in the same city or hospital; this may be due to time and study conditions in addition to number of collected samples, however, the incidence of *Acinetobacter* infections has risen significantly and continuously worldwide, this bacterium became important nosocomial pathogens. MDR strains are difficult to treat and associated with significant morbidity and mortality⁽³⁰⁾. This may because of this bacterium is an organism of low virulence but enabling it to cause infections⁽³¹⁾.

The major source of isolates in the present study was ICU. Out of 85 samples from ICU, 11 (13%) *Acinetobacter* isolates were obtained, this result

correspond with a study in India by Patwardhan *et al.* ⁽³²⁾. The rising incidence of *Acinetobacter* infection in the intensive care unit (ICU) may be due to their ability to develop resistance to multiple classes of antibiotics. In addition, it can infect virtually any body site ⁽³³⁾.

This study suggests that contaminated medical devices in our hospitals may become sources of outbreaks of MDR organisms like *Acinetobacter*. Contamination of these medical devices recorded as sources of outbreaks worldwide ⁽⁷⁾.

Isolates showed high resistance rates to first-, second-, third- and fourth generation cephalosporin, antibiotic combinations, aminoglycosides and nitrofurantoin. This is an expected result in *Acinetobacter* which recorded in local studies ^(34, 35). One of the most unwelcomed features of *Acinetobacter* spp. is the ability to develop multiple resistance mechanisms against several major antibiotic classes that mediated by mobile genetic elements like conjugative plasmids, integrons, insertion sequences and transposons ⁽³⁶⁾. Resistance mechanisms that are expressed frequently in nosocomial strains of *Acinetobacter* include production of enzymes like β -lactamases, alterations in cell-wall channels (porins), and efflux pumps ⁽³⁷⁾. Extended spectrum cephalosporinase AmpC are predominantly restricted to the genus *Acinetobacter* and designated as *Acinetobacter*-derived cephalosporinases (ADCs) ⁽³⁸⁾ that is typically hydrolyze penicillins and narrow- and extended-spectrum cephalosporins but not carbapenems ⁽³⁹⁾.

Antibiotic susceptibility testing revealed that 11(68.8%) isolates were quinolon resistant. In Iraq, quinolone antibiotic is widely used; the increased use of these antibiotics may lead to increasing the resistance to these antimicrobials. In the past quinolon antibiotics have shown good activity to *Acinetobacter* in Iraq, for instance in 2003, Al-Shekri ⁽³⁵⁾ stated that 91% *Acinetobacter* isolates were susceptible to ciprofloxacin in Hilla city, however, results high rate of Quinolone resistance in current study agree with Abd AL-Kareem ⁽³⁴⁾ and Chaiwarith *et al.* ⁽³²⁾.

Out of the 11 tested isolates, 4 (36.4%) isolates carried *aac(6')-Ib-cr* gene. plasmid mediated quinolon resistance (PMQR) genes have already been detected in all populated continents and in most clinically common Enterobacteriaceae. Among these genes, *aac(6')-Ib-cr* seems to be more prevalent ⁽⁴⁰⁾, in Iraq this gene was previously recorded in clinical isolates of *E. coli* in Najaf city ⁽⁴¹⁾. There are little information in the distribution PMQR genes in non-enteric bacteria, to our knowledge this is the first report of *aac(6')-Ib-cr* gene in *Acinetobacter*. Until now, among non-enteric bacteria, only *qnrS* in *Aeromonas* ⁽⁴²⁾ and *qnrA* in *Acinetobacter* ⁽²³⁾ PMQR genes were identified. This identification of plasmid-mediated gene outside Enterobacteriaceae highlights a possible diffusion of those resistance determinants within gram negative rods.

Conclusion

Acinetobacter is highly resistant to fluoroquinolone antibiotic in Al-Diwaniya city and this is the first report of *aac(6')-Ib-cr* gene in *Acinetobacter*.

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