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Comparison of OmpA Gene-Targeted Real-Time PCR with the Conventional Culture Method for Detection of *Acinetobacter baumanii* in Pneumonic BALB/c Mice

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ABSTRACT

Background: *Acinetobacter baumannii* is an important pathogen in health care and is responsible for severe nosocomial and community-acquired pneumonia. To design novel therapeutic agents, a mouse model for *A. baumannii* pneumonia is essential. **Methods:** We described a mouse model of *A. baumannii* using clinical and 19606R standard strains for developing a quantitative real-time PCR (qRT-PCR) for rapid identification of *A. baumannii* infection from lung tissues of BALB/c mice. **Results:** To infect the mice, three doses of bacteria (0.5×10^8, 1×10^8, and 1.5×10^8 cfu/ml) were used. Lung tissues were cultured and compared with *ompA* gene. Clinical isolates had better positive results at day three with the highest dose than 19606 strain either in culture (4 versus 3) or in qRT-PCR (5 versus 4). However, qRT-PCR detection was 100%, the specificity was 70%, and the positive predictive value was 27%. **Conclusion:** The qRT-PCR detection of *A. baumannii* in the BALB/c mice model has a higher sensitivity than the culture method. ***DOI: 10.29252/ibj.23.2.159***

Keywords: *Acinetobacter baumanii,* OmpA, qRT-PCR Pneumonia, BALB/c mice

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INTRODUCTION

Acinetobacter baumannii is regarded as one of the most important opportunistic pathogens of increasing clinical importance over the course of the last three decades[1,2]. *A. baumannii* infection has recently emerged as a major cause of health care-associated (hospital- and community-acquired) infections worldwide. Pneumonia is the most frequent clinical presentation of *A. baumannii* infections[3]. This bacterial agent is identified as the fifth most common pathogen in Intensive care units (ICUs) in developed and developing countries[2,4,5]. The high rate of antibiotic resistance observed for *A. baumannii* isolates is attributed to its ability to employ a number of virulence factors such as OmpA[6,7]. OmpA is highly conserved among bacterial species, and in *A. baumannii*, it has been associated with a variety of interesting biological properties in *in vitro* model systems[8,9]. OmpA has also been associated with antimicrobial resistance in related pathogens[10]. The frequent existence of multidrug-resistant, extensive drug-resistant, and pandrug-resistant *A. baumannii* isolates revived the use of colistin, as an old polymyxin

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antibiotic[11-14]. Recently, colistin-resistant *A. baumannii* isolates are augmenting, which represents extremely alarming phenomenon[15]. Therefore, there is an urgent need for the development of novel therapeutic approaches.

Animal models are essential for the progress towards developing new therapeutics and vaccines and play fundamental roles in the evaluations of efficacy and safety of the new products before entering clinical trials. During the past years, the mice models of *A. baumannii* pneumonia have been developed and used widely[3]. In these models, several inbred strains of mice such as C57BL/6 and BALB/c as well as A/J strains have been used. Although these strains are likely to demonstrate a more homogeneous response to infection, they may not exactly mirror the variable responses that may take place in humans[16-21]. To recognize the infected mice and verify the production of infectious models, researchers apply diagnostic methods. Current diagnostic techniques rely on culture-based approaches, which are time-consuming and dependent on individual interpretation. In addition, the sensitivity of culture method is not high enough[22]. Nowadays, the real-time PCR is being used for sensitive detection of different viral[23] and bacterial infections[24], which improves the early detection of these diseases.

The aim of this study was to optimize a quantitative real-time PCR (qRT-PCR) method for rapid identification of *A. baumannii* in infected lung tissues of BALB/c mice using a clinical isolate and the strain 19606 of *A. baumannii*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A CR-XDR *A. baumannii* isolated from burn-wound infection was used in this study[25]. This strain is resistant to amikacin, cefepime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, levofloxacin, minocycline, tetracycline, tobramycin, and trimethoprim-sulfamethoxazole, but it is susceptible to ampicillin-sulbactam (Table 1). The phenotype of *A. baumannii* is defined as XDR, which is consistent with the International Expert Proposal for Interim Standards Guidelines[26]. *A. baumannii* ATCC 19606R was incorporated in the study as a colistin and an imipenem-susceptible reference strain (Table 1). Fresh brain heart infusion broth (Merck, Darmstadt,

Table 1. Two multidrug-resistant *A. baumannii* strains were used and the minimum inhibitory concentration (MIC) of 13 antimicrobial drugs for these strains were determined

Antibiotics	Types of strain/MIC (µg/ml)	
	Clinical isolate	19606R (standard) strain
B-Lactamase inhibitors		
Ampicillin-sulbactam	4/2 (S)	32/16 (R)
Cephems		
Ceftazidime	64 (R)	32 (R)
Cefepime	64 (R)	32 (R)
Carbapenems		
Imipenem	16 (R)	2 (S)
Lipopeptides		
Colisitin	16 (R)	2 (S)
Aminoglycosides		
	32 (R)	16 (R)
Amikacin	128 (R)	64 (R)
Tobramycin	32 (R)	16 (R)
Tetracyclines		
Minocycline	32 (R)	16 (R)
Tetracycline	32 (R)	16 (R)
Fluoroquinolones		
Ciprofloxacin	8 (R)	4 (R)
Levofloxacin	16 (R)	8 (R)
Folat pathway inhibitors		
Trimethoprim-sulfamethoxazole	8/152 (R)	4/76 (R)

(R) resistant; (S) susceptible

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Germany) bacterial cultures, in an aerobic atmosphere in the logarithmic growth phase (4–5 hours) at 37 °C, were adjusted to a concentration of 1.0×10^6 colony forming units (CFU)/mL, as verified by both spectrophotometry (OD_{600} 0.01–0.02 nm) and colony counting[27].

A. baumannii isolate confirmation

The isolate was identified as *A. baumannii* using API 20NE system (bioMérieux, Marcy-l'Etoile, France) and was later confirmed by the detection of *bla*OXA-51-PCR, to differentiate between *Acinetobacter calcoaceticus* and *Acinetobacter genomic species*[28,29]. The clinical isolate was stored at -20 °C in CRYOBANK™ (Copan Diagnostics Inc., Canada) until further use.

Mice

Eight- to 12-week-old, specific-pathogen free, male or female BALB/c mice, with 25–35 g in weight, were purchased from the Pasteur Institute of Iran (Karaj, Iran). Animals had free access to food and water except during experimental procedures. All studies were performed in accordance with the institutional ethical guidelines for the care and use of laboratory animals, and the protocol was approved by Tehran University of Medical Sciences, Tehran, Iran[30].

Intranasal *A. baumannii* inoculation

For intranasal inoculation, fresh inocula were prepared for each experiment from frozen stocks of the *A. baumannii* isolate as previously mentioned by van Faassen *et al.*[31]. Mice were anesthetized by intraperitoneal (i.p.) injection of 12.5 mg/kg (5 µl) xylazine and 80 mg/kg (25 µl) ketamine and then inoculated intranasally with appropriate numbers of various *A. baumannii* isolates in 50 µl of saline. For evaluation of infection using real-time PCR, 180 mice were assigned to four groups (5 mice in each group) using three doses (0.5×10^8, 1×10^8, and 1.5×10^8 cfu/ml) of bacteria in 50 µl for three days.

Conventional bacterial culture of pneumonic mice lung

Five mice of each group were sacrificed 24 hours after days 1, 2, and 3 post inoculation. The lung (about 0.36 g) was removed and segmented and then homogenized under the sterile condition. Next, 1 ml saline was added to the homogenized tissue, and 100 µl was cultured on Muller-Hinton agar (MHA) and then incubated at 37 °C for one day. One part of tissue was removed for qRT-PCR, and the rest was used for the conventional culture.

Evaluation of the *A. baumannii* OmpA gene expression by qRT-PCR

DNA extraction from lung tissue

A commercial kit (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, CA, USA) was used in accordance with manufacturer's recommendations to extract DNA. About 4 g of each tissue was dissected using a sterile scalpel blade and placed in a stomacher bag containing 20 ml of Earle's balanced salt solution. The sample was homogenized with a blender at high speed for 60 s. The resultant supernatant was stored at -20 °C until it was used for DNA extraction. To extract DNA, 100 µl of liquid homogenate was mixed with 180 µl of ATL buffer (Tissue Lysis Buffer), supplied with the kit, and then the kit's spin-column protocol for "purification of total DNA from animal tissues" was used.

Primers

Primers used for qRT-PCR, previously published by McConnell *et al.*[32] are as follows: OmpA-F (TCTTGGTGGTCACTTGAAGC) and OmpA-R (ACTCTTGTGGTTGTGGAGCA) as well as 16sr RNA-F (ACTCCTACGGGAGGCAGCAGT) and 16sr RNA-R (TATTACCGCGGCTGCTGGC). Sequences were checked for specificity using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were optimized using temperature gradients, and the PCR products were visualized on 1% agarose gel electrophoresis. The equivalent Tm value for *OmpA* gene (Tm: 79.4 ± 0.4 °C) was also detected when the positive control strain (*A. baumanii*; ATCC 19606 standard strain) was tested with qRT-PCR.

qRT-PCR

All real-time PCRs were performed on an ABI 7300 qRT-PCR detection system (Applied Biosystems, Foster City, CA, USA). Real-time qPCR amplification was carried out using the Qiagen Rotor-Gene SYBR Green Kit. A standard curve (using at least 5 standards) as well as positive, negative, and no template controls were included in every run. For SYBR green-based reactions, the conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. At the end of each reaction, a melting curve (from 50 to 99 °C) was performed. In order to rule out the false-negative results due to DNA losses during DNA extraction or the presence of PCR inhibitor in the template, human herpes virus 6 (HHV6) DNA (obtained from the Department of Virology, Tehran University of Medical Sciences, Tehran, Iran) was sed as an internal control. Mice were scarified at days 1, 2, and 3 post inoculation with three sub-lethal

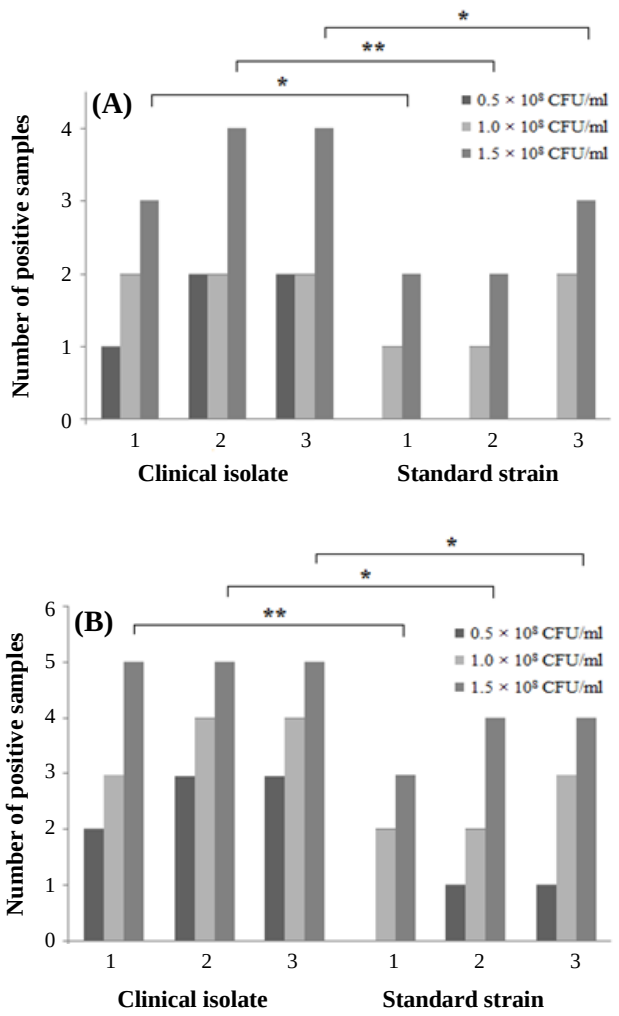

Fig. 1. Positive results of *A.baumannii* in the samples of lung tissues of pneumonic mice. Mice were inoculated with three different doses of clinical isolate and standard strain (19606R), and numbers of colonies and *OmpA* genes were measured on days 1, 2, and 3 post inoculation. (A); Number of colonies detected in the traditional tissue culture. (B); Number of *OmpA* gene detected by qRT-PCR directly from lung tissue. Twenty mice were used for each dose of bacteria, and five mice were used in each group (total 180 mice). * and ** represent $p < 0.05$ and $p < 0.01$, respectively.

concentrations of the bacteria (0.5×10^8, 1×10^8, and 1.5×10^8 CFU/ml), and the tissue was evaluated with qRT-PCR OmpA expression.

Statistical analysis

Data were presented as means ± standard deviation (SD) for each group. Differences were assessed by student's *t*-test by one-way ANOVA. Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

Establishment of qRT-PCR assays

The linearity and limits of detection of the assays were determined with serial 10-fold dilutions of ATCC 19606R DNA from 10^1 to 10^6 copies/µL. The limit of detection for the target DNA was 10 copies per 20 µL reaction volume. The assays correlated well for OmpA ($r^2 = 0.994$). Intra- and inter-assay repeatability was tested in triplicate for each dilution within the same run, and each concentration was repeated three different times to assess the reproducibility of the qRT-PCR assays. The coefficient of variation of intra-assay and inter-assay were low and in the ranges of 0.06%–0.48% and 0.08%–4.40%, respectively. Intra- and inter-assay coefficient of variation of less than 4.2% confirmed the high repeatability of the assays. Samples were considered positive if a threshold cycle was reached during the 35 cycles.

Comparison of conventional bacterial culture results with qRT-PCR

Since *OmpA* sequence of different strains of *A. baumannii* are similar, the standard curve was used to determine the existence of bacterial genomic DNA in the lung tissue. On third day post infection, the lungs of the infected mice in conventional culture showed the highest positive number when the highest inoculation dose (1.5×10^8 CFU/ml) was used; however, clinical isolate had more positive results than the standard strain (Fig. 1A). The direct qRT-PCR had most sensitivity and rapid detection of *OmpA* gene expression. The number of positive results in direct qRT-PCR was higher in comparison with conventional cultures, in all three bacterial doses on each day after inoculation (Fig. 1B).

DISCUSSION

This report is the first observational study to assess the clinical utility of qRT-PCR on specimens from the respiratory tract of a mouse model for detection of *A. baumannii*. The previous results from patient samples in the ICUs demonstrated that *A. baumannii* was identified on 8.7% of cases using bacterial culture[33]. In our study, we showed that the positive bacterial culture in three different doses were 0-80% of bacteria in the lung of mice inoculated with *A. baumannii*, whereas qRT-PCR could detect a higher rate (10-100%) of the bacteria (as OmpA). We also demonstrated a high sensitivity and negative predictive value of direct qRT-PCR detection for identification of

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A. baumannii. The results revealed that in both methods (conventional culture and qRT-PCR), the highest dose (1.5×10^8 CFU/ml) of the bacterial inoculation had the best positive outcomes in all three days post inoculation, especially in relation to the clinical isolate of *A. baumannii*[22].

Using positive culture of *A. baumannii*, as the gold standard, the sensitivity of qRT-PCR detection was 100%, the specificity was 70%, the positive predictive value was 27%, and the negative predictive value was 100% among the samples collected from *A. baumannii* pneumonic mice. We observed a high frequency of samples that were negative by the conventional bacterial culture but positive by qRT-PCR. One possibility is that some Acinetobacter strains that are detected by qRT-PCR could not able to grow on Acinetobacter medium[24]. The application of qRT-PCR was limited by the low positive predictive value for the following possibilities. First, negative culture results could have been obscured by immune system of mice in some cases. As such, the dead bacterial remnants would fail to grow on culture, but the qRT-PCR detects the presence of genomic DNA in the samples. Second, more sensitive nature of qRT-PCR detection might result in recognizing a small number of *A. baumannii*, which might not be detected in culture[23]. Third, the PCR might be attributed to a false-positive results, possibly indicating the existence of bacterial DNA that is not associated with viable bacteria[32]. However, the inclusion of a negative PCR control minimized this possibility. Fourth, more sensitive qRT-PCR might detect a small number of *A. baumannii*, which are not responsible agent for pneumonia.

In conclusion, our study indicated that qRT-PCR is a sensitive method for diagnosis of *A. baumannii* in pneumonic animal models. However, further studies are required to probe the potential role of this approach.

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CONFLICT OF INTEREST. None declared.

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