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In vitro and *in vivo* Evaluation of Antibiotic Combination against Imipenem Resistant *Acinetobacter baumannii* Strains Isolated from Bangladeshi Patients

Bhuiyan Mohammad Mahtab Uddin[1], Ritu Saha[2,*], Zubair Ahmed Ratan[3,4], Surovi Era Suchi[5], S. M. Shamsuzzaman[6]

[1]Department of Microbiology, Enam Medical College, Dhaka, Bangladesh,
[2]Department of Microbiology, Bashundhara Ad-din Medical College, Dhaka, Bangladesh,
[3]School of Health and Society, University of Wollongong, Australia,
[4]Department of Biomedical Engineering, Khulna University of Engineering and Technology, Khulna, Bangladesh,
[5]Department of Microbiology, Ad-din Sakina Women's Medical College, Jeshore, Bangladesh
[6]Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh
*Corresponding author: ritu86.smc@gmail.com

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Abstract Acinetobacter baumannii is becoming a common etiological agent of nosocomial infections resulting in septicemia, meningitis, endocarditis, pneumonia, wound, and urinary tract infections within the healthcare setting. There are currently no defined optimal therapies available for multidrug-resistant (MDR) Acinetobacter baumannii infections. We evaluated the efficacy of imipenem, ceftazidime, amikacin and their combinations against imipenem resistant A. baumannii in experimental rat models. We also detected MBL encoding genes such as blaNDM-1, blaVIM and blaIMP and ESBL encoding genes such as blaCTX-M-15 and blaOXA-1 genes by Polymerase chain reaction (PCR). MBL encoding genes such as blaNDM-1 (83.33%), blaVIM (66.67%) and blaIMP (41.67%) and ESBL encoding genes such as blaCTX-M-15 (16.67%) and blaOXA-1 (12.50%) were detected among imipenem resistant Acinetobacter baumannii by PCR. In vitro activities of imipenem, ceftazidime, amikacin, ciprofloxacin, tigecycline and their combinations were tested using agar dilution method. The proportions of synergy observed in imipenem-ceftazidime, imipenem-amikacin, imipenem-ciprofloxacin and amikacin-tigecycline combinations were 25%, 54.17%, 12.50% and 41.67% respectively in vitro. Rat septicaemic models were evaluated using the imipenem resistant A. baumannii strain. Rats were treated with three antimicrobials and their combinations. For the rat model, the efficacies of imipenem, amikacin and imipenem plus ceftazidime and imipenem plus amikacin were assayed. In the septicaemic rat model, compared to the control group, (i) imipenem alone, (ii) ceftazidime alone (iii) imipenem plus ceftazidime (iv) imipenem plus amikacin showed increased sterile blood culture (50%, 16.67%, 66.67% and 83.33%). Imipenem plus amikacin or amikacin plus tigecycline may be appropriate for the treatment against imipenem resistant Acinetobacter baumannii infections.

Keywords: *Acinetobacter baumannii, imipenem resistant, antibiotic combination, in vitro and in vivo efficacy*

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1. Introduction

Serious nosocomial and community-acquired infection by Acinetobacter baumannii is gradually increasing [1]. Pneumonia, bacteremia, urinary tract infections and surgical site infections being the most important infections caused by this organism [2]. It is currently the most important isolate from gram negative sepsis in immunocompromised patients posing risk for high mortality [3].

The well-known ability of A.baumannii to acquire resistance to almost all groups of available antibiotics leads to serious problems in the management of infections caused by multi-drug resistant (MDR) A. baumannii infections [2,4] and In these cases, carbapenems have been considered the treatment of choice. However, increasing numbers of carbapenem-resistant A. baumannii isolates have been reported worldwide [1,5], prompting the search for other therapeutic options.

The purpose of this study was to make an effective combination of antibiotics for these multidrug resistant organisms and to compare the in vitro and in vivo activity

of imipenem, a drug clinically effective against A. baumanii, with ceftazidime and amikacin. The efficacy of monotherapy was compared with combined treatment with amikacin. For these experiments we have developed a new Acinetobacter experimental sepsis model In immuocompetent rats.

2. Methods

2.1. In vitro Study

We conducted a cross-sectional study in the Department of Microbiology of Dhaka Medical College, Dhaka, Bangladesh during July 2014 to June 2015. This research protocol was approved by the research review committee (RRC) and ethical review committee (ERC) of Dhaka Medical College. Twenty four isolates resistant to imipenem by disk diffusion technique following Clinical and Laboratory Standards Institute [CLSI] guidelines were tested for efficacy of antibiotic combination [6].

2.1.1. Identification of Imipenem Resistant A. Baumannii Isolates

Samples collected from different sources were inoculated on MacConkey agar media and Blood agar media. Non-lactose fermenting colonies on MacConkey agar were identified as *Acinetobacter baumannii* if they were gram-negative coccobacilli, oxidase negative, non-motile, indole and urease negative, citrate positive and grew at 41°C and 44°C [7].

Imipenem resistant *A. baumannii* were identified by disk diffusion technique using commercially available antibiotic disks (Oxoid Ltd, Basngstoke, United Kingdom). *Escherichia coli* ATCC 25922 was used for quality control [6].

2.1.2. Molecular Characterization of MBL and ESBL Producers

The presence of MBL genes such as blaNDM-1, blaIMP and blaVIM ESBL & encoding genes such as blaCTX-M-15 and blaOXA-1 among the imipenem resistant isolates was detected by polymerase chain reaction (PCR). To prepare bacterial pellets, a loop full of bacterial colonies was inoculated into a Falcon tube containing trypticase soy broth. After incubation overnight at 37^{0}C, the Falcon tubes were centrifuged at 4000 ×g for 10 minutes, after which the supernatant was discarded. A small amount of sterile trypticase soy broth was added into the Falcon tubes with pellets and mixed evenly. Then an equal amount of bacterial suspension was placed into 2 to 3 to microcentrifuse tubes. The microcentrifuse tubes were then centrifuged at 4000 ×g for 10 minutes and the supernatant was discarded. The microcentrifuse tubes containing bacterial pellets were kept at -20^{0}C until DNA extraction. Bacterial DNA was extracted by the boiling method [8]. The following pairs of previously used primers were used to yield PCR products: for blaNDM-1-GCGCAACACAGCCTGACTTT(forward), CAGCCACC AAAAGCGATGTC (reverse), for blaIMP- GGAAT AGAGTGGCTTAATCTC(forward), CCAAACYACTAS GTTATCT (reverse) and for blaVIM- GAT GGT GTT TGG TCG CAT A (forward), CGA ATG CGC AGC ACC AG (reverse), for blaCTX-M-15- CACACGTGGAATTT AGGGACT(forward), GCCGTCTAAGGCGATAAACA (reverse) and for blaOXA-1-ACCAGATTCCAACTTT CAA(forward), TCTTGGCTTTTATGCTTG (reverse) [9].

The following cycling parameters were used: initial denaturation at 95°C for 10 minutes, then 30 cycles of denaturation at 95°C for one minute, annealing at 63°C (for blaNDM-1), 52°C (for blaIMP), 52°C (for blaVIM) for 45 seconds, extension at 72°C for one minute and 30 seconds, and a final extension at 72°C for 10 minutes. The amplified DNA were loaded into a 2% agarose gel, electrophoresed at 100 volts for 30 minutes, stained with 1% ethidium bromide, and visualized under UV light.

2.1.3. Minimum Inhibitory Concentration (MIC)

MIC was performed by the agar dilution method. MICs were determined by using dilutions of individual antibiotics incorporated into Mueller Hinton agar (Oxoid Ltd, Basngstoke, United Kingdom). Seven doubling dilutions each antibiotic were prepared. To obtain 104 cfu/spot on the agar surface, one microlitre of 10 times diluted 0.5 McFarland turbidity of test inoculums were placed on Mueller Hinton agar plates. After incubation at 35°C overnight, the lowest concentration of antibiotic impregnated Mueller-Hinton agar showing no visible growth on agar medium was considerd the MIC of that drug of that strain. Escherichia coli ATCC 25922 was used as control strain. The MIC was defined as the lowest concentration of antibiotic at which no growth was visible to the naked eye.

2.1.4. Antibiotic Combination Testing

Twenty four isolates with clear resistance to imipenem (MIC, >4 µg/ml) were selected for the combination studies. Combinations of imipenem with amikacin, ceftazidime and ciprofloxacin and combination of tigecycline and amikacin were examined by agar dilution method. Twofold serial dilutions of antibiotics were prepared from two fold higher dilutions of MICs upto four fold lower dilutions of MIC. In evaluating the combination effect, synergy was present by the agar dilution method when there was a fourfold or greater reduction in the MICs of both antibiotics. A reduction of less than fourfold in the MICs of both antibiotics was considered additive. Indifference was found when neither drug exhibited a decrease in MIC, and an increase in the MIC was considered antagonism. Testing for synergy by the agar dilution technique is based on inhibitory rather than bactericidal endpoints [10].

2.2. *In vivo* Study

The experiments were performed in immunocompetant male and female rats weighing 40-50 grams, obtained from ICDDR,B breeding house, Dhaka, Bangladesh.

Rats were infected by intraperitoneal entry of 125 µl of approximately 108 cfu/ml bacterial inoculums through intraperitonally. Bacterial inoculums were obtained through a 24 hours subculture of Acinetobacter baumannii in MacConkey agar media at 37°C. The animals were observed for 72 hours and the survival rates were recorded every 12 hours, blood samples were taken as detailed below. All the blood samples were processed for microbiological studies.

2.2.1. Antimicrobial Treatment

To evaluate the effectiveness of the different treatment regimens, at first thirty six (36) rats were divided into six groups with six rats in each group and the groups were regarded as group A, B, C, D, E and F respectively. Group A, B, C, D and E were inoculated with bacterial inoculums but group F were not inoculated with bacterial inoculums. Group E were only inoculated with bacterial inoculums but did not receive antimicrobial treatment, regarded as positive control group. Group F were not inoculated with bacterial inoculums but received antimicrobial treatment, regarded as negative control group. Group A, B, C, and D received antimicrobial treatment after 4 hours inoculation of bacterial inoculums in following treatment regimens over 72 hours.

Group A - imipenem only, im, 120 mg/kg/day, twice daily.

Group B - amikacin only, im, 15 mg/kg/day, twice daily.

Group C – Imipenem plus amikacin Imipenem im, 120 mg/kg/day, twice daily Amikacin im, 15 mg/kg/day, twice daily

Group D – imipenem plus ceftazidime Imipenem im, 120 mg/kg/day, twice daily Ceftazidime im, 100 mg/kg/day, twice daily

The first dose of every antibiotic was administered 4 hours after inoculation of the organisms. In order to confirm that these drugs were not directly toxic to the animals, group F (negative control) were given each antibiotic for 72 hours. The infected animals were observed for 72 hours of treatment and the cumulative survival rates were recorded every 12 hours. Blood samples were taken as described below [11].

2.2.2. Microbiological Study

After 72 hours of antibiotic treatment, blood samples were collected from rat by cardiac puncture aseptically. At first, upper part of the chest was shaved by razor, then washed with hexisol, povidon iodine and finally 70% alcohol. After palpating the cardiac pulsation with the finger pulp, 21G syringe needle was introduced through the skin in the heart of rat blindly. For blood culture, 1.5 ml of each rat's blood were collected and then inoculated in sterile conical flask with 15 ml of trypticase soya broth and incubated for 24 hours at 37°C. On the next day, 100 µl of each sample was plated on sheep blood agar plates and incubated for another 24 hours at 37°C [9]. The results of the blood cultures were expressed as positive or negative [11].

3. Result

In vitro tests

Out of 28 isolated Acinetobacter baumannii strains, 24 were imipenem resistant detected by disk diffusion technique. Twenty four (100%) MBL producers were dected by PCR, of which 20 (83.33%) were positive for blaNDM-1, 16 (66.67%) for blaVIM and 10 (41.67%) for blaIMP. Out of twenty four imipenem resistant Acinetobacter baumannii strains, 4 (16.67%) were positive for blaCTX-M-15 and 3 (12.50%) were positive for blaOXA-1.

The MIC of imipenem of these isolates ranged from ≥256 µg/ml to 16 µg/ml. MIC of other drugs such as amikacin, ceftazidime, ciprofloxacin and tigecycline were ranged from≥2048 µg/ml to 16 µg/ml, ≥1024 µg/ml to 128 µg/ml, ≥128µg/ml to 4 µg/ml and 8 µg/ml to 1 µg/ml respectively.

Using different antibiotic combinations against imipenem resistant Acinetobacter baumannii isolates, showed different types of synergy. While combining imipenem and amikacin, among imipenem resistant twenty four Acinetobacter baumannii isolates, 54.17% showed synergistic effect (four fold reduction of MIC), 20.83% showed additive effect (two fold reduction of MIC) and 25% showed indifferent effect (no reduction of MIC). In case of imipenem and ceftazidime combination, 25% showed synergistic effect, 16.67% showed additive effect and 58.33% showed indifferent effect. While combining imipenem plus ciprofloxacin and amikacin and tigecycline, showed 12.50% and 41.67% showed synergisctic effect respectively (Table 1).

Table 1. Comparison of synergism between use of antibiotics in different combinations in vitro (n=24)

Combinations	Synergy	Additive	Indifferent	Antagonism
Imipenem + Ceftazidime	6 (25.00)	4 (16.67)	14 (58.33	0 (0.00)
Imipenem + Amikacin	13 (54.17)	5 (20.83)	6 (25.00)	0 (0.00)
Imipenem + Ciprofloxacin	3 (12.50)	3 (12.50)	18 (75.00)	0 (0.00)
Amikacin + Tigecycline	10 (41.67)	6 (25.00)	8 (33.33)	0 (0.00)

In vivo tests

All the rats in the positive control group were bacteraemic and all the rats in the negative control group were sterile. In the group treated with only imipenem and only amikacin, 50% and 83.33% rats were bacteraemic respectively after 72 hours of treatment. On the other hand in the group treated with imipenem plus amikacin and another group treated with imipenem plus ceftazidime, 16.67% and 33.33% rats were blood culture negative respectively (Table 2).

Table 2. Result of antibiotic therapy on the clearance of Acinetobacter baumannii from the blood of rats

Group	Blood Culture Positive n (%)
Poitive control (6)	6 (100.0)
Negative control (6)	0 (0.00)
Imipenem (6)	3 (50.00)
Amikacin (6)	5 (83.33)
Imipenem + Amikacin (6)	1 (16.67)
Imipenem + Ceftazidime (6)	2 (33.33)

4. Discussion

Acinetobacter baumannii is well-known for its potential to be resistant to many commonly used antimicrobial agents, including penicillins, cephalosporins, monobactams, aminoglycosides and furoquinolones. Infections resulting

from highly resistant *A. baumannii* isolates, for which there are limited therapeutic options, can lead to a high fatality rate. Carbapenems are effective antimicrobial agents against *A. baumannii*, but the emergence of strains with reduced susceptibility to carbapenems has often been described [12,13]. Therefore, the main goal of this study was to compare the *in vitro* and *in vivo* efficacy of imipenem alone or in combination with amikacin in the treatment of *A. baumannii* infections, using a new experimental model of immunocompetent rats. Owing to the frequency of imipenem resistant strains and the need to find new therapeutic approaches, we have compared these antibiotics, used frequently in infections caused by this organism, with different combination.

In this study, *in vitro*, while combining imipenem with amikacin against imipenem resistant *A. baumannii*, 54.17% showed synergism, 20.83% showed additive effect, 25% showed indifferent effect and no antagonism was observerd. A study [14] showed 42 % synergism while combining imipenem with amikacin against carbapenem resistant *A. baumannii* which is almost similar to present study. In the present study, while combining imipenem with ceftazidime against imipenem resistant *A. baumannii*, 25% showed synergism, 16.67% showed additive effect, 58.33% showed indifferent effect and no antagonism was observerd. No data was available to compare the efficacy of this combination against *A. baumannii*. A study [15] showed 93% synergism while combining imipenem with cefotaxime against *Nocardia asteroids* which is in contrast to the present study. The higher percentage of synergism in that study might be due to the fact that imipenem and cefotaxime might have acted at different sites of the cell wall synthesis or bound to different penicillin binding proteins.

In the present study, *in vitro*, while combining imipenem with ciprofloxacin against imipenem resistant *A. baumannii*, 12.50% showed synergism, 12.50% showed additive effect, 75% showed indifferent effect and no antagonism was observerd. In accordance with the present study, a study [14] reported 16% synergism while combining imipenem with ciprofloxacin against imipenem resistant *A. baumannii*. Our present study showed, while combining tigecycline with amikacin *in vitro* against imipenem resistant *A. baumannii*, 41.67% showed synergism, 25% showed additive effect, 33.33% showed indifferent effect and no antagonism was observerd. A study by Principe *et al.* [16] reported 8.3% synergism while combinig tigecycline with amikacin, which is less from the present study. This might be due to the fact that susceptibilities of *Acinetobacter* spp. against various antimicrobials are considerably different among countries, centres and even among different wards of the same hospital [17].

The present study observed 25% synergism with the combination of imipenem plus ceftazidime, 54.17% with imipenem plus amikacin, 12.50% imipenem plus ciprofloxacin and 41.67% with tigecycline plus amikacin. Montero *et al.* [18] showed that, imipenem and aminoglycosides combination was the best alternative for carbapenem resistant *A. baumannii*.

Present study observed periodic observation of rats after antibiotic therapy on survival of rats. All the rats of every group were survived. Hernandez *et al.* [11] showed that, in case of imipenem and amikacin combination treated cases 14% mice died and in case of only imipenem treated cases 10% mice died after 72 hours of antibiotic treatment. But present study showed that, no rat were died. This might be due to no use of any porcine mucin in the present study but porcine mucine was used in previous study. Porcine mucin increases the virulence of the organism. Even no immunosuppressive agent like cyclophosphomide was used in the present study to rats become neutropenic. But in a study by Johnson *et al* [19] cyclophosphomide was used as immunosuppressive agent to rats become neutropenic and significant number of rats were died in different groups of rats in that study.

The best *in vivo* results appeared in the group treated with imipenem and amikacin combination. Bacterial clearance of the rats from blood sample was 83.33% with the combination of imipenem and amikacin treatment. Present study also observed 66.67% clearance of organism from blood while combiming imipenem and ceftazidime, 16.67% with amikacin alone and 50% with imipenem alone. A study [11] showed 100% clearance of organism from blood of the rats while combining imipenem and amikacin.

In this study, out of 24 imipenem resistant *Acinetobacter baumannii*, 24 (100%) carbapenemase encoding genes were detected by PCR. Of them, 20 (83.33%) were positive for *bla*NDM-1, 16 (66.67%) were positive for *bla*VIM and 10 (41.67%) were positive for *bla*IMP. Previous study by Farzana *et al.* [6] reported 31 (88.57%) MBL producers out of the 35 imipenem resistant bacteria The frequency of MBL producers in the study of Farzana *et al.* was almost similar to the present study.

Kumarasamy *et al.* [20] have recently reported the emergence and spread of 180 cases of patients infected with bacteria carrying the NDM-1 encoding gene from Pakistan, India and the UK. Now this study showed that NDM-1 genes are also found in Bangladesh among the patients infected with imipenem resistant organisms. Kumarasamy *et al.* [20] also suggested that these organisms were acquired from a local source in Asia.

The rapid spread and dissemination of these multidrug-resistant bacteria worldwide represents a major public health problem, thus the US Centers for Disease Control and Prevention (CDC) has recently planned to add NDM-1 producing MDR bacteria as agents of communicable diseases and hospitals must immediately report any suspect cases, particularly those for which the patient received medical treatment in India or Pakistan. The aim of this work was to develop a rapid real-time polymerase chain reaction (PCR) assay to detect the NDM-1 encoding gene in bacteria. Chen *et al.* [21] showed that this $bla_{\text{NDM-1}}$ positive *Acinetobacter baumannii* strain was susceptible to several fluoroquinolone antibiotics and to polymyxin B though our study showed very high percentage of NDM-1 encoding bacteria were resistant to fluoroquinolone.

In *Acinetobacter baumannii,* six IMP variants belonging to three different phylogroups have been identified and reported namely IMP-1 in Italy, Japan and South Korea; IMP-2 in Italy and Japan [1]. We also found *bla*IMP in significant amount (41.67%).

There are only few studies that have documented VIM type of MBL in *Acinetobacter.* VIM-2 producing *Acinetobacter* spp. have been isolated in the Far East [22]

and in Germany [23], while the VIM-1 determinant has been reported only in Greece [24].

In conclusion, the experimental model permits the study of the *in vivo* activity of antimicrobial drugs in *A. baumannii* bacteremia in immunocompetent rats. The studies made *in vivo* agree with the results obtained *in vitro*. Combination of imipenem and amikacin was the most effective treatment. The second best effective combination is imipenem and ceftazidime *in vivo* (rat model) and tigecycline plus amikacin is the second best combination *in vitro*.

5. Conclusion

The combination of two or more antimicrobials may provide a better effect. The present study reported both the *in vitro* and *in vivo* efficacy of combination of two drugs against imipenem resistant *A. baumannii*. Outcome of combinations of more than two drugs can also be evaluated. Prompt and rapid detection of MBL producers will prevent their spread and *in vitro* resistance pattern of this bacterial strain which will guide the clinicians to use appropriate antibiotics, also the results of combinations will guide for treatment options against imipenem resistant isolates.

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