Original Research

XDR-*Klebsiella pneumoniae* isolates harboring bla_{OXA-48}: *In vitro* and *in vivo* evaluation using a murine thigh-infection model

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Impact statement

The present study aimed to evaluate the effectiveness of various antibiotics both *in vitro* and *in vivo* using murine animal model either alone or in combination against various strains of extensively drug-resistant (XDR) *Klebsiella pneumoniae*, life-threatening pathogens of relevant medical importance isolated from febrile neutropenic pediatric cancer patients. This work also emphasizes how to select the appropriate antibiotics options and help the physicians to choice the appropriate antibiotic for the treatment of such superbugs (extensively drug-resistant (XDR) *Klebsiella pneumoniae*). The results showed that *in vitro* dual carbapenem combination of ertapenem with meropenem had shown synergistic effect against all of the tested XDR isolates. Antibiotic combinations of dual carbapenems and meropenem plus colistin showed synergism in 100% and 75% of the testes isolates, respectively. Results of the *in vivo* evaluation, colistin alone had significantly reduced bacterial count while its combination with meropenem was not superior to monotherapy.

Abstract

Blood stream infection with extensively drug-resistant-carbapenamase producing *Klebsiella (K.) pneumoniae* usually represents a major threat with medical challenges among hospitalized cancer patients with poor functional status and underlying diseases. Accordingly, the aim of the study was to evaluate the efficacy of different antibiotics either alone or in combinations against extensively drug-resistant-OXA-48 producing *K. pneumoniae* clinical isolates that were previously recovered from febrile neutropenic pediatric cancer patients. The antimicrobial activity of amikacin, gentamicin, colistin, ertapenem, imipenem, meropenem and tigecycline was assessed by broth microdilution method. The results revealed that all the tested OXA-48 producing *K. pneumoniae* isolates exhibited extensively drug-resistant phenotype and all of them were susceptible to tigecycline. Checkerboard method was used to determine the fraction inhibitory concentration index, to further classify the effect of antibiotic combination as synergistic, additive, indifferent, or antagonistic effect. The results revealed that *in vitro* dual carbapenem combination of ertapenem with meropenem had shown synergistic effect against all of the tested isolates. Additionally, synergistic effect of meropenem with colistin was detected among three of four isolates tested. Herein we investigated the *in vivo* activity of colistin, meropenem alone and in combination in a rat thigh infection model. The results showed that addition of meropenem to colistin was not effective at reduction of bacterial count as compared to colistin alone at 24 h post treatment. Accordingly, we can conclude that *in vitro* antibiotic combinations of dual carbapenems (ertapenem plus meropenem) and meropenem plus colistin showed synergism in 100% and 75% of the tested isolates, respectively. Colistin alone had significantly reduced bacterial count while its combination with meropenem was not superior to monotherapy in murine thigh infection model.

Keywords: Carbapenemases, *Klebsiella pneumoniae*, antibiotic combination, murine thigh model, extensively drug-resistant, bla_{OXA-48}

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Introduction

Carbapenem resistance among Gram-negative bacteria is a cause of significant health concern due to their powerful ability to express multidrug resistance or extensive drug resistance phenotypes particularly among critically ill patients with significant comorbidities.[1] Worldwide,

the production of carbapenamases among *Klebsiella pneumoniae* has become the most important carbapenem resistance mechanism.[2] The OXA-48 (oxacillinase) carbapenamase was initially identified among *K. pneumoniae* from Turkey and then had widely spread as a source of nosocomial outbreaks in Mediterranean countries including Egypt.[3,4]

Carbapenemase producing bacteria are usually associated with generalized resistance to β-lactam group including carbapenems, penicillin and cephalosporins in addition to aminoglycosides and quinolones classes.[5–7] Regarding the futility of the previously mentioned antibiotics against carbapenemase producers, clinicians are looking for salvage treatment such as polymyxins and tigecycline. Unfortunately, the use of colistin or tigecycline alone as monotherapy was associated with appearance of heteroresistance strains and increased rate of death, respectively.[8,9] To by-pass this problem, experts had recommended combinational therapy as a way to overwhelm the spread of resistance with maximum antimicrobial efficacy.[10,11] Additionally, the clinical studies had reported on improved clinical outcomes with combination therapy even if the tested isolates were resistant to individual drugs.[12]

In the present study, we have evaluated the *in vitro* and *in vivo* antimicrobial activity of various antibiotics alone or in combination against OXA-48 carbapenamase producing *K. pneumoniae*.

Materials and methods

Bacterial isolates and antimicrobial agents

The study was performed on four previously isolated and characterized carbapenamase producing *K. pneumoniae* isolates (coded KP151, KP188, KP189 and KP190) each harboring bla_{OXA-48} encoded gene.[13] These isolates were recovered from febrile neutropenic pediatric cancer patients in Egypt. The study was approved by the hospital Ethics Committee and Faculty of Pharmacy ethical committee Nr. 72 where both informed and written consents were obtained from parents of patients after explaining the study purpose. The isolates were recovered from blood specimens collected from the respective patients. Due to the absence of reference strain of *K. pneumoniae*, the reference strain *E. coli* ATCC 25922 as representing member of lactose fermenter *Enterobacteriaceae* was used as a quality (susceptible) control for susceptibility testing as recommended by the CLSI guidelines.

Antibiotic susceptibility test by disk diffusion

This test was determined by Kirby–Bauer method using 19 antimicrobial agents disks including amikacin (AK, 30), amoxicillin/clavulanic acid (AMC, 30), aztreonam (ATM, 30), cefepime, (FEP, 30), cefotaxime (CTX, 30), ceftazidime (CAZ,30), ceftriaxone (CRO, 30), ciprofloxacin (CIP, 5), colistin, (CT, 10), doripenem (DOR, 10), ertapenem (ETR, 10), fosfomycin (FF, 50), gentamicin (CN, 10), imipenem (IMP, 10), polymyxin B (PB,300 U), rifampicin (RA,5), tetracycline (TE,30), tigecycline (TGC,15), sulphamethoxazole/trimethoprim (SXT,1.25/23.75) were tested. The antimicrobial disks were obtained from Bioanalyse Lab, Turkey and Oxoid, England. Different classes of antibiotics including β-lactam group namely 3rd, 4th generation cephalosporins and three carbapenems (including doripenem; ertapenem and, and imipenem) in addition to aminoglycosides, tetracyclines, quinolones phosphonic acid derivative, and polymyxins were tested to further classify the phenotype of recovered isolates.

Isolates that showed acquired resistance to at least one agent in all antimicrobial categories and remain susceptible to only one or two of the antimicrobial categories were considered extensively drug-resistant (XDR) pathogens.[1,14,15] Due to the lack of availability of meropenem disk at time of conducting study, the susceptibility pattern of this antibiotic had been quantitatively determined by calculating the MIC by micro-broth dilution which is more accurate and reliable than disc susceptibility.

Amikacin, colistin, ertapenem, gentamicin, imipenem, meropenem, and tigecycline powder were purchased from Sigma-Aldrich, St. Louis, MO for the determination of the minimum inhibitory concentration (MIC) by micro-broth dilution method, antimicrobial combination by checkerboard technique, and for *in vivo* evaluation in an animal model. Briefly, stock solutions of antibiotics were freshly prepared by dissolving tested agents in sterile distilled water before each experiment. The most common prescribed antibiotic as previously described in literature and in our previous experiments (Disc sensitivity) that still retain activity against XDR *K. pneumoniae* has been evaluated either alone or in combination both *in vitro* using MIC and *in vivo* using a murine animal model.

Determination of MIC by broth microdilution method

The MICs of the tested antibiotics were determined by broth microdilution method according to the Clinical Laboratory Standard Institute (CLSI) guidelines.[14] Double strength Mueller-Hinton broth (CAMHB; Oxoid, England), different antibiotic stock solutions, and the tested organisms adjusted at 10^7 cells/mL were prepared. The wells of the microtiter plate were filled with 100 µL of CAMHB and thereafter, 100 µL of antibiotic stock dilution were added to the first well. A new pipette tip was used to mix the added antibiotic with broth media and 100 µL were drown from the first well and transferred to the next well to achieve two-fold serial dilutions and this was repeated for the subsequent wells to achieve the tested dilution range. After dilution, 5 µL of the adjusted inoculum were added to each well to the give final count of 10^5 cells/mL and thereafter, the plates were incubated without shaking according to the CLSI guidelines at 37°C from 18 to 24 h.[14] The last two wells in each row were used as controls: one of them contained the broth and the tested isolate, with absence of antibiotic (positive control) and the other contained the broth alone (negative control). The European committee on Antimicrobial susceptibility tests (EUCAST) and CLSI breakpoints were used for the determination of colistin and the other tested antibiotics, respectively.[14,15]

Evaluation of antibiotic combination by checkerboard method

Each microtiter plate contains a mixture of two antimicrobial agents at concentration ranging from 1/8 MIC to 4 × MIC of the tested isolate to determine the magnitude of synergy and antagonism. Each row in the plate contained 50 μL of the same concentration of the first antimicrobial agent and the concentration in each subsequent row decreased by half. Similarly, each column in the plate contained 50 μL of the same concentration of the second antimicrobial agent and concentration in each subsequent column decreased by half. Finally, the microtiter plate was then inoculated with diluted tested culture (10^6 cfu/mL). The fractional inhibitory concentration index (FICI) has been determined as previously described.[16] The FICI = FIC of antibiotic A + FIC of antibiotic B. The FIC of antibiotic A = MIC of antibiotic A in combination/MIC of antibiotic A alone. Similarly, FIC of antibiotic B = MIC of antibiotic B in combination/MIC of antibiotic B alone. Interpretation of result was as follows: FICI $\leq$ 0.5 (synergism), > 0.5–1 (additive), > 1–4.0 (indifference), and > 4 (antagonism).[16]

Evaluation of antibiotics alone and combinations in a Wistar rat thigh infection model

Healthy male Wistar rats, weighing 90–110 g, were obtained from the animal house of the National Research Centre, Cairo, Egypt. Animals were maintained in accordance with the regulations of the ethical committee of the National Research Centre which gave its consent in accordance with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee. The whole study was approved by the hospital Ethics Committee, Faculty of Pharmacy, Ain Shams University ethical committee Nr. 72. Thigh infection model was developed by intramuscular injection of right thigh of each rat by 0.3 mL freshly prepared bacterial suspension of *K. pneumoniae* (isolate code, KP151) at a density of 1×10^8 CFU/mL (0.5 McFarland). Rats were randomly divided into six groups (six rats/group) receiving monotherapy, combinational therapy, and no treatment for 24 and 48 h observation. The following antibiotic doses as reported by earlier by Fan *et al.*[17] were used for injection: colistin at 20 mg/kg every 8 h, meropenem at 200 mg/kg every 8 h, and imipenem at 120 mg/kg every 8 h. After 24 and 48 h of treatment, the three rats from each group were sacrificed and their thigh muscles were aseptically excised. Thereafter, the thigh muscles were homogenized and the number of colony forming unit (CFU) was determined after 10-fold serial dilution of the homogenates. Synergy of a combination therapy was indicated by a $\geq$ 2 log10 CFU/mL decrease in comparison with the single drug, while antagonism was defined as >2 log10 CFU/mL increase.[17] The statistical analysis was examined using the GraphPad Prism software.

Table 1. Antibiogram analysis of the four XDR-*K. pneumoniae* isolates.

Antimicrobial agent	XDR-*K. pneumoniae* isolate (IZ mm)			
	KP151	KP188	KP189	KP190
AK	10/R	0/R	11/R	14/R
AMC	0/R	0/R	0/R	0/R
ATM	0/R	0/R	0/R	0/R
CTX	0/R	0/R	0/R	0/R
CAZ	0/R	0/R	0/R	0/R
CRO	0/R	0/R	0/R	0/R
FEP	0/R	0/R	0/R	0/R
CIP	0/R	0/R	0/R	0/R
SXT	0/R	16/S	17/S	23/S
TE	0/R	0/R	0/R	0/R
IMP	11/R	0/R	0/R	0/R
ETR	0/R	0/R	0/R	0/R
DOR	0/R	0/R	0/R	0/R
CT	12/S	0/R	10/R	0/R
PB	11/R	0/R	10/R	0/R
FF	0/R	0/R	0/R	0/R
RA	0/R	0/R	0/R	0/R
CN	0/R	0/R	0/R	0/R
TGC	17/S	22/S	24/S	26/S

AK: amikacin; AMC: amoxicillin/clavulanic acid; ATM: aztreonam; FEP: cefepime; CTX: cefotaxime; CAZ: ceftazidime; CRO: ceftriaxone; CIP: ciprofloxacin; CT: colistin; DOR: doripenem; ETR: ertapenem; FF: fosfomycin; CN: gentamicin; IMP: imipenem; PB: polymyxin B; RA: rifampicin; TE: tetracycline; TGC: tigecycline; SXT: sulphamethoxazole/trimethoprim; IZ mm: inhibition zone diameter in mm.

Table 2. MIC of various antibiotics (μg/mL) against OXA-48 carbapenamase producing *K. pneumoniae* isolates.

Isolate code	AK	CN	CT	ETR	IMP	MEM	TGC
KP151	64	1024	2	1024	128	256	1
KP188	256	256	512	1024	512	1024	1
KP189	1024	8	64	4	32	256	1
KP190	256	512	256	1024	128	256	1

AK: amikacin; CN: gentamicin; CT: colistin; ETR: ertapenem; IMP: imipenem; MEM: meropenem; TGC: tigecycline.

Results

Antimicrobial susceptibility testing

The antimicrobial susceptibility test results determined by the Kirby-Bauer disk diffusion method of the four OXA-48 carbapenamase producing *K. pneumoniae* isolates are shown in Table 1. Interpretive criteria for disc diffusion susceptibility testing were according to the CLSI guidelines (Table S1).

The MICs of amikacin, gentamicin, colistin, ertapenem, imipenem, meropenem, and tigecycline against the four OXA-48 carbapenamase producing *K. pneumoniae* isolates are shown in Table 2. According to CLSI and EUCAST MIC breakpoints, the tested isolates were resistant to all tested antibiotics except tigecycline with the exception of isolate KP151 that showed susceptibility to colistin in addition to tigecycline.

Evaluation of double antibiotic combination

Synergy between different antibiotics was evaluated by checkerboard method as shown in Table 3. Synergistic

Table 3. The FICI of different antibiotic combinations of OXA-48 carbapenamase producing *K. pneumoniae* isolates.

Isolate code	CT + MEM FICI/Interpret	ETR + MEM FICI/Interpret	IMP + CN FICI/Interpret	MEM + CN FICI/Interpret	CT + AK FICI/Interpret
KP151	0.25/Synergy	0.3/Synergy	1.25/Indifference	1.25/Indifference	2/Indifference
KP188	1/Additive	0.09/Synergy	1.5/Indifference	1.75/Indifference	4/Indifference
KP189	0.5/Synergy	0.5/Synergy	1.5/Indifference	1.25/Indifference	1/Additive
KP190	0.5/Synergy	0.24/Synergy	0.75/Additive	0.75/Additive	0.5/Synergy

Notes: The FIC of antibiotic A = MIC of antibiotic A in combination/MIC of antibiotic A alone.
The FIC of antibiotic B = MIC of antibiotic B in combination/MIC of antibiotic B alone.
The FICI was the sum of FIC of tested antibiotic.
The result of FICI was interpreted as follows: FICI ≤ 0.5, >0.5–1, >1–4.0, and >4 indicates synergism, additive, indifference, and antagonistic effects, respectively.[16]
CT+ MEM: colistin plus meropenem; ETR+ MEM: ertapenem plus meropenem; IMP+CN: imipenem plus gentamicin; MEM+CN: meropenem plus gentamicin; CT+ AK: colistin plus amikacin.

Table 4. *In vivo* efficacy of different antibiotics after 24 and 48 h treatment.

Group	Description	Viable count after 24 h CFU/mL	Mean value	Viable count after 48 h CFU/mL	Mean value
1 control	Normal rats without infection	31×10^3 30×10^3	30.5×10^3	55.8×10^3 39×10^3	47×10^3
2 control	Inject 0.3 mL of bacterial suspension[a] and vehicle	160×10^7 166×10^7	163×10^7	220×10^7 189×10^7	204×10^7
3	Inject 0.3 mL of bacterial suspension, colistin 20 mg/kg every 8 h	216×10^2 135×10^2 140×10^2	163×10^2	92×10^2 65×10^2 96×10^2	71×10^2
4	Inject 0.3 mL of bacterial suspension, meropenem 200 mg/kg every 8 h	57×10^6 101×10^6 168×10^6	108×10^6	16.8×10^5 66×10^5 25×10^5	91×10^5
5	Inject 0.3 mL of bacterial suspension, combine meropenem and colistin	132×10^2 40×10^2 35×10^2	69×10^2	60×10^2 55×10^2 5×10^2	40×10^2
6	Inject 0.3 mL of bacterial suspension, Imipenem	39×10^5 160×10^5 109×10^5	102×10^5	36×10^4 100×10^4 57×10^4	64×10^4

[a]Bacterial suspension in all tested groups was adjusted to match 0.5 McFarland turbidity standard.

effects of two carbapenems (ertapenem and meropenem) were detected in all the tested isolates. Additionally, synergistic effects of colistin and meropenem combination were observed for up to 75% of isolates. The dual combination of colistin with meropenem, imipenem with gentamicin, meropenem with gentamicin, and colistin with amikacin had shown additive effect among 25% of tested isolates. Antagonism was not observed with any of the tested combinations. Colistin and meropenem combination was further *in vivo* evaluated as they had shown promising FICI (=0.25), while other combination had shown FICI ranging from 0.3 to 2 against KP 151.

In vivo evaluation in Wistar rat thigh infection model

The efficacy profile of single and dual antibiotic combination over 24 and 48 h is shown in Table 4. The results revealed that colistin alone as a monotherapy or with combination with meropenem as a combined therapy showed a statistical significant decrease ($P < 0.05$) in bacterial counts of the tested *K. pneumoniae* (isolate KP151) at 24 and 48 h post treatment. However, meropenem or imipenem alone were not effective at reduction of bacterial count as compared to colistin alone at 24 or 48 h post treatment.

Discussion

Carbapenem-resistant *K. pneumoniae* usually exhibit multidrug or extensive drug-resistant phenotype that minimizes treatment options. Unlikely, the latter phenotype remains susceptible to only one or two antimicrobial categories in all classes of the antimicrobial agents. The antibiogram analysis of three carbapenems including, doripenem, ertapenem, and imipenem was evaluated using the disk sensitivity according to the CLSI guidelines. However, due to the lack of availability of meropenem disk at the time of conducting study, the antibiotic susceptibility pattern of this antibiotic had been quantitatively determined by calculating the MIC by micro-broth dilution which is more accurate and reliable than disc susceptibility. According to literature and our results obtained from the disc sensitivity, the most common antibiotics that still retain activities against XDR *K. pneumoniae* have been evaluated both *in vitro* and *in vivo* either alone or in combination.[18–20] However, we are looking forward to expand our investigation for other antibiotic combinations in the near future. Accordingly, we evaluated the effect of combining dual antibiotics by checkerboard method. Colistin-based combination therapy with carbapenem was a popular strategy

employed against many carbapenamase producers. Our results revealed that combination of colistin with meropenem showed synergy against 75% of the tested isolates. Synergy could be attributed to the ability of colistin to bind to the bacterial membrane through electrostatic interaction with lipid A moiety of lipopolysaccharide. Hence, the permeability of carbapenem through perturbed outer membrane is increased.[21,22] However, clinicians should powerfully expect the development of colistin resistance during colistin treatment in patients with persistent or relapsing carbapenamase producing *K. pneumoniae* as reported in many studies.[18,19,23] This finding is alarming, proofing that previous exposure to polymyxins could be an important risk factor for the development of imminent heteroresistant phenotype. Antibiotic sensitivity of tigecycline was determined by both disk diffusion and microbroth dilution method. The results revealed that the four tested isolates were sensitive to tigecycline and therefore, tigecycline alone is considered as a mainstay antibiotic for the treatment of *K. pneumoniae* OXA-48 producers and therefore it was not tested in combination with other antibiotics.

Additionally, double combination of carbapenems by checkerboard method was also evaluated. Our data demonstrated enhanced synergistic effect against all the tested OXA-48 *K. pneumoniae* producing isolates despite being non susceptible to ertapenem and meropenem. The enhanced benefit of the dual combination approach could be attributed to the enzyme preferential affinity and thus ease of hydrolysis of ertapenem versus meropenem. Thus, upon administration of ertapenem with another carbapenem, ertapenem will act as a suicidal inhibitor and the enzymes will be readily consumed, leaving high concentration of other carbapenem in vicinity of the tested isolate.[24,25] For better correlation of results, rat thigh infection model was used to determine the efficacy of using monotherapy versus combinational therapy against OXA-48 carbapenamase producing *K. pneumoniae* (isolate KP151). The murine thigh infection model was chosen for its sensitivity, ease of performance, and reproducibility to initially examine antimicrobial efficacy among mammalian system. The isolate was selected due to its notable resistance pattern towards β-lactam group (ceftriaxone, cefotaxime, cefepime, imipenem, meropenem, and doripenem), and aminoglycosides (amikacin and gentamicin). Notably, control group of rats without infection had shown a mean value of 30.5×10^3 cfu/mL, reflecting checkpoints for murine thigh microbiota. Our results also revealed that colistin monotherapy significantly decreased bacterial counts by more than four log cycles when compared with those observed in control group receiving no treatment. Despite *in vitro* reduced sensitivity expressed by elevated MIC, colistin monotherapy or colistin-meropenem combination showed statistical significant decrease in the bacterial count (P 0.0128) of the tested *K. pneumoniae* (isolate KP151) at 24 and 48 h post treatment using the *in vivo* murine thigh model. This could be more likely attributed to integrity of immune response in the experimental animal model. Colistin monotherapy appeared to be more effective in reducing cfu/mL compared to meropenem or imipenem monotherapy. This finding was in agreement with Fan *et al.*[17] and Pachón-Ibáñez *et al.*[26] that reported on effectiveness of colistin in reducing bacterial count of *Acinetobacter baumannii* by approximately 97.1% and three log cycles, respectively. However, the addition of meropenem to colistin did not significantly reduced bacterial count as compared to colistin alone in our study. Similar results were also reported by Cai *et al.*[27] on ineffectiveness of combing colistin with meropenem in intraperitoneal murine infection model. This urgently shows that for the treatment of XDR *Klebsiella* species, new antibiotic combination should be addressed. Additionally, in the current study, there was no correlation between the *in vitro* synergism determined by checkerboard and the *in vivo* animal model outcome. Such discrepancies in results could be related to the concentration of antibiotics at infection site, rate of tissue diffusion, and the susceptibility of bacteria. Hence, knowledge on the antibiotics pharmacokinetics and pharmacodynamics properties will be crucial to optimize dose regimen in critically ill patients.[28,29] Additionally, despite listed publications that deal with carbapenem resistance still, these studies are diverse in context of tested bacteria, resistance genes, antibiotic used, and final outcomes. For example, a retrospective cohort study revealed that combination therapy of colistin with tigecycline or meropenem versus colistin monotherapy against XDR-*Acinetobacter baumannii* was associated with better clinical outcome (reduced mortality and intensive care unit stay).[9] However, another study reported that intravenous colistin combination therapy versus colistin monotherapy against MDR Gram negative bacteria was not associated with lower mortality rate among patients.[10] Other studies conducted on carbapenem-resistant *K. pneumoniae* revealed that colistin and meropenem combination showed synergistic effect by checkerboard,[12] and ertapenem containing double carbapenem was associated with microbiological success observed among 79% of patients[24] and that double carbapenem therapy for patients with bacteremia was associated with 67% clinical success and with 100% microbiological eradication.[25]

In conclusion, the *in vitro* studies by checkerboard method had shown promising synergistic effects by combining dual antibiotics, above all double carbapenem regimen namely ertapenem and meropenem followed by colistin with meropenem. Moreover, future attempts will be undertaken for the *in vivo* testing of other antibiotics combination that showed promising synergistic effects *in vitro* using the checkerboard assay. On the other hand, *in vivo* study in Wistar thigh infection model revealed that addition of meropenem to colistin was not superior to colistin monotherapy.

Authors' contributions: NK conducted the experiments and wrote the first draft of the manuscript. ME helps in conduction of the experiments, statistics and in revising the manuscript. WE, MM and KA supervised the whole study and revising the manuscript.

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SUPPLEMENTAL MATERIAL

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