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Highlights:

- We evaluated a practical laboratory method of testing for clinically significant synergy between CAZ/AVI+ATM in NDM producing Enterobacterales using an E-test/disc method.
- Using broth dilution method, 33/43 (77%) isolates were ATM resistant (median [range] MIC=56 [16 – 512] mg/L).
- Addition of CAZ/AVI restored the ATM breakpoint (MIC <4mg/L) in 29/33 (89%) of resistant isolates using broth dilution.
- The E-test/disc method correlated with findings from broth dilution in 35/43 (81%) of cases.
- E-test/disc sensitivity was 77% and specificity 85%. Positive predictive value was 92% and negative predictive value 61%.

A practical laboratory method to determine ceftazidime-avibactam-aztreonam synergy in patients with New Delhi Metallo-beta-lactamase (NDM) producing Enterobacterales infection.

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Running Title: CAZ/AVI+ATM Synergy Testing

Abstract

Background: In response to infection with New Delhi Metallo-beta-lactamase (NDM) producing Enterobacterales , combination antimicrobial therapy with ceftazidime/avibactam (CAZ/AVI) plus aztreonam (ATM) has been explored. This study evaluated a practical laboratory method of testing for clinically significant synergy between CAZ/AVI+ATM in NDM producing Enterobacterales.

Methods: Minimum inhibitory concentration (MIC) of clinical NDM producing isolates were determined for ATM alone and CAZ/AVI+ATM using broth dilution. Restoration of ATM breakpoint following the addition of CAZ/AVI was explored. A CAZ/AVI E-test/ATM disc method was compared to broth dilution.

Results: Of 43 isolates, 33/43 (77%) isolates were ATM resistant (median [range] MIC=56 [16 – 512] mg/L). Addition of CAZ/AVI restored the ATM breakpoint (MIC <4mg/L) in 29/33 (89%) of resistant isolates. Overall, the E-test/disc method correlated with findings from broth dilution in 35/43 (81%) of cases. E-test/disc sensitivity was 77% and specificity 85%. Positive predictive value was 92% and negative predictive value 61%.

Conclusion: CAZ/AVI+ATM demonstrated significant synergy in most ATM resistant NDM producing Enterobacterales. The E-test/disc method is a quick, reproducible, and reliable method of testing for clinically relevant synergy in the microbiology laboratory.

Keywords:: Ceftazidime-avibactam, Aztreonam, Synergy, New Dehli Metallo-beta-lactamase, Combination treatment

Introduction

Enterobacterales expressing Class B metallo-beta-lactamase (MBL) enzymes pose a significant therapeutic challenge[1,2]. MBLs, such as the New Delhi MBL (NDM), are being detected at an increasing rate in clinical isolates in many areas of the world[1,2]. MBLs are often plasmid mediated and are acquired alongside other extended-spectrum beta-lactamase (ESBL) and non-beta-lactamase resistance mechanisms. Therefore, presence of MBLs tend to confer resistance to nearly all beta-lactam antibiotics, including the carbapenems[2,3]. Furthermore, isolates often tend to be resistant to fluoroquinolones and aminoglycosides, limiting therapeutic options to agents with high rates of toxicity and variable pharmacokinetics (PK), such as colistin [2,3].

A potential solution to MBL producing Enterobacterales is the development of combination therapy. The monobactam aztreonam (ATM) is resistant to hydrolysis by MBLs, but susceptible to hydrolysis by serine Class A, C, and D ESBL's. In contrast, ceftazidime-avibactam (CAZ-AVI), a third-generation cephalosporin combined with a beta-lactamase inhibitor, has broad activity against serine beta-lactamases, but is hydrolysed by MBLs. The combination of ATM with CAZ-AVI has been demonstrated to have a synergistic effect against MBLs in-vitro with supporting clinical trials demonstrating in-vivo efficacy of ATM-AVI[4–10].

To support the clinical use of CAZ/AVI+ATM, practical laboratory methods to identify patients who may benefit from combination treatment are required. Potential methods of screening for in-vitro synergy between CAZ-AVI and ATM for MBL producing Enterobacterales have been proposed including broth dilution, antimicrobial gradient strip (E-test) and disc diffusion methods[4–6,9–12]. Whilst a synergistic effect, often defined as a four-fold reduction in observed minimum inhibitory concentration (MIC), is reported; this fails to determine whether this combination can be applied in clinical practice. The interpretation of MIC requires the application

of breakpoints that consider antimicrobial pharmacokinetics and pharmacodynamics (PK-PD) and other host factors that may influence the outcome from treatment.

We aimed to develop a practical laboratory method to screen for clinically relevant synergy between CAZ-AVI+ATM in NDM producing isolates using an E-test/disc method compared to gold standard broth dilution.

Materials and methods

Bacterial isolates:

At Imperial College Healthcare NHS Trust, London, UK carbapenemase producing organisms from clinical and screening isolates are categorised and stored for future use (Ethics Number 21/HH/6538). All isolates from October to December 2020 were reviewed with NDM-type producing Enterobacterales selected. In total, 61 isolates were identified with 43 included following removal of duplicates and missing samples.

Susceptibility testing:

Isolates had antimicrobial susceptibility determined using EUCAST recommended disc diffusion, Phoenix identification methods (BD Phoenix), and antimicrobial gradient strip (E-test, bioMerieux, France) methods. Phenotypic ESBL was confirmed using ceftazidime (10ug) and cefpodoxime (10ug) discs with and without clavulanic acid as per EUCAST guidance[13]. Presence of NDM-type carbapenemase was confirmed using NG-Test Carba5 (NG biotech, France).

Broth microdilution and synergy testing:

Broth microdilution using Muller Hinton Broth (Thermofisher Scientific) was performed using described methods[14]. MIC was determined for ATM (Bristol-Myers Squibb Pharmaceuticals Ltd)

alone and in the presence of CAZ/AVI (Pfizer). ATM was tested at concentrations of 0.5-256mg/L. CAZ/AVI was used in its clinical formulation with the concentration fixed in each well at 4mg/L for the AVI component and 16mg/L for the CAZ component. Only ATM concentration was varied in the CAZ/AVI+ATM wells. Microtiter plates were incubated at 37^{o}C and read at 18-hours. Plates were read manually, with MIC defined as the first non-turbid well observed. Synergy was defined using two methods; 1) a four-fold decrease in MIC between ATM alone and CAZ/AVI+ATM, and 2) clinical synergy with MIC below the ATM breakpoint as per EUCAST definitions (susceptible, S ≤ 1mg/L; susceptible with optimised dosing (intermediate) 1-4mg/L, resistant, R > 4mg/L)[15].

Modified E-test / disc diffusion method:

CAZ/AVI+ATM synergy were determined using the CAZ/AVI E-test/ATM disc diffusion method. Innocula with turbidities of 0.5 McFarland, determined using spectrophotometry (range 0.08-0.13), were plated on Muller Hinton agar (Thermo Scientific, UK). A CAZ/AVI E-test with fixed AVI concentration at 4mg/L were placed on the agar plate. An ATM disc (30ug; Thermo Scientific, UK) was placed at 15mm from centre of the strip to centre of the disc. It was placed at the level of the CAZ/AVI breakpoint (8mg/L). To test reproducibility of the method, triplicate plates were set up for 11/43 (25%) isolates.

Plates were incubated and read at 16-18 hours. Two approaches to defining synergy were explored. First, a quantitative approach with the zone radius on both sides of the disc measured and converted into an estimated disc diameter for ATM alone or CAZ/AVI+ATM. Zone diameters were compared to EUCAST zone size for ATM with synergy defined as restoration of an estimated zone diameter consistent with reported breakpoints. Second, a qualitative approach was taken with an inverse-D defined as demonstration of synergy. This qualitative approach was extrapolated as the inverse observation of inducible clindamycin resistance that

is often screened for as part of routine antimicrobial susceptibility testing in the laboratory. **Figure 1** demonstrates the E-test/disc set up with defined observations based on the presence and absence of synergy.

Statistical methods

All data were reported descriptively. Sensitivity, specificity, positive predictive value, and negative predictive value of the E-test/disc method were determined using a 2x2 table with broth dilution taken as the gold standard. S and I breakpoints were classified as susceptible as they indicate that treatment would be appropriate according to breakpoints for ATM.

Results

Of 43 confirmed NDM producing isolates in the study, *Escherichia coli* (15/43; 35%), *Enterobacter cloacae* (16/43; 37%), and *Klebsiella species* (8/43; 19%) predominated. **Table 1** describes the isolate characteristics. ESBL phenotype were present in 36/43 (84%) with the remaining 7/43 (16%) demonstrating AmpC phenotype. Meropenem resistance (MIC > 8mg/L) was observed in 19/43 (44%) isolates, with 19/43 (44%) classified as intermediate (MIC 2–8mg/L).

Aztreonam susceptibility testing

For ATM (single drug) susceptibility testing, 6/43 (14%) isolates were classified as susceptible (observed median [range] MIC = 0.5 [0.25-0.5]mg/L), 4/43 (9%) intermediate (median [range] MIC = 3 [2-4]mg/L) and 33/43 (77%) resistant (median [range] MIC = 56 [16 – 512 mg/L). Using the quantitative E-test/disc method, 13/43 (30%, median radius [range] = 18 [14-19]mm) isolates were classified as ATM susceptible and 1/43 (2%, radius = 12mm) intermediate. For the 29/43 (67%) ATM resistant isolates the median [range] disc radius was 6.5 [0-10]mm.

Aztreonam/ceftazidime-avibactam synergy testing by broth microdilution

Using broth dilution, synergy was observed with the addition of CAZ/AVI to ATM in 32/43 (74%) isolates. Addition of CAZ/AVI to ATM did not affect the MIC of isolates that were already ATM susceptible. All four ATM intermediate isolates were classified as susceptible with addition of CAZ/AVI (median [range] MIC = 0.25 [0.25]mg/L). Among the 33 ATM resistant isolates, 22/33 (67%) were susceptible with addition of CAZ/AVI (median [range] MIC = 0.25 [0.25-1]mg/L), 7/33 (21%) were classified as intermediate (median [range] MIC = 2 [2-4]mg/L), and 3/33 (9%) remained ATM resistant (median [range] MIC = 16 [16-512]mg/L).

Aztreonam/ceftazidime-avibactam synergy testing by disc/E-test

CAV/AVI+ATM radius classified all 14/43 (33%) ATM susceptible/intermediate isolates as susceptible (median radius [range] = 18 [15-26]mm). Of the 29/43 ATM resistant isolates, 27/29 (93%, median radius [range] = 15 [13-20]mm) were classified as susceptible. One (3%) was classified as intermediate (radius = 11mm) and one (3%) remained resistant (radius = 10mm).

Method comparison

Table 1 provides a comparison of broth dilution to the E-test/disc methods. For ATM, the disc radius measurement demonstrated 33/43 (77%) concordance in breakpoint estimates (S/I/R) for ATM alone. Of the 10 discordant results, 4/10 (40%) were either ATM intermediate or susceptible using broth and E-test/disc methods. For CAZ/AVI+ATM all 32/43 isolates that were susceptible on broth dilution correlated with E-test/disc radius estimation. The 7/43 isolates classified as intermediate on broth dilution were susceptible on disc radius estimation. Of the 4/43 CAZ/AVI+ATM resistant isolates, 1/4 (25%) was resistant, 1/4 (25%) intermediate, and 2/4 (50%) susceptible.

Using the E-test/disc qualitative method, synergy was demonstrated in 23/43 (53%) isolates. No synergy were demonstrated in 16/43 (37%) isolates. The remaining 4/43 (9%) isolates appeared to demonstrate CAZ/AVI sensitivity with three of these also demonstrating synergy with ATM. On comparison with broth synergy there was concordance in results in 35/43 (81%) tests. Compared to broth dilution, the E-test/disc qualitative method has a sensitivity of 77% and specificity of 85%. Positive predictive value was 92% with the negative predictive value 61%.

Discussion

We demonstrate a rapid, practical laboratory method of screening for clinically relevant synergy between CAZ/AVI+ATM in NDM producing Enterobacterales using an E-test/disc methodology. For ATM resistant isolates, clinically relevant synergy with restoration of ATM breakpoint (MIC ≤ 4mg/L) was observed in 29/33 (88%) of resistant isolates following combination with CAZ/AVI. Good correlation was observed between broth dilution and E-test/disc method, suggesting that the E-test/disc method looking for the inverse-D sign is an appropriate screening tool synergy between these two agents.

Rapid molecular diagnostics can provide information on the presence of resistance genotypes, such as NDM gene detection, but fail to support the delivery of targeted antimicrobial therapy[16]. Targeted therapy relies on the rapid determination of in-vitro antimicrobial susceptibility to describe the organism phenotype. To date, methods have been proposed to demonstrate in-vitro synergy for CAZ/AVI+ATM in MBL producing Enterobacterales. However, many of these have failed to correlate the demonstration of synergy with clinical breakpoints for ATM. One study recently compared the use of disc stacking, gradient strip stacking, and gradient strip crossing in 10 MBL producing Enterobacterales comparing them using CLSI breakpoints[12]. The study demonstrated good overall performance of gradient strip based synergy testing with 82-94% categorical agreement using these methods. Disc stacking demonstrated worse agreement

of 43%[12]. Our results using the inverse-D method demonstrate similar categorical agreement for the demonstration of clinically relevant synergy with 81% agreement to broth dilution using EUCAST derived breakpoints in a large sample of 43 real-world isolates. The consideration of organism breakpoint is of increasing importance when considering treatment of NDM producing infections[17]. Breakpoints take into account in-vitro measures (or correlates) of MIC, the likelihood of achieving therapeutic drug concentrations within the patient, and clinical evidence of outcomes from the treatment of infection[18]. To date, evidence suggests that ATM in combination with AVI can be successful in the management of NDM producing Enterobacterales infection, but uncertainty remains in terms of optimal dosing strategies, PK-PD targets for combination therapy, and therefore when this combination should be considered[3,7,10,19]. Development of practical laboratory screening tools for synergy may help bridge this gap.

This study had several limitations. Only Enterobacterales producing NDM were included. No clear breakpoints for ATM/AVI or CAZ/AVI+ATM are defined. We inferred organism susceptibility based on the ATM breakpoints alone[15]. Whilst this is likely a conservative approach, it means that an area of technical uncertainty remains. We used clinical isolates that had not undergone full genotypic profiling. However, the use of real-world laboratory methods of phenotyping mirrors how this test will be used in clinical practice.

In conclusion, CAZ/AVI+ATM restored the breakpoint for ATM resistant NDM producing isolates included within this study. The E-test/disc method provides a rapid, practical laboratory approach for real-world screening for CAZ/AVI+ATM synergy and may support its targeted use in patients with NDM producing Enterobacterales infection.

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Declarations

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Competing Interests: TMR has consulted for/ received speaker fees from Sandoz (2020), bioMerieux (2021-2022), & Roche diagnostics (2021).

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All other authors have no potential conflicts of interest to declare.

Ethical Approval: Ethical approval for the collection and use of bacterial products was obtained under regional ethics committee reference number 21/HH/6538.

Contribution statement

TMR, XG, HD, and FD planned and designed the study. FD obtained ethical approval as required for the study. TMR, IBT, and RM identified clinical samples and undertook all experiments described within the study. AM, LSPM, MA, & MG supported with organisation and equipment / reagent procurement. TMR undertook primary data analysis and

drafted the initial version of the manuscript. All authors contributed significantly to the revision of the manuscript prior to submission for publication. All authors agreed for submission of the manuscript in its current format.

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request where not presented in the manuscript,

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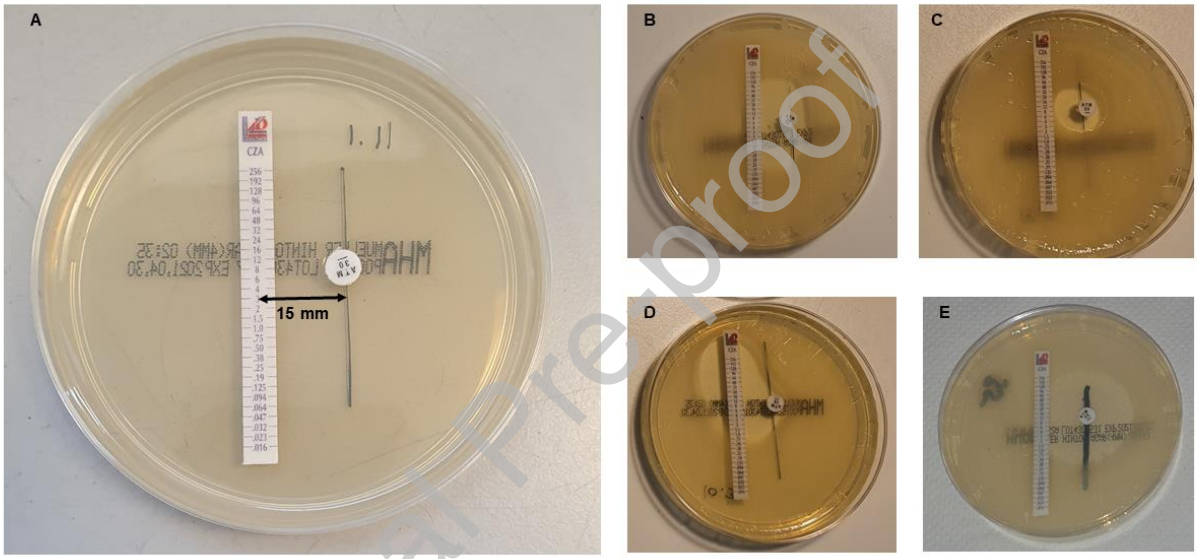
Table 1. Characteristics of clinical and screening NDM producing isolates obtained between October and December 2020 used within the study

Sp.	Broth ATM MIC (mg/L)	Broth ATM S/I/R	Broth CA-ATM MIC (mg/L) [$]	Broth CA-ATM S/I/R [$]	ATM disc radius (mm)	ATM S/I/R	CA-ATM disc radius (mm)	CA-ATM S/I/R	Reverse-D
ECLO	0.25	S	0.25	S	12	I	15	S	No Synergy
CFRE	512	R	512	R	7.5*	R	15*	S	Synergy
ECOL	512	R	2	I	5*	R	15*	S	Synergy
ECLO	512	R	1	S	6.5*	R	15*	S	Synergy
ECOL	512	R	0.25	S	16	S	20	S	No Synergy
KPNE	0.25	S	0.25	S	16	S	26	S	No Synergy CAZ-AVI S
ECLO	32	R	16	R	10*	R	15*	S	Synergy CAZ-AVI S
ECLO	32	R	0.5	S	5*	R	15*	S	Synergy CAZ-AVI S
KPNE	16	R	0.5	S	5*	R	15*	S	Synergy CAZ-AVI S
KPNE	0.5	S	0.5	S	19	S	20	S	No Synergy
ECOL	75	R	16	R	8*	R	11*	I	No Synergy
ECOL	512	R	1	S	18	S	15	S	No Synergy
ECOL	512	R	2	I	5	R	14	S	Synergy
KPNE	512	R	2	I	6.5*	R	15*	S	Synergy
ECOL	512	R	16	R	0	R	10*	R	No Synergy
ECLO	16	R	0.25	S	8	R	15	S	Synergy
CFRE	16	R	0.25	S	15	S	18	S	No Synergy
ECLO	32	R	0.25	S	4	R	13	S	Synergy
KOXY	2	I	0.25	S	19	S	18	S	No Synergy
ECLO	512	R	2	I	8	R	18	S	Synergy
ECOL	512	R	1	S	4.5*	R	15*	S	Synergy
ECOL	2	I	0.25	S	18	S	23	S	No Synergy
ECLO	36	R	1	S	14	S	15	S	No Synergy
ECLO	250	R	4	I	18	S	15	S	No Synergy

ABAU	32	R	0.25	S	3	R	15	S	Synergy
KPNE	250	R	0.25	S	6	R	19	S	Synergy
ECLO	0.25	S	0.25	S	18	S	16	S	No Synergy
ECOL	16	R	0.25	S	9	R	15	S	Synergy
ECOL	256	R	4	I	0	R	15	S	Synergy
ECOL	0.25	S	0.25	S	18	S	22	S	No Synergy
KPNE	0.25	S	0.25	S	18	S	18	S	No Synergy
ECOL	125	R	0.25	S	9	R	19	S	Synergy
KPNE	32	R	0.25	S	9	R	20	S	Synergy
ECOL	512	R	4	I	4	R	14	S	Synergy B/L
ECLO	0.25	R	0.25	S	4	R	18	S	Synergy
CFRE	75	R	0.25	S	7	R	18	S	Synergy
ECLO	75	R	0.25	S	9	R	19	S	No Synergy
ECLO	75	R	0.25	S	9	R	18	S	Synergy
ECLO	75	R	0.25	S	10	R	16	S	Synergy
ECLO	4	I	0.25	S	19	S	16	S	No Synergy
ECLO	36	R	0.25	S	10	R	15	S	Synergy
ECOL	4	I	0.25	S	10	R	15	S	Synergy
ECOL	512	R	0.25	S	5*	R	15*	S	Synergy

Legend: ECOL = *Escherichia coli*, ECLO = *Enterobacter cloacae*, KPNE = *Klebsiella pneumoniae*, CFRE = *Citrobacter freundii*, ABAU = *Acinetobacter baumanii*, KOXY = *Klebsiella oxytoca*, R = resistant, I = Intermediate / Susceptible with optimal dosing, S = susceptible, * = triplicate repeat (average radius), $ = fixed concentration of ceftazidime/avibactam (C/A) at 16mg/4mg throughout with variable aztreonam (ATM) concentration from 0.5 – 256mg/L.

Figure 1. Example of ceftazidime/avibactam E-test – aztreonam disc method used within this study.



Legend: a) E-test/disc set up with ceftazidime-avibactam E-test and aztreonam disc (30 ug) placed 15 mm apart; b) Synergy demonstrated by reverse-D; c) No synergy; d) ceftazidime-avibactam sensitive with aztreonam synergy; e) aztreonam sensitive with no synergy.