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Original Research Article

Study of *bla*KPC and their Antimicrobial Susceptibility Profile from Clinical Isolates in a Tertiary Hospital in Central India

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ABSTRACT

Background
Antibiotics are a limited resource. The wonder drugs of the 20th century "antibiotics" have become a limited resource. The golden age of antibiotic discovery, when the rate of discovery of new molecules kept pace with bacterial innovation, is now a distant memory. The last decade has witnessed a celestial rise in Carbapenem resistan*ce*. Antimicrobial resistance(AMR) is a worldwide problem and is crossing international boundaries spreading with ease and remarkable speed adding considerable, avoidable costs to the already overburdened healthcare system. *Klebsiella pneumoniae* and *Escherichia. coli* are the most common and important pathogens in the *enterobacteriacae* group causing various nosocomial and community acquired infections including bacteremia, pneumonia, liver abscess, and urinary tract infections.

Aim
To study production of Class A carbapenemases-*Klebsiella pneumoniae* carbapenemases (KPC) in clinical isolates of *E.coli* and *K.pneumoniae*and their antimicrobial susceptibility profile. Further, study co-existing β – lactamases : Class A β -lactamases -extended spectrum β –lactamases (ESβL), Class B β -lactamases - metallo β -lactamases (MβL) and Class C β -lactamases - AmpC in KPC producing clinical isolates of *E.coli* and *K.pneumoniae* by inhibitor based disc potentiation method.

Method
The current study was conducted in a teaching institute which is also a tertiary care hospital. Consecutive, non-duplicate isolates were included from varied in- patient samples from January 2013 to July 2014. A total of 168 isolates of *Escherichia coli*(n=53) and *Klebsiella pneumoniae*(n=115)from sterile site infections were studied. The tests performed included

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antimicrobial susceptibility tests; detection of KPC carbapenemases and co-existence of other beta lactamases like Metallo-betalactamases, extended-spectrum b-lactamases (ESBL) and AmpC b-lactamases.

Result

In this study, all isolates of *E.coli* and *K.pneumoniae* high resistance to all classes of cephalosporins. Resistance to gentamycin and co-trimoxazole ranged from 32-50% it was least among antimicrobials tested for both organisms. ESβL was the most common beta-lacatamse detected, followed by MβL and AmpC in our study. KPC's were produced by 13.21% *E.coli* and 7.83 % *K.pneumoniae* isolates. Overall co-production of multiple β-lacatamases was common in our study

Conclusion

The shortage of new antimicrobial agents on the immediate horizon suggests enhanced adherence with effective infection control measures, detect and target therapy and antibiotic stewardship to curb patient-to-patient transmission and to reduce the selection of multidrug-resistant bacteria,

Key words: Antimicrobials, carbapenem resistance, *bla*KPC

INTRODUCTION

AMR has profound implications for patient treatment outcomes. New ideas are often based on the recognition of old truths. Microorganisms though diminutive, are formidable foes Infections caused by resistant organisms are more difficult to treat, often requiring more toxic or less effective antibiotics, leading to increased morbidity and mortality. Resistant infections can also cause delays in treatment, either through postponing therapy sessions due to ongoing infections or through the direct toxicity of second-line antimicrobials. These delays can allow disease to progress and reduce the overall effectiveness of treatment regimens sometimes leading to death.

Carbapenems, first introduced in 1980, are potent antibiotics for the treatment of infections due to multi-resistant gram-negative bacteria.(1) The genes of KPC β- lactamases (*bla*KPC) that encode KPCs are present on transferable plasmids, quickly disseminated along with other resistant determinants and readily shared among pathogens.(3,4) KPC's confer resistance to all beta lactam agents including penicillins, cephalosporins, monobactams, carbapenems, oxyimino-cephalosporinsand cephamycins.(4,5) The hallmark of this general increase is the unbridled dissemination of carbapenem resistance genes, namely CTX-M extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpC beta-lactamases, Metallo beta lactamases, *Klebsiella pneumoniae* carbapenemases (KPC) and oxacillinases.

The optimal treatment of infections caused by KPC producing bacteria is not well established and data on clinical outcomes is sparse.(6,7) Currently the main issues are to provide practical recommendations on detection, treatment and prevention of KPC infections in different resource settings.(8,9) Limited data is available on presence of KPC's from India. In India KPC's have been reported from Pondicherry(10), Varanasi, Imphal, Jaipur.(11-17)Limited data is available from our region. In view of these observations the Present study was taken with an aim to reveal the existence if any, of KPC producing clinical isolates of *E.coli* and *K.* pneumoniae in our region. In addition an attempt was made to provide practical phenotypic detection method information for reliable and easy detection and treatment options that may be useful to the clinicians at the bedside in resource constraint settings.

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AIM

To study production of Class A carbapenemases-*Klebsiella pneumoniae* carbapenemases (KPC) in clinical isolates of *E.coli* and *K.pneumoniae*, theirTo d antimicrobial susceptibility profile. Further, study co-existing β – lactamases: Class A β -lactamases -extended spectrum β –lactamases (ESβL), Class B β -lactamases - metallo β -lactamases (MβL) and Class C β -lactamases - AmpC in KPC producing clinical isolates of *E.coli* and *K.pneumoniae* by inhibitor based disc potentiation method.

MATERIAL AND METHODS

The current study is a prospective observational study was conducted in a government teaching institute which is also a tertiary care hospital in central India. Consecutive, non-duplicate isolates were included from varied from in- patient samples were included from January 2013 to July 2014. A total of 168 isolates of *Escherichia coli*(n=53) and *Klebsiella pneumoniae*(n=115)from sterile site infections were studied. The tests performed included antimicrobial susceptibility tests; detection of KPC carbapenemases and co-existence of other beta lactamases like Metallo-betalactamases, extended-spectrum b-lactamases (ESBL) and AmpC b-lactamases.

Antimicrobial susceptibility testing[18,19] Each isolate was tested for antimicrobial susceptibility by Kirby Bauer disc diffusion method on Muller Hinton agar (MHA) which was prepared from dehydrated base supplied by Himedia, Mumbai. Following antimicrobial discswereused ampicilin(10µg), cefazolin(30µg), cefoxitin(30µg), meropenem(10µg), ertapenem(10µg), gentamycin(10µg), cefepime(30µg), cefotaxime(30µg), cotrimoxazole(1.25/23.75µg), ceftazidime(30µg), aztreonam(30µg), ciprofloxacin(5µg).

Preparation of substrate - inhibitor combination discs[20-24] Meropenem disks were supplemented with four different β-lactamase inhibitors: Dipicolinic acid(DPA) Obtained from Himedia pvt ltd, Mumbai, Ethylene diamine tetra acetic acid(EDTA obtained from ranchem chemicals,Mumbai), extra pure Phenylboronic acid (Sisco research private ltd,mumbai) and cloxacillin powder(osper pharmaceuticals, Mumbai). The final amounts of β-lactamase inhibitor in the disks were 1000 µg of DPA, 730 µg of EDTA, 400 µg of PBA and 750 µg of cloxacillin. EDTA, PBA and cloxacillin were dissolved in sterile water. Ten microliters of the stock solution of EDTA, cloxacillin, Dipicolinic acid were dispensed on meropenem disks to achieve desirable concentration. A 10µl drop from stock solution of each inhibitor delivered-1000µgof DCA, 750µg of 0.5MEDTA, 400µg of PBA and 750µg of Cloxacillin respectively on each meropenem disc. The substrate inhibitor combination discs were freshly prepared and were used within one hour. Plates of Muller Hinton agar, the medium was dispensed in 21cm glass plates to a depth of 4mm. Each batch of medium was checked for its sterility by incubating the plate at 37 °C for 24 hours. A total of 18 discs were placed on a single 21 cm plate to generate data for epidemiological significance. However clinical reporting of antimicrobial susceptibility followed CLSI 2013.

i) ESBL production .Combined disk diffusion method was done using cefotaxime CTX (30µg) and ceftazidime CAZ (30µg) disc alone and in combination with clavulanic acid disc (30/10µg). A >=5mm diameter increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs. the zone diameter of the agent when tested alone confirms ESBL production by the strain. Both cefotaxime and ceftazidime discs alone

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and in combination were used simultaneously to improve the sensitvity of ESβL detection. **Control** :Positive – *Klebsiella pneumoniae* ATCC 700603 Negative - *Escherichia coli* ATCC 25922.

ii) Detection of Class C β- lactamases- AmpC(Jacoby 2005, Giske et al, 2011) AmpC was detected in cefoxitin resistant (zone diameter<14mm) isolates by inhibitor based disc potentiation method using inhibitors- Phenylboronic acid (400µg) and Cloxacillin (750µg). Production of AmpC was considered when both criterias mentioned below were fulfilled. Interpretation- A response of≥5mm increase in zone diameter for meropenem supplemented with phenylboronic acid(400µgm) compared to the zone diameter of meropenem alone. A response of ≥5mm increase in zone diameter for meropenem supplemented with Cloxacillin(750 µgm) compared to the zone diameter of meropenem alone. **Control** :Negative - *Escherichia coli* ATCC 25922.

iii) Detection of Class B carbapenemases-metallo- β–lactamases(MβL) (Rai et al 2011, Giske et al 2011)MβL'swere detected by inhibitor based disc potentiation method using inhibitors- EDTA(750µgm) and Dipicolinic acid(DCA, 1000µg).Two inhibitors were used to improve the sensitvity of MβL detection. MβLproduction was considered when eithercriteria was fulfilled. Interpretation-A response of ≥5mm increase in zone diameter for meropenem supplemented with EDTA (750µgm) compared to the zone diameter of meropenem alone.[2] A response of ≥5mm increase in zone diameter for meropenem supplemented with Dipicolinic acid(1000 µgm) compared to the zone diameter of meropenem alone. **Control**: Positive: (Local laboratory isolate*Pseudomonas aeruginosa* 006159/12) confirmed by MIC reduction method.Negative:*Escherichia coli* ATCC 25922.

iv) Detection of *Klebsiella pneumoniae* carbapenemases (KPC)[(25-27)] KPC's were detected by inhibitor based disc potentiation method using inhibitors-Phenylboronic acid-(PBA, 400µgm) and cefotaxime/ceftazidime with clavulanic acid(30/10µg)(Tsakris et al 2011,Giske et al 2011). Production of KPC was considered when both criterias mentioned below were fulfilled. Interpretation. A response of ≥5mm increase in zone diameter for meropenem supplemented with Phenylboronic acid(400µgm) compared to the zone diameter of meropenem alone. A response of ≥5mm increase in zone diameter for either cefotaxime/ceftazidime tested in combination with clavulanic acid as compared to the zone diameter when tested alone. **Control:** Positive-*Klebsiella pneumoniae* ATCC BAA-1705 (Microbiologics, USA)Negative - *Escherichia coli* ATCC 25922.

v) <u>Co-production of MBL and KPC</u>: Production of both KPC and MBL enzymes was considered when the growth-inhibitory zone diameter around the meropenem disk with both PBA and EDTA/DCA was increased ≥5 mm compared with the growth-inhibitory zone diameter around the disk containing meropenem alone.[(28,29)]

β- lactamase	**CAZ/ CTX**	**ETP**	**CX**	**CA** response	**PBA** response	**EDTA** response	**DCA** response	**CLOX** response
ESBL	*R	**S	S	Yes	No	No	No	No
AmpC	R	S	R	No	Yes	No	No	Yes
Carbapenemase ClassA(KPC)	R	R/↓S	S	Yes	Yes	No	No	No
Carbapenemase	R	R	R	No	No	Yes	Yes	No

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Class B(MBL)								

Table 1: Testing algorithm for detection of β-lactamases

*R-Resistant. **S-SensitiveCAZ -ceftazidime, CTX-cefotaxime, CX-cefoxitin, ETP-ertapenem, CA-Clavulanic acid, PBA-phenyl boronic acid, DCA-dipicolinic acid, EDTA-Ethylene diamine tetra acetic acid, CLOX-cloxacillin.

Determination of minimum inhibitory concentration by agar dilution method for polymyxin, tigecyline, meropenem and colistin. The performance and interpretation of result was as per EUCAST guidelines 2014 and CLSI 2013 for quantitative determination of resistance.

Statistical analysis

Comparison of response of dipicolinic acid (DCA) and ethylene diamine tetra acetic acid (EDTA) was done by chi square test. (STRATA version 10.1, 2009 software used for statistical analysis.Anitmicrobial susceptibility testing results were interpreted as per CLSI 2013 guidelines.

RESULTS

Isolation of *E.coli* and *Klebsiella pneumoniae* was most commonly from pus. Of the 53 isolates of *E.coli* studied, their isolation was 39.62% from pus, 15.09% each from ascitic fluid and blood, 11.32% from pleural fluid, 5.67% each from drain fluid and tracheal aspirate, 7.54 % from CSF. Similarly of the 115 *K.pneumoniae* studied, their isolation was 39.91% from pus, 23.48% from pleural fluid, 19.13% from blood, 7.83% from ascitic fluid, 6.96% from tracheal aspirate, 6.09% from CSF, 2.60% from drain fluid. isolates of *E.coli* and *K.pneumoniae* co existence of multiple beta -lactamase was common.

S.no	Specimen	*E.coli* no (%)	*K.pneumoniae* no (%)
1	Pus	21 (39.62)	39 (33.91)
2	Pleural fluid	6 (11.32)	27 (23.48)
3	Blood	8 (15.09)	22 (19.13)
4	Ascitic fluid	8 (15.09)	9 (7.83)
5	Tracheal aspirate	3 (5.67)	8 (6.96)
6	CSF	4 (7.54)	7 (6.09)
7	Drain fluid	3 (5.67)	3 (2.60)
	TOTAL	53(100)	115(100)

Table 2: Specimen wise distribution of clinical isolates of Escherichia coli (n=53) and Klebsiella pneumoniae (n=115)

Drug resistance Overall resistance to antimicrobial was high in both *E.coli* and *K.pneumoniae* . Both organisms showed 100% resistance to ampicillin and cefazolin. As many as 85-100% isolates showed resistance to third generation (cefotaxime and ceftazidime) and fourth generation(cefipime). Amongst the penemes ertapenem showed higher resistance than meropenem in both. Though resistance to gentamycin and co-trimoxazole ranged from 32-50% it was least among antimicrobials tested for both organisms.ESβL production was found in 71.70% and 78.26% isolates of *E.coli* and *K.pneumoniae* respectively.

ESβL production	*E.coli* no (%)	*K.pneumoniae* no (%)

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ESβL producers	38(71.70)	90 (78.26)
ESβL non producers	15(28.30)	25(21.74)
TOTAL	53(100)	115(100)

Table 3: Phenotypic detection Class A β-lactamases (ESβL) in clinical isolates of E.coli (n=53) and K. pneumoniae (n=115)

AmpC production was found in 15.09% and 16.52% isolates of *E.coli* of *K.pneumoniae* respectively. Metallo β-lactamase (MβL) production was found in 33.96% and 51.30% isolates of *E.coli* and *K.pneumoniae* respectively.

AmpC production	***E.coli* no. (%)**	***K.pneumoniae* no (%)**
AmpC producers	8(15.09)	19(16.52)
AmpC non producers	45(84.91)	96(83.48)
TOTAL	53(100)	115(100)

Table 4: Phenotypic detection of Class C β-lactamases (AmpC) in isolates of E.coli (n=53) and K. pneumoniae (n=115)

Isolate showing positive response to either EDTA/DCA were considered as MβL producer.

Positive response to EDTA was found in 28.30% and 49.56% isolates of *E.coli* and *K.pneumoniae* respectively. Positive response to DCA was found in 33.96% and 51.30% isolates of *E.coli* isolates of *K.pneumoniae* respectively. Positive response to both EDTA and DCA was found in 28.30% and 49.56% isolates of *E.coli* isolates of *K.pneumoniae* respectively. DCA was found to additionally detect 3 isolates of E.coli and 2 isolates of K.pneumoniae. However the difference was not found to be significant with p value at 0.7272 and 0.8541 for *E.coli* and *K.pneumoniae* respectively.

S.no	**Bacterial isolate**	**Positive Response to *EDTA no(%)**	**Positive response to** DCA no(%)**	**Positive Response to both EDTA&DCA no(%)**
1.	*E.coli*	15(28.30)	18(33.96)	15(28.30)
2.	*K.pneumoniae*	57(49.56)	59(51.30)	57(49.56)

Table 5: Phenotypic detection of Class B β-lactamases-MβL in clinical isolates of E.coli (n=53) and K. pneumoniae (n=115)

*EDTA-Ethylene diamine tetra acetic acid. **DCA-Dipicolinic acid.

KPC production	***E.coli* no (%)**	***K.pneumoniae* no (%)**
KPC producers	7 (13.21)	9 (7.83)
KPC non producers	46 (86.79)	106 (92.17)
TOTAL	53(100)	115(100)

Table 6: Phenotypic detection of Class A β-lactamases (KPC) in clinical isolates of E.coli and K. pneumoniae (n=115)

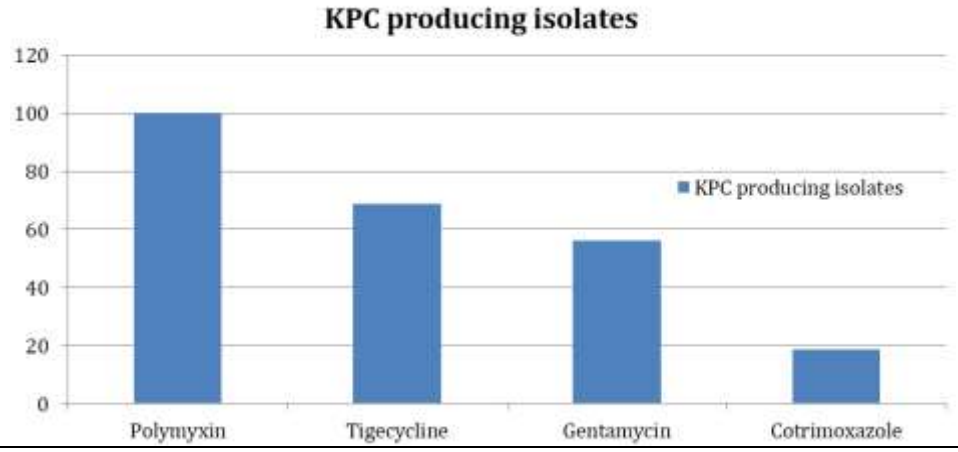

Figure 1: Antimicrobial susceptibility profile of KPC producing isolates E.coli (n=7) and K. pneumoniae (n=9)

S.no	Beta lactamase	*E.coli* no. (%)	*K.pneumoniae* no. (%)
1	ESβL alone	18(33.96)	27(23.48)
2	AmpC alone	1(1.88)	-
3	MβL alone	3(5.66)	5(3.48)
4	ESβL+AmpC	6(11.32)	17(14.78)
5	ESβL+MβL	13(24.53)	44(38.26)
6	MβL+AmpC	-	-
7	ESβL+AmpC+MβL	1(1.88)	2(1.74)

Table 7. Co-production of other β-lactamases in KPC producing isolates of E.coli Production of KPC alone was seen in 85.71 % and 55.56 % in isolates of E.coli and K.pneumoniae respectively. (Table-4)KPC+MβL production was seen in 14.29 % and 44.44% in isolates of E.coli and K.pneumoniae respectively.

DISCUSSION

Resistance to monobactams can be due to hydrolysis by ESβL's, efflux and cephalosporinases hyperproduction which could be the reason for high resistance to azetreonam 81.13 % in *E.coli* and 83.47% in *K.pneumoniae* in our study. Our study is in agreement with Giske et al. Nagaraj et al 2012 studied carbapenem resistant clinical isolates of *Escherichia coli* and *K. pneumoniae* of which 75% were *bla*NDM positive .Nagdeo et al2012 reported MβL production in *Klebsiella* 30.69%. (Table 5) MβL production in *E. coli* .Our findings are comaparable with Rai et al, Wadekar et al,Nagdeo et al.[10-17]In our study resistance to meropenem was 71.69% in *E.coli* and 92.17% in *K.pneumoniae*. KPC production was found in 13.21% and 7.83% isolates of *E.coli* isolates of *K.pneumoniae* respectively. All KPC producing isolates were found to be multi drug resistant.In isolates of *E.coli* and *K.pneumoniae* co existence of multiple beta -lactamase was common. In our study resistance to ciprofloxacin was 84.90% and 84.34 % in isolates of *E.coli* and *K.pneumoniae* respectively. Overall only tigecyline, gentamycin, polymyxin B and cotrimoxazole were found to be effective against KPC producing isolates. KPC+MβL production was found to be 14.29 % and 44.44% in isolates of *E.coli* and *K.pneumoniae* respectively(Table 7). ESβL+MβL production was seen in 24.53 % and 38.26% in isolates of *E.coli* and *K.pneumoniae* respectively. ESβL and AmpC co-production was seen in 11.32% isolates of *E.coli* and 14.78% isolates of *K.pneumoniae.*

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Being a tertiary center we receive transfer in cases / referrals from other hospitals and we observed complex co-existence of KPC/MβL/ESβL/ AmpC β- lactamases in various permutation and combinations. In present study overall ESβL, Amp-C, and MβL production was found to be higher in *K. pneumoniae* isolates. KPC production was seen in 13.21% and 7.83% isolates of *E.coli* isolates of *K.pneumoniae* respectively. Sensitivity of KPC producing isolates was found to be 100% to polymyxin B, 68.75% to tigecycline, 56.25% to gentamycin and 18.75% to cotrimoxazole. KPC+MβL production was seen in 14.29 % and 44.44% in isolates of *E.coli* and *K.pneumoniae* respectively.

Prolonged hospitalizations and transfer between hospitals, ICU stay, age, comorbidities, immunosuppression, presence of prosthetic devices (e.g. indwelling catheters and central lines), and previous antibiotic administration (multiple courses and/or prolonged use) are known risk factors. Organ transplant recipients and patients receiving hemodialysis or chemotherapy are particularly vulnerable. Antibiotic use has been identified as the strongest risk factor for colonization. MIC of meropenem showed a varied range but showed good concordance with the disc diffusion results Sensitivity to Polymyxin B was found to be 100%, tigecycline 68.75%, gentamicin 56.25% and 18.75% to cotrimoxazole .Similar observation have been made by Parveen et al upadhyay et al, Nayak et al and Singh et al.Some sensitvity to cotrimoxazole in KPC producing isolates was also observed by Nayak et al.[10-17]Efforts are needed to regulate rational antibiotic therapy,impart an attitudinal change in prescribers, generate meaningful data for epidemiological, therapeutic and infection control purposes.[30]

Carbapenems albeit expensive are the achilles heel in the armamentarium against serious gram negative infections due to the stability of these agents against majority of β-lactamases. Presence of carbapenemases does not always produce a resistant phenotype on conventional disc diffusion or automated susceptibility-testing methods. Detection tests are still evolving, hindered by the heterogeneity of both enzymes and hosts, which confers different levels of carbapenem susceptibility. (Thompson 2010).Although there is room for improvement and our study needs molecular support, the best current methods must be used. Optimal use of microbiology laboratories is essential to combat the spread of multiple antibiotic-resistant pathogens. The phenotypic can be employed in any laboratory settings where molecular diagnostic techniques are not available to detect this important mechanism of antimicrobial resistance.[2,5,30]

CONCLUSION

In conclusion, All antibiotic use, whether warranted or not, places selection pressure on bacteria. Antibiotic resistance to useful classes of antibiotics including the β-lactams, aminoglycosides and quinolones has emerged and this has been increasingly reported.[3,17] In all isolates of *E.coli* and *K.pneumoniae* high resistance to all classes of cephalosporins was observed. Resistance to gentamycin and co-trimoxazole ranged from 32-50% it was least among antimicrobials tested for both organisms. ESβL was the most common beta-lacatamse detected, followed by MβL and AmpC in our study. KPC's were produced by 13.21% *E.coli* and 7.83 % *K.pneumoniae* isolates. Overall co-production of multiple β- lacatamases was common in our study.

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