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Full Length Research Paper

Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species in Pakistan

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The aim of this study was to check for the antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) of extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species. All methods used in this study were according to the standard criteria of NCCLs. It was shown that there was high rate of resistance, which was alarming for health practitioners of Pakistan.

Key words: Antibiotics, multiple antibiotic resistances (MAR), extended spectrum β-lactamase (ESBL).

INTRODUCTION

Antimicrobial drugs are of two types. One is the natural antimicrobials which are produced by living organisms. Some antimicrobials are synthetic derivatives of the microbial substance which act as antibiotics. Cephalosporin and penicillin belong to such antibiotics which are produced by fungi. The other type is the chemical antimicrobials which are completely synthetic. Such type of antimicrobial compounds is more toxic to human cells when compared with the natural antibiotics. There is a designated concentration of each antimicrobial drug, and this concentration is approved by NCCLs and the health protection agency (NCCLs, 2005; HPA, 2006). A recommended concentration of the drug is not completely safe for humans.

Antimicrobial drugs are classified according to their mechanism of action, for example, cell wall inhibiting, cell membrane inhibiting, protein synthesis inhibiting and nucleic acid inhibiting. In Microbiology diagnostic laboratory, after identification of bacteria, the next important step is susceptibility testing with the panel of different antibiotics. This panel is designed according to NCCLs criteria. Generally, there is Kirby-Bauer disc diffusion method which is used in laboratories using Muller Hinton media. The resistance, sensitivity and intermediate pattern of antibiotics are done according to zone size which is mentioned in the supplement provided by NCCLs. The antibiotic disc is placed 30 mm away from each other. In this way, we can avoid synergism or antagonistic effect of antibiotics by mixing the antibiotics with each other (Cheesbrough, 2001). In this study, we analyzed the antibiotic resistance patterns according to the calculation results of multiple antibiotic resistance indices. This gives us an idea about the present situation of the antibiotic resistance in Pakistani society.

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Abbreviation: MAR, Multiple antibiotic resistance.

MATERIALS AND METHODS

Panel of antimicrobial drugs used

The panel used in this study consists of 18 antibiotics which belong to three major groups of antibiotics according to the mechanism of action. This panel was designed according to NCCLs guidelines (NCCLs, 2002) and was commonly used in majority of the Pakistani clinical settings (Table 1). All clinical samples which came from citi laboratory and research, Lahore, were processed. Isolation and identification of strains were done using the biochemical pattern

Table 1. Mechanism of action and groups of antibiotics used in the study.

Mechanism of action	Inhibitor of Protein synthesis	Code	Concentration
Antibiotic group	Aminoglycosides		
Antibiotics	Amikacin	AK	30 µg
	Gentamycin	CN	10 µg
Mechanism of action	**Inhibitor of cell wall synthesis**		
Antibiotic group	**i)Beta-lactam antibiotics Penicillin**		
Antibiotics	Ampicillin	AMP	10 µg
	Carbencilin	CAR	100 µg
Antibiotic group	**ii)Beta-lactam antibiotics Cephalosporin**		
Cephalosporin I	Cephredine	CE	30 µg
Cephalosporin II	Cefuroxime	CXM	30 µg
Cephalosporin III	Cefoperazone	CFP	75 µg
	Ceftazidime	CAZ	30 µg
	Ceftriaxone	CRO	30 µg
	Ceftriaxone	CRO	30 µg
	Ceftizoxime	ZOX	10 µg
Antibiotic group	**iii)Beta-lactamase inhibitor**		
	Augmentin	AMC	20/10 µg
	Tazocin	TZP	100/10 µg
Antibiotic group	**Monobactams**		
	Aztreonam	AZT	30 µg
Antibiotic group	**Carbapenems**		
	Imipenem	IMP	10 µg
	Meropenem	MEM	10 µg
Mechanism of action	**Inhibitor of Nucleic acid**		
Antibiotic group	Sulfonamide		
Antibiotic	Cotrimoxazole	SXT	5 µg
Antibiotic group	**Fluroquinolones**		
	Ciprofolxacin	CIP	5 µg
	Norfloxacin	NOR	10 µg
Antibiotic group	**Urinary tract antiseptic**		
	Nitofurontoin	FN	300 µg

recommended by NCCLs guidelines (NCCLs, 2002). Extended spectrum β-lactamase (ESBL) production was confirmed using health protection agency criteria via double disc synergism combination test and E-test (HPA, 2006).

Multiple antibiotic resistances (MAR) calculation

Multiple antibiotic resistance index (MAR) is helpful in analyzing health risk, and is used to check the antibiotic resistance. In this study, 18 antibiotics were used and are represented as (b), while that particular isolate is resistant to 10 antibiotics. These resistant antibiotics are represented as (a), then its MAR is calculated as a/b, which means that in this particular case, MAR is 10/18 = 0.55. However, if MAR is going to be calculated from such a sample site, where we took many isolates from, then this formula is changed. In such a case, we take a/(b.c) formula for calculating MAR index. Here, (a) represents the aggregate resistance of antibiotics to all isolates, while (b) represents the total number of antibiotics and (c) stands for the number of isolates from the specimen site. The second case is generally used in the environmental sampling.

RESULTS

In this study, the first case was used in clinical samples, because there was only one isolate from one site of the specimen which was according to our selection criteria. This is the general selection criteria, but when there are more than three antibiotics showing resistance, we can calculate the MAR. The value of MAR index (0.200) differentiates the low and high risk. If the value is

Table 2. Multiple antibiotic resistance (MAR) indices of bacteria.

MAR index	Percent frequency of MAR index (%)	
	E. coli	*Klebsiella* species
0	7.2	11.4
0.1	9.6	13.6
0.2	9	11.4
0.3	4.8	7
0.4	13.7	9
0.5	25	25
0.6	12.1	7
0.7	43.1	12
0.8	0.8	4.5
0.9	0	0
1.0	0	0

between 0.200 and 0.250, it becomes a very risky phase where there are equal chances that MAR may fall in the high risk and low risk phases (Krumperman et al., 1983). MAR is considered as a good tool for risk assessment. This also gives an idea of the number of bacteria showing antibiotic resistance in the risk zone in the study's routine susceptibility testing. This MAR index also recommended that all isolates, somehow, originated from the environment where antibiotics were over used (Paul et al., 1997) (Table 2).

Generally, β-lactamase producing isolates showed resistance to the penicillin group, as well as the cephalosporin group. These groups are commonly used in the susceptibility panel, as they are widely used antimicrobial drugs. *E. coli* and *Klebsiella* isolates showed MAR index in the risk zone and they were resistance to some antibiotics of cephalosporin group than they were when they were further advanced for phenotypic detection test of β-lactamses (Table 2).

Antibiotic resistance pattern of *E. coli* and *Klebsiella* with cell wall, protein and nucleic acid inhibiting drugs

Antibiotic resistance pattern was studied with cell wall, protein and nucleic acid inhibiting drugs. β-Lactamase producing *E. coli* showed maximum resistance over 90% with AMP, CAR, CE, CXM, CRO and CAZ, while β-lactamase producing *Klebsiella* showed 100% resistance with CAR, CE, CXM and CRO, but CAZ and AMP showed the resistance of both strains to be almost similar and was above 90%. CFP showed resistance more than 80% both in *E. coli and Klebsiella*, but in the case of AMC, the resistance pattern in *E. coli* was above 50%. However, it did not clearly show sensitive zones; instead intermediate sensitive zones were more when compared with sensitive zones. In *Klebseilla*, this drug had more than 60% resistance, but here again, intermediate sensitivity was 20%. ZOX had above 60% resistance in *E. coli*, but above 90% in *Klebsiella.* MEM and IMP were considered as the therapy of choice, because there were very rare cases of MEM resistance, but no resistance was found with IMP in both types of strains. TZP also had good results with very little resistance in the case of *E. coli* but not in *Klebsiella* (Table 3). In the case of protein inhibiting drugs, CN and AK was used. CN has more than 80% resistance in *Klebsiella* but more than 60% in *E. coli.* On the other hand, AK responded well and there was very little resistance in *E. coli*, but not in *Klebsiella* (Table 4). In nucleic acid inhibiting drugs, CIP and SXT showed more than 80% resistance, while FN and NOR showed more than 90% resistance in *E. coli*. In *Klebsiella,* CIP had more than 70% resistance, but SXT, FN and NOR had resistance more than 90%. The overall resistance pattern presents that there is a very high resistance rate only for few drugs left as treatment of choice. The other important information which we got from these data was that *Klebsiella* had more resistance percentage than almost all drugs when compared with *E. coli.* Although, there was no marked difference, still some drugs seemed useless for β-lactamase producing *Klebsiella* infection therapy (Table 5).

Antibiotic resistance pattern according to gender classification

In *E. coli* infections, AMP, CAR, CE, CXM, CRO, CAZ, FN and NOR all had resistance more than 90% both in males and females. AMC showed nearly 70% resistance in both males and females. ZOX had less resistance in males, that is, 68% when compared with females which had 75% resistance. IMP and MEM had no resistance, while AK and TZP had resistance close to 10% in both genders. CN showed more resistance in males with a resistance of more than 65%, whereas CN resistance in females was 52%. However, CIP and SXT had a resistance of more than 85% in both genders. There were only two drugs, ZOX and SXT, in the case of *E. coli* infections which had different resistance patterns on the basis of gender group (Figure 1). In *Klebsiella* infections, AMP had more resistance in males (100%) when compared with females (85%) (Figure 2). Some antimicrobial drugs had no difference in resistance, such as AMC and CN, which were above 80%, but in CAR, CE, CXM, CRO, FN and NOR, it was 100%. There was 100% resistance in males in CFP and 65% in females. In the case of CAZ and ZOX, there was 80% resistance in males and 100% resistance in females. In females, there was little resistance in MEM, but none in males. Moreover, there was no resistance in any gender with IMP, TZP and AK. CIP had more resistance in males when compared with

Table 3. Resistance pattern of *E. coli* and *Klebsiella species* against cell wall inhibiting antibiotics.

Organism	Antibiotic resistance (%)											
	AMP	AMC	CAR	CE	CFP	CXM	CRO	CAZ	ZOX	IMP	MEM	TZP
E. coli	95.5	53	100	96	84	94	98.8	90	63	0	0	2
Klebsiella	90	60	100	100	80	100	100	92	90	0	10	0

See Table 1 for the full name of the various antibiotics.

Table 4. Resistance pattern of *E. coli* and *Klebsiella species* against protein inhibiting antibiotics.

Organism	Antibiotic resistance (%)	
	AK	CN
E. coli	4	61
Klebsiella.	0	80

See Table 1 for the full name of the various antibiotics.

Table 5. Resistance pattern of *E. coli* and *Klebsiella species* against nucleic acid inhibiting antibiotics.

Organism	Antibiotic resistance (%)			
	CIP	FN	NOR	SXT
E. coli	82	96	100	85
Klebsiella.	70	100	100	90

See Table 1 for the full name of the various antibiotics

females, while in SXT; there was more resistance in females when compared with males. Nonetheless, CFP, CAZ, ZOX, MEM and CIP had different resistance profiles on the basis of gender classification.

Antibiotic resistance pattern according to age group

Antibiotic resistance pattern was checked according to different age groups both in *E. coli* and *Klebsiella* β-lacatmase infections. The age groups were prepared with the difference of ten years. The preparation was started from 0+ to 90+. In *E. coli* infection, AMP had a maximum resistance of 100% in the age group of 70+ to 90+, while in AMP; minimum resistance was in the age group of 0+ to 30+ with almost 90% resistance. In AMC, there was more resistance in the age group of above 70, while in the case of the age group from 30 to 60+, the resistance was almost 50%. CAR, CE, CXM, CRO, FN, NOR and CAZ were resistant equally, regardless of the age group. CFP and ZOX showed gradual increase in resistance with increasing age group. IMP had no resistance in any age group, but MEM and TZP had some resistance in the 70+ to 90+. However, CN, CIP and SXT also had gradual changes in resistance with increasing age (Table 6).

Klebsiella infection was not found in 0+, 80+ and 90+ age groups. In AMP, there was more than 80% resistance in the 10+ and 20+ age groups, while more than 90% resistance was found in the 30+ to 70+ age groups. In AMC, the age group below 30+ had less than 60% resistance, while that above 30+ had about 65% resistance. CAR, CE, CXM, CRO, CAZ, FN and NOR had a resistance of over 90% in all age groups because β-lactamase had a resistance with the cephalosporin group. CFP had less than 80% resistance but up to 40% and above 80% resistance in the remaining age groups. IMP had no resistance but MEM, TZP and AK showed resistance in the 60+ and 70+ age groups. CN showed less than 80% resistance in the 10+, 20+ and 30+ age groups, but more than 80% resistance in other age groups. CIP had less than 70% resistance in the 10+ and 20+ age groups, and more than 70% resistance in the 30+, 40+ and 50+ age groups. Besides, more than 80% was found in the 60+ and 70+ age groups. SXT had more than 80% resistance in the 10+ to 50+ age groups, but the resistance increased to more than 90% in the 60+ and 70+ age groups (Table 7). The resistance pattern of antibiotic presented that there was gradual increase in the resistance with old age. Majority of the infection made patient at old age to be immnuo-compromised, which resulted in more resistance of antibiotics. β-Lactamase enzyme also made many drugs resistance, such as cephalosporin group, which was generally useful in routine infections. A similar case was found with the penicillin group, which was the drug of choice in the routine infection, but became useless in β-lactamase infections.

DISCUSSION

Antibiotic susceptibility testing is a very important step in diagnostic microbiology where we can diagnose which therapy pattern was recommended. The importance of susceptibility pattern becomes more important with the detection of β-lactamase enzyme. This is well known that production of this enzyme increases resistance of the

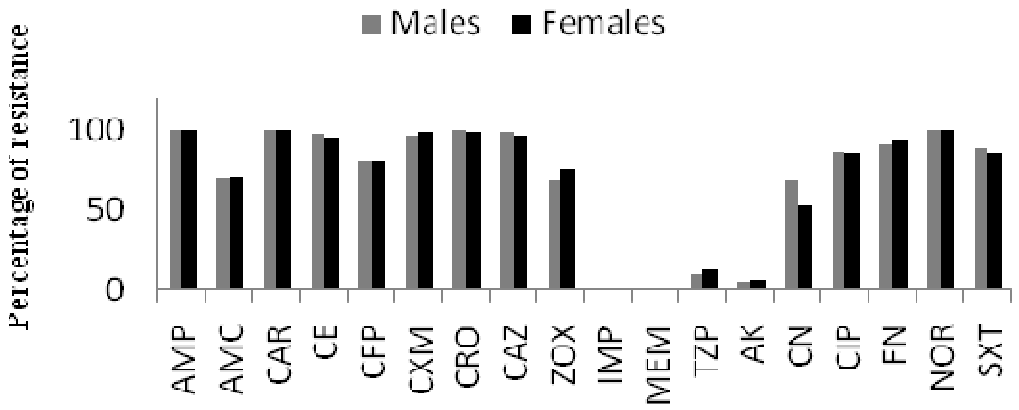

Figure 1. Antibiotic resistance in *E. coli* according to gender classification. See Table 1 for the full name of the various antibiotics.

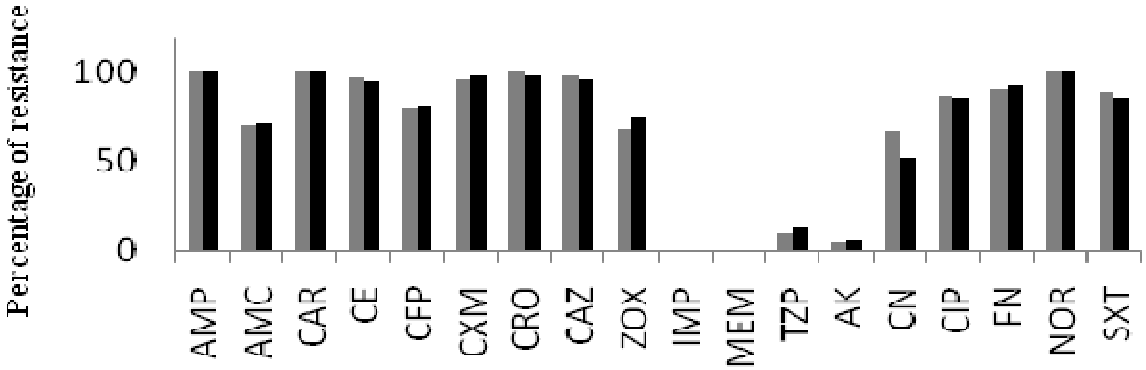

Figure 2. Antibiotic resistance in *Klebsiella species* according to gender classification. See Table 1 for the full name of the various antibiotics.

antibiotic panel. The selection of *E. coli* and *Klebsiella* was done by performing regular ESBL phenotypic detection tests, such as Double disc synergism test, combination test and E-test. Further selection of resistance isolates was done according to MAR calculation, in places where it was checked that *E. coli* and *Klebsiella* had multiple antibiotic resistance index below 0.2. Then cephalosporin resistances were checked, before advancing the analyzing of antibiotic susceptibility pattern of β-lactamase producing isolates. The resistance pattern was evaluated according to gender classification and age group. It was shown that many antibiotics had more resistance in females, but some drugs were more resistant in males (Figures 1 and 2). The infection of *E. coli* and *Klebsiella* also had some antibiotic resistance differences. Some antimicrobial drugs had more *E. coli* resistance, while in some cases, *Klebsiella* showed more resistance patterns. There were few cases of *Klebsiella* infection, but with the increasing antibiotic resistance pattern. In this study, the results of amikacin, tazocin (Piperacillin/tazobactam), meropenem and imepenem was very good. A study was conducted in India, which showed meropenem as the best option for therapy against Gram negative bacilli. Although, there was high rate of resistance against cephalosporin drugs, the infection causing Gram negative bacilli was a very alarming condition (Goel et al., 2009). Imepenem and meropenem showed almost 98 to 100% sensitivity, while in Tazocin, 90% sensitivity was studied with different Gram negative bacilli which had high antibiotic resistance (Joly-Guillou et al., 2010). A study was conducted at King Fahd Hospital, Saudi Arabia, and it showed that meropenem was 95.8% sensitive, amikacin was 93.7% sensitive and imipenem was 91.7% sensitive in ESBL producing *E. coli*, while in ESBL producing *Klebsiella*, meropenem was 94.4% sensitive followed by gentamicin and piperacillin-tazocin which was 88.9% and amikacin and ciprofloxacin, which was 83.3%. The susceptibility of these two ESBL producer isolates were greatly reduced in β-lactamase inhibitor combinations and trimethoprim-

Table 6. Antibiotic resistance pattern in *E. coli* infection according to age groups.

Antibiotic	Age group									
	0+	10+	20+	30+	40+	50+	60+	70+	80+	90+
AMP	90	92	90	95	94	94	95	100	100	100
AMC	25	30	20	47	38	45	42	70	82	85
CAR	100	99	98	100	100	100	100	100	100	100
CE	100	90	94	100	96	100	100	98	99	100
CFP	79	85	82	85	90	87	89	85	94	98
CXM	95	92	96	89	90	100	100	96	97	100
CRO	98	99	100	95	94	98	94	99	100	100
CAZ	95	98	95	94	100	95	97	100	99	96
ZOX	45	58	68	69	74	80	84	79	80	81
IMP	0	0	0	0	0	0	0	0	0	0
MEM	0	0	0	0	0	0	0	10	5	1
TZP	0	0	0	0	1	0	0	1	4	2
AK	0	2	0	0	0	1	4	0	0	2.5
CN	67	55	42	68	72	75	66	69	84	70
CIP	80	86	79	85	77	82	69	95	91	88
FN	98	99	97	100	100	100	100	100	100	100
NOR	100	92	100	97	100	100	100	99	99	100
SXT	85	88	89	82	76	77	87	89	78	85

See Table 1 for the full name of the various antibiotics.

sulphametoxazole (Al-Zahran and Akhtar, 2005). In this study's case, the imepenem, meropenem, amikacin and tazocin had very good sensitivity, although, variability with other drugs, such as augmentin, cefoperazone, ceftizoxime, gentamycin and ciprofloxacin, were present.

In another study conducted in Pakistan, the susceptibility pattern of the urinary tract infection causing *E. coli* was studied. The susceptibility pattern of imepenem was 98%, while meropenem was 97%. Gentamicin had a sensitivity of 48%, while ciprofloxacin was 35% and co-trimoxazole was 17%. They also concluded that multi-drug resistant and ESBL producing *E. coli* was in large proportion in this region (Ullah et al., 2009). The pattern of sensitivity was also affected with the type of infection, as ESBL producers had high rate of resistance to cephalosporin and penicillin groups when compared to non ESBL producers. Similarly, the rate of resistance increased in certain special conditions like nosocomial infections and in immnuo-compromised patients. Particularly, in old age, there was more resistance of drugs. In this study, it was found that drug resistance was high in the age group of above 50+, but this was not the case with all drugs, since majority of the drugs had no effect with the change in age or gender. Such drugs were resistance in all cases, because of β-lactamase. Some drugs had little variability depending on the change made in the age group and gender classification. A study was conducted in which it was discussed that with the change in age group, the susceptibility pattern was somewhat affected. Similarly, with the change in gender, there was effect on the susceptibility pattern, but that definitely depended on the site of infection. As in the urinary tract infections, there was more prevalence found in females. Similarly, more drug resistance was seen in female UTI cases. This study was conducted in Pakistan on diversity of bacterial pathogens of UTI and susceptibility pattern of drugs used in therapy of infections. They found that UTI was the leading infection in females and there was high resistant rate with different drugs. It was also mentioned that with older age, the resistance of drugs increased, while in young age, the resistance pattern was less common (Bashir et al., 2008). In another study conducted at Aga Khan University, Karachi presents the data which also support the results of this study that with the extreme age groups, the presence of ESBL and high drug resistance is more significant. They explained that these extreme age groups are under 5 years and above 60 years. In ESBL producing isolates, cross resistance with non β-lactam drugs were also frequently seen, such as flouoroquinolones, aminoglycosides and co-trimoxazole (Jabeen et al., 2005).

Another study was conducted at Fauji Foundation Hospital, Rawalpindi. The data were studied for two years starting from 2004 to 2006. According to the results, they found that ESBL was more common in females with UTI (64.3%). In males, the most prevalent ESBL infection site

Table 7. Antibiotic resistance pattern in *Klebsiella species* according to age groups.

Antibiotic	Age group									
	0+	10+	20+	30+	40+	50+	60+	70+	80+	90+
AMP	-	80	88	91	90	95	98	99	-	-
AMC	-	55	57	59	60	64	65	65	-	-
CAR	-	97	96	100	100	100	100	100	-	-
CE	-	98	99	97	100	100	100	100	-	-
CFP	-	77	75	76	78	80	84	85	-	-
CXM	-	97	98	96	100	100	100	100	-	-
CRO	-	97	100	98	100	100	100	100	-	-
CAZ	-	94	100	95	100	100	98	95	-	-
ZOX	-	87	85	92	91	95	100	100	-	-
IMP	-	0	0	0	0	0	0	0	-	-
MEM	-	0	0	0	0	0	5	7	-	-
TZP	-	0	0	0	0	0	8	13	-	-
AK	-	0	0	0	0	2	5	5	-	-
CN	-	67	75	78	80	81	80	85	-	-
CIP	-	65	67	70	74	71	80	84	-	-
FN	-	99	98	97	100	100	100	100	-	-
NOR	-	100	100	99	100	100	100	100	-	-
SXT	-	80	85	87	80	84	90	92	-	-

See Table 1 for the full name of the various antibiotics.

was pus samples (35.7%). ESBL enteric Gram-negative bacilli were common in the 60+ age group, followed by the 40+ age group. There was less percentage of ESBL in the 0+ and 30+ age groups. In females, ESBL producers were common in middle age and later in the 50+ age group (Mumtaz et al., 2010). These results also showed that UTI was the most prevalent infection produced by ESBL isolates in females. In males, wound swab/pus swab was the most prevalent site of infection related to ESBL producers. Similarly, more antibiotic resistances were common in females, but some drugs like SXT, CAZ and ZOX had more resistance in males in the case of *Klebsiella* infection. In *E. coli* infection, CAZ, SXT and CN had high resistance in males, while ZOX had high resistance in females.

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