Carbapenem Resistant *Escherichia coli* and *Klebsiella pneumoniae* in Community Wastewaters of Dhaka, Bangladesh

By

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
B.Sc in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled **"**Characterizing the Environmental Spread of Carbapenem Resistant Escherichia coli and Klebsiella pneumoniae in Community Wastewaters of Dhaka, Bangladesh**"** submitted by
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Abstract

Background: Carbapenems are beta-lactams that include some of the most widely used antibiotics worldwide, with inhibition of its treatment posing an alarming risk to healthcare. Resistance to this drug is determined by the production of beta-lactamases which are encoded by genes such as NDM-1, IMP, VIM, OXA-48 etc. The detection of such carbapenem resistant bacterial species has been observed in both hospital and community wastewaters of Dhaka, Bangladesh. As microorganisms from sewage can find its way to drinking water, the spread of these microorganisms into community wastewater lines creates the possibility of infections that are challenging to treat. As such, this study was conducted on community wastewater samples to thoroughly monitor and determine the distribution of carbapenem-resistant bacteria in different community regions at differing distances.

Materials and Methods: Wastewater samples were collected from 8 sites in Dhaka and transferred to the laboratory. Bacterial cultures were grown on selective agar media for isolation of *Escherichia coli* and *Klebsiella pneumoniae* colonies. Antibiotic resistance profiles of colonies were determined with Kirby-Bauer disc diffusion testing with PCR identification of beta-lactamase genes.

Results: From this study it can be perceived that, 48.84% *K. pneumoniae* which were either imipenem or meropenem resistant or both of antibiotic resistant, most of them carried the *blaNDM* gene which is the most common gene responsible for coding the carbapenemase enzyme known as metallo beta-lactamase that makes bacteria resistant to a broad range of beta-lactam antibiotics. But, even though the prevalence of carbapenem resistant *E. coli* is higher (51.16%), *blaNDM* gene carriers were less in comparison to *K. pneumoniae*.

Keywords: Carbapenem, Antibiotic, Dhaka, *blaNDM*, *E. coli*, *K. pneumoniae*, Community Wastewater

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List of Acronyms

PBPs	Penicillin Binding Proteins
E. coli	*Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
VIM	Verona integron-encoded metallo-β-lactamase
IMP	Imipenemase
NDM	New Delhi metallo-β-lactamase
EMB	Eosin Methylene Blue
MHA	Mueller Hinton Agar
PCR	Polymerase Chain Reaction

Chapter 1

Introduction

Beta-lactam antibiotics are amongst the most frequently used antimicrobial agents. These antibiotics act by inhibiting a set of transpeptidase enzymes, also known as penicillin binding proteins (PBPs) that are essential for the synthesis of the peptidoglycan layer of the bacterial cell wall (Sauvage E *et.al* 2008). There are different beta-lactams, and they are classified into groups based on the core ring structure which is the 3-carbon and 1-nitrogen ring (beta-lactam ring) that is highly reactive. These groups include- Penicillins, Cephalosporins, Carbapenems, Monobactams, and Beta-lactamase inhibitors (Pandey *et.al* 2021).

Among these, the carbapenems have the greatest range of activity against both Gram-negative and Gram-positive bacteria and act by acylation of PBPs, thus inhibiting peptidase reactions involved in bacterial cell wall formation. Antibiotics within the carbapenem group include imipenem, meropenem, doripenem, ertapenem, etc., which confer varying spectrums of potency against different microbial species (Papp-Wallace *et.al* 2011). Despite the clinical success of these drugs, the emergence of β-lactamase enzymes poses a threat to the treatment of such infectious diseases. B-lactamases, produced by bacteria, are β-lactam hydrolysing enzymes that have evolved over time, acquiring new genes and characteristic hydrolysis mechanisms, that have developed resistance to even carbapenem antibiotic activity (Bonomo R. A. 2017). Other mechanisms of resistance to carbapenems and beta-lactams can occur in Gram-positive bacteria through mutation-derived changes of their PBPs, while Gram-negatives commonly utilize processes such as changes in the number of outer membrane proteins that prevent entry of B-lactams, or overexpression of efflux pumps that decrease susceptibility to these drugs (Bonomo R. A. 2017; Meletis, G 2016; Meletis G. *et.al* 2012; Walsh, 2010; Poirel *et al*. 2007).

Beta lactamases include four main classes of agents, of which class B include metallo-beta-lactamases that are further subdivided into B1, B2 and B3 based on a series of signature traits (Palzkill.T 2013). Among these, B1 subdivision contains all the important acquired MBLS like the Verona integron-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM), Imipenemase (IMP) etc. which exhibits broad spectrum activity by catalyzing the hydrolysis of a broad range of beta lactam drugs including the carbapenems.

All these β-lactamases have been found worldwide in many different genera of *Enterobacteriaceae* that includes *Escherichia coli* and *Klebsiella pneumoniae* (Bradford P. A. 2001). Especially in Bangladesh, carbapenem resistant *E. coli* are found to be present in water samples from all settings. A study focusing on sampling water from 7 regions in Bangladesh found 68% of all sampling sites to be contaminated with NDM-1 producing bacteria (Toleman *et al*, 2015). A study conducted on wastewater in Dhaka, Bangladesh, has found 71% of samples from hospital adjacent areas and 12% of samples from community areas to be positive for blaNDM-1 along with other beta-lactamase producing genes. It also showed that *K. pneumoniae* was the most prevalent amongst NDM-1 positive isolates followed by *E. coli* and *Acinetobacter spp*. (Islam *et al*. 2017).

As such, this project has been formulated to find out the prevalence of beta-lactamase producing *E. coli* and *K. pneumoniae* in community wastewaters. This will build upon previous knowledge on the spread of such pathogenic organisms in community areas where cross-contamination may occur with drinking water causing difficult to treat infections. Thus, our project wishes to create awareness among the communities by enlightening on our detrimental antibiotic usages and confined infrastructure of the Dhaka city.

Chapter 2

Methods and Materials

2.1 Sample Collection and Processing

Samples were collected in 3 batches within the time period of September-October 2021. This included regions selected by convenience sampling from 8 different locations within Dhaka City. These include Gulshan, Banani, Banani DOHS, Kafrul, Kachukhet, Mohakhali DOHS, Shapla Housing, and Baitul Aman Housing areas. It was ensured that sample collection points were not associated with hospitals and were within a 500m radius that had no healthcare facilities within it. Samples were collected in sterilized tubes and transported to the laboratory. Samples were processed by centrifugation at 3000rpm for 5mins to remove the debris and obtain the supernatant.

2.2 Sample Processing and Bacterial Isolation

10-fold dilution was performed 6 times on all the samples and following standard spread-plate method those were plated upon Chromogenic UTI Agar media, EMB Agar media and MacConkey Agar Media. All the plates were incubated for 18-24 hours at 37℃ for growth of colonies. Colony count was carried out and recorded. Species Identification was conducted by observation of the colony's morphological features on selective media. For instance: due to the presence of chromogenic substrates in the UTI agar media *E. coli* produces purple-magenta colonies whereas *K. pneumoniae* produces blue to purple, mucoid colonies. Similarly, on EMB agar media *E. coli* produces purple with black center and green metallic sheen whereas *K. pneumoniae* give rise to pink, mucoid colonies; and finally on MacConkey agar, growth of *E. coli* could be observed via production of pink to red colonies. Isolates were then streaked upon Nutrient Agar plates for further analysis.

2.3 Antibiotic Resistance profile of the Selected *E. coli* and *Klebsiella pneumoniae*

Among the isolates, the *E. coli* and *Klebsiella pneumoniae.* were selected for detailed antibiogram study by Kirby-Bauer disc diffusion susceptibility testing, using the commercially available standard disks (Oxoid, UK) of Meropenem (10 µg/ml), Imipenem (10 µg/ml), Cefepime (30 µg/ml), Colistin (10 µg/ml), Gentamicin (10 µg/ml), Amikacin (30µg/ml), Piperacillin/Tazobactam (100/10 µg/ml), Amoxycillin (30 µg/ml), Ciprofloxacin (5 µg/ml). A lawn culture of the test organisms onto a Mueller-Hinton agar plate was performed. Upon incubation for 18-24 hours at 37℃ the MHA plates were observed and the resistance profiles were compared following CLSI standards (2021) and eventually the data were stored.

2.4 Extraction of DNA from the Resistant spp.

For the extraction of the resistant bacterial DNA, 'Boiling method' was performed due to its simplicity, cost effectiveness and short handling time. The resistant bacterial species were grown in LB broth overnight which later went through a series of centrifugation and washing steps before finally the cells were incubated at 95℃ for 15 minutes, and immediately cooled on ice for 10 minutes. The DNA rich supernatant was collected and stored at -20℃.

2.5 PCR Amplification and Nucleotide Sequencing

The intact DNA extracts were used as a template for PCR amplification of MBL producing genes. The genes were amplified with the following primers:

Primer target type	Target Gene	Primer Sequences	References
Metallo-Beta - Lactamase	blaNDM	NDM-F 5'-ACCGCCTGGACCGATGACCA -3' NDM-R 5'-GCCAAAGTTGGGCGCGGTTG -3'	[Devi, L. S. 2020]
	blaIMP	IMP-F 5′-GAAGGCGTTTATGTTCATAC-3′ IMP-R 5′-GTATGTTTCAAGAGTGATGC-3′	[Solanki,R *et.al* 2014]
	blaVIM-2	VIM-2-F 5'-AAAGTTATGCCGCACTCACC-3' VIM-2-R 5'-TGCAACTTCATGTTATGCCG-3'	[Farzana *et al* 2003]

Table 01: Primer sequences for detection of metallo-beta-lactamase coding genes.

The PCR reaction was carried out in a final volume of 25µl, containing 4µL DNA template, 5µL nuclease free water, 4µL primers (2µM each) and 12µL EmeraldAmp® GT PCR Master Mix (2X). PCR amplification was performed using Thermocycler (Applied Biosystem-2720 thermal Cycler). The amplification program was setted up according to the references. Aliquots of PCR products analyzed by electrophoresis in 1.2% (w/v) agarose gel. Electrophoresis was performed using 1× Tris–acetic acid and EDTA (TAE) buffer stained with EtBr and run at a constant voltage of 85V for 70 min. The DNA bands were visualized under UV and images were acquired and stored.

Chapter 3

Results

3.1 Antibiotic Susceptibility Profiling

A total of 110 isolates were obtained and differentiated into 2 genera; *E. coli* (59), *K. pneumoniae* (51). All the Enterobacterial isolates were subjected to nine antibiotics impregnated in disks and their resistance profiles were recorded. It was observed that Amoxicillin had the highest incidence among the strains (92%), followed by Cefepime (50%) and Ciprofloxacin (34.86%). The strains recorded least resistance to Colistin (4.58%) and Gentamycin (8.18%). Significant resistance could be observed in case of Piperacillin/Tazobactam (27.27%), Amikacin (18.34%), Imipenem (17.27%) and Meropenem (10.9%).

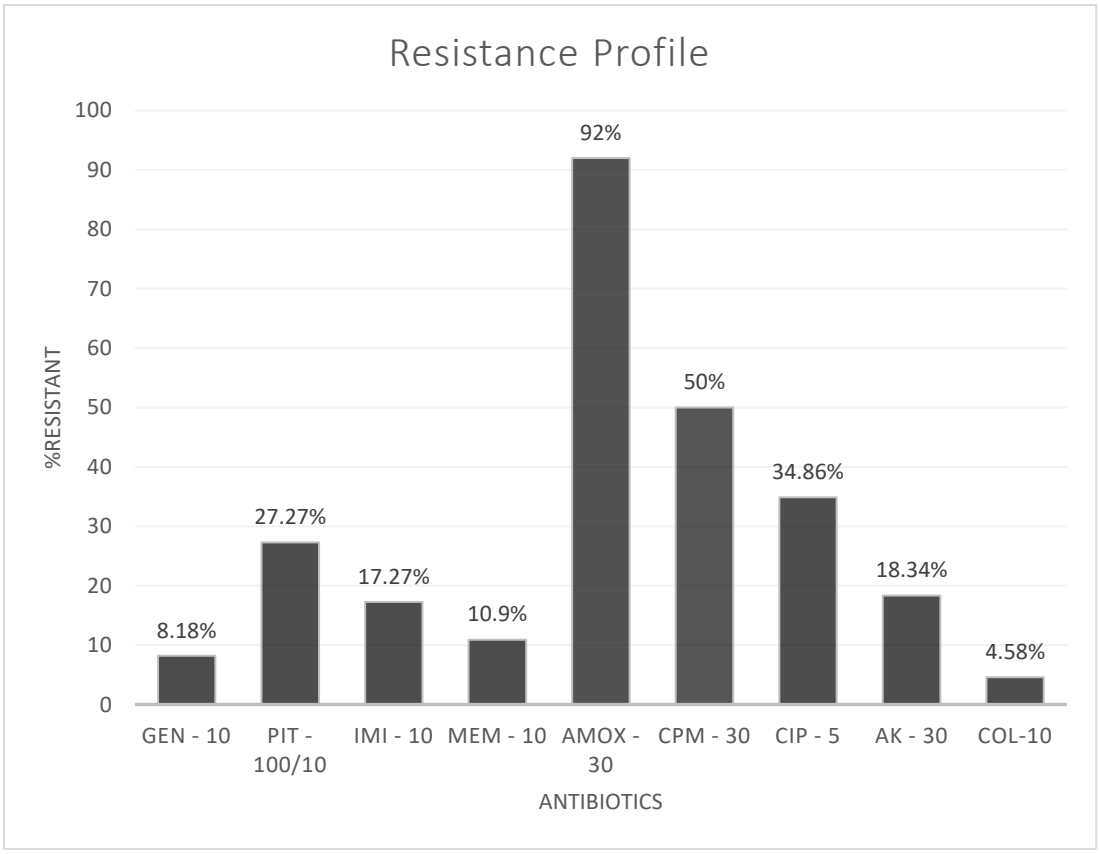

Figure 01: Percentage of drug resistance among all the 110 Enterobacterial strains.

Each of the 2 enterobacterial genera recorded multiple drug resistance; however, it was observed that drug resistance between *E. coli* and *K. pneumoniae* isolates was not significantly different (P>0.05).

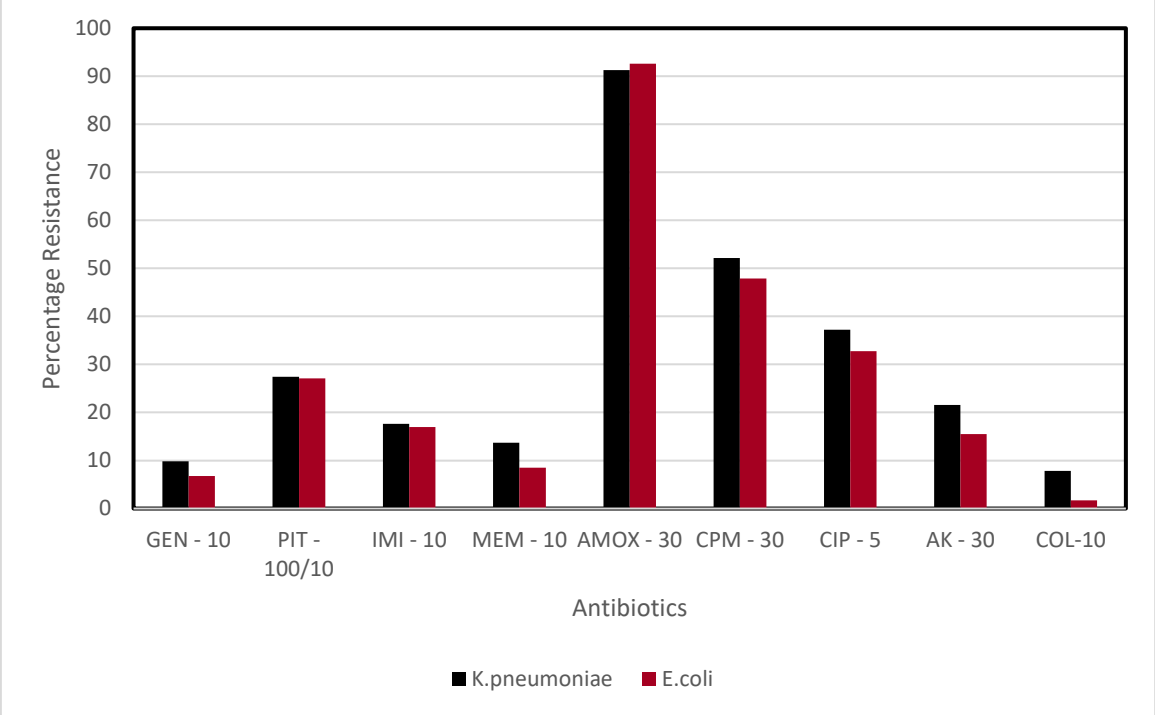

Figure 02: Percentage of multidrug resistance among the two bacterial genera

Although Colistin recorded highest sensitivity among *E. coli* and *K. pneumoniae*, the carbapenem antibiotics showed notable sensitivity among the strains too. Moreover, except the Gentamicin and Amoxicillin, the order of sensitivity among all other antibiotics was as follows; *E. coli* > *K. pneumoniae*. But overall, the antibiotic sensitivity was not significantly different among the two enterobacterial genera (Table 01).

Antibiotic	***% Sensitivity***	
	E. coli	*K. pneumoniae*
Gentamycin	72.88	76.47
Piperacillin/Tazobactam	44.07	39.22
Imipenem	67.79	60.79
Meropenem	84.75	82.35
Amoxicillin	03.70	6.52
Cefepime	35.42	15.22
Ciprofloxacin	24.14	15.68
Amikacin	55.17	43.14
Colistin	100.0	92.15

Table 02: Percentage of isolates sensitivity to antibiotics

E. coli and *Klebsiella* were most resistant to Amoxicillin-92.59% & 91.30%, Cefepime-47.91% & 52.17% and Ciprofloxacin-32.75% & 37.25%. These strains were least resistant to Colistin-1.72% & 7.84% and Gentamycin-6.78% & 9.80%. The rest of the antibiotics, especially the Carbapenems recorded relatively average but notable resistance among these isolates (Table 02).

Antibiotic	***% Resistance***	
	E. coli	*K. pneumoniae*
Gentamycin	06.78	09.80
Piperacillin/Tazobactam	27.12	27.45
Imipenem	16.95	17.65
Meropenem	08.47	13.73
Amoxicillin	92.59	91.30
Cefepime	47.91	52.17
Ciprofloxacin	32.75	37.25
Amikacin	15.52	21.57
Colistin	01.72	7.84

Table 03: Percentage of isolates resistance to Antibiotics

3.2 Distribution of Carbapenemase Coding Genes

To characterize the dissemination of carbapenem resistant bacteria, the isolates that showed resistance towards Meropenem and Imipenem antibiotics were stored for further molecular testing. The carbapenem resistant 43 isolates were subjected to *blaNDM, blaIMP* and *blaVIM-2* primers for carbapenemase coding gene detection. It was observed that among the resistant *E. coli* strains 31.82% were *blaNDM* positive. On the other hand, 39.09% resistant *K. pneumoniae* carried *blaNDM* gene. However, none of the species were found to be carrying *blaIMP* and *blaVIM-2* genes.

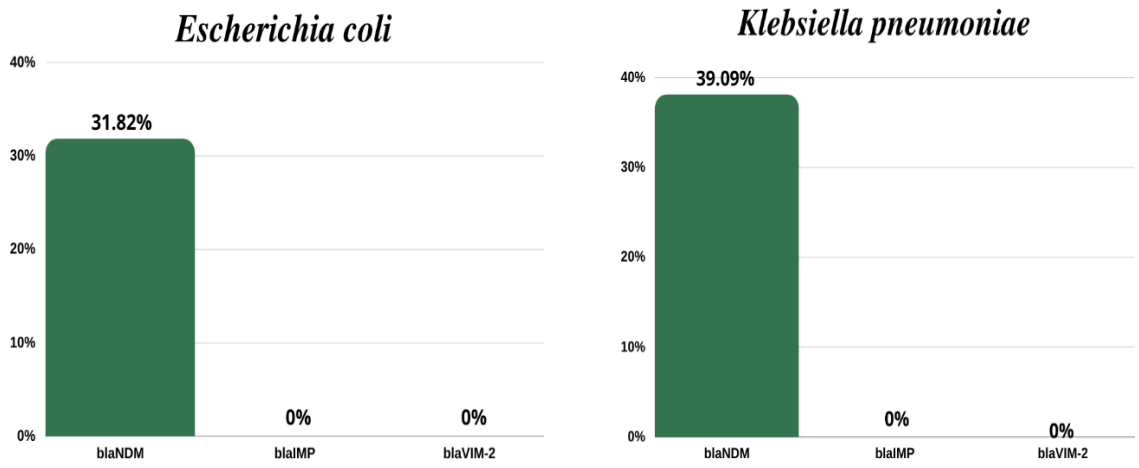

Figure 03: PCR results positive for carbapenemase coding gene products.

From this study it can be perceived that, 48.84% *K. pneumoniae* which were either imipenem or meropenem resistant or both of antibiotic resistant, most of them carried the *blaNDM* gene which is the most common gene responsible for the carbapenemase enzyme known as metallo beta lactamase that makes bacteria resistant to a broad range of beta-lactam antibiotics. But, even though the prevalence of carbapenem resistant *E. coli* is higher (51.16%), *blaNDM* gene carriers were less in comparison to *K. pneumoniae*.

Chapter 4

Discussion

In the recent years, Dhaka has experienced a staggering increase in impervious areas, creating obstruction to natural drainage pattern, and reducing detention basins which ultimately have created shortened runoff concentration-time and as a result, waterlogging problems. Every year during the monsoon, Dhaka Water Supply and Sewerage Authority (WASA) starts digging up the roads flouting rules to eradicate such problems and ultimately our bad community drainage, sewage from overflowing sewerage and latrines mix up with the rainfall runoff and drinking water lines causing waterborne diseases. Through this study it has been revealed that, community sewage sample is quite heavily loaded with multiple drug resistant enteric bacteria and can be a plausible source of contamination to other waterbodies. Multidrug resistant *E. coli* and *K. pneumoniae* strains in this study is particularly significant because they are associated with different gastrointestinal diseases acquired through contaminated water. Though the antibiotics of β-lactam and carbapenems group are the common antibiotics used to treat such infections caused by Gram-negative group of bacterial pathogens, but developing countries like Bangladesh still lacks in complete investigations regarding carbapenemase producing gram negative bacteria especially *E. coli* and *K. pneumoniae.* Recently, the prevalence of carbapenem resistant *E. coli* in urban wastewater samples has been found to be 8% (Asaduzzaman, M. 2020). In another study, the percentage of *K. pneumoniae* resistant to carbapenems has been found to be 79.2% (Sakkas, H *et.al* 2019). In our Study we have also found out that, *K. pneumoniae* was the most prevalent amongst NDM-1 positive isolates followed by *E. coli* (39.09% and 31.82%).

Given the severe threat that multidrug resistant bacteria represent to public health, timely and rigorous actions are required to limit their spread. One tool for achieving that goal should be

the prevention of contamination of the aquatic environment. Despite the fact that antibiotics are essential in the treatment of infectious and other diseases, the widespread development of antibiotic resistance in community waste water necessitates the continued search for new innovative bioactive compounds. In conclusion, findings of the current study reveal a notable percentage prevalence and dissemination of *E. coli* & *K. pneumoniae* harboring various carbapenemase genes in community of Bangladesh. Suitable and adequate control measures from awareness to prescription should be adopted and future research needs to be aimed at establishing both the exposure risks associated with community water and the relative contributions of different types of contamination sources and factors influencing variation in the prevalence of enteric bacteria in community water which may ultimately play a significant role in restraining the spread and transmission of antibiotic resistance from different sources.

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