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Original Research Article

Plasmid profiling of multidrug resistant isolates of *E. coli, Klebsiella* and *Pseudomonas* and antimicrobial potential of *Allium sativum* L. (garlic) against these isolates

Modi - Parekh Kavita*, Bhat Ankush and Joshi Parnal

Department of Microbiology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune -16, India

**Corresponding author*

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Plasmid, Drug resistance, Garlic, Antibacterial, ESKAPE

A B S T R A C T

ESKAPE pathogens like *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* spp. capable of 'escaping' the biocidal action of majority of drugs, are instrumental for increase of antimicrobial resistance. Local studies are required, due to wide regional differences in the pattern of drug resistance. Plasmids carry genes conferring resistance to one or more antibiotics have the ability to transfer between bacterial species and thus accelerate dissemination of multiple drug resistance. Herbal preparations of garlic have been reported to exhibit antibacterial activity against Gram negative bacteria. The objective of the present study was to carry out the plasmid profiling of multidrug resistant isolates and to test the antibacterial potential of raw aqueous garlic extract on these isolates. Twenty five multidrug resistant isolates were included in this study. Plasmid isolation was carried out by alkaline lysis method by Birnboim and Doly. Plasmids were detected in 64% percent of isolates. Average zone of inhibition to aqueous garlic extract ranged from 17 mm to 30 mm for *E. coli*, 11 mm to 35 mm for *Pseudomonas* and 14 mm to 23 mm for *Klebsiella*, respectively. Results suggested that garlic has a potential to control pathogenic multidrug resistant *E. coli*, *Pseudomonas* and *Klebsiella*. Further studies however are required to explore its potential as an effective antimicrobial and plasmid curing agent.

Introduction

Multidrug resistance (MDR), defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories, is a major global health problem (CDC, 2006). Of particular concern, are Gram negative pathogens like *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* nicknamed ESKAPE, due to their ability to "escape" antibiotic treatment using various resistance mechanisms. So acute is the problem of drug resistance, that we are fast approaching a post-antibiotic era, where simple infections will be challenging and costly to treat (Jack NP *et al.*, 2013).

Newer antibiotics are nowhere in sight, leaving us with few therapeutic options like reviving old antibacterials like colistin and fosfomycin, or using a modified existing class like doripenem (Giamarellou H and Poulakou G, 2009). Carbapenems remain the last-resort to treat severe infections, in the wake of increasing resistance to 3rd generation cephalosporins, as reported for *E. coli* and *K. pneumoniae*. The problem is further accelerated by 54% resistance to carbapenems in *K. pneumoniae* as reported from some areas of the world (WHO, 2014). *Pseudomonas* has an unique niche amongst the nosocomial infections, due to various mechanisms of both innate and acquired drug resistance.

These mechanisms are often present simultaneously, conferring combined resistance to many strains (Lambert PA, 2002)**.** This opportunistic pathogen, leads to serious infections like fulminative septicemia, meningitis or pneumonia, worldwide in hospitalized patients (Livermore DM, 2002).

Plasmids are dispensible, extra chromosomal elements which carry antibiotic resistance genes. They are capable of transfer by conjugation between bacterial species and are thus involved in transfer of antibiotic resistance genes across different bacteria. (Bennett PM, 2008). They have been reported to cause resistance to cephalosporins, fluoroquinolones and aminoglycosides (Maria, 2014). Studying the pattern of plasmids can thus give an insight to understanding acquired drug resistance patterns. Given the background of increasing drug resistance, development of novel antimicrobial compounds, from natural sources is the need of the hour. The antimicrobial activity of garlic (Allium sativum L) has been extensively studied (Kyung KH, 2012).

Aqueous extracts of garlic are particularly rich in allicin and account for approximately 75% of garlic-derived sulphinates (Daynea, 2014). Allicin and other thiosulphinates reportedly react with cysteine to abolish antimicrobial activity. However the exact mechanism(s) through which allicin and other garlic compounds inhibit or kill bacteria are elusive.

Thus, there is a great need for formulations from natural sources to be used as adjuncts to conventional antimicrobial agents. This study thus aims to study the plasmid profiles of drug resistant isolates of *E. coli*, *Klebsiella and Pseudomonas*. The study also explores the potential of aqueous garlic extract as an antimicrobial agent against these isolates.

Materials and Methods

Bacterial isolates

A total of twenty five clinical isolates, five of P*seudomonas*, fourteen of *E. coli* and six of *Klebsiella* were included in this study. Isolates were obtained from a local leading hospital, in Pune, Maharashtra, India. Isolates were maintained as glycerol stocks at -20° C. Biosafety precautions were taken while handling clinical isolates.

Identification of clinical bacterial isolates

Morphological and biochemical testing of isolates were performed according to Bergey's Manual of Determinative Bacteriology 9th Edition to confirm the *E.coli*, *Pseudomonas* and *Klebsiella* isolates.

Plasmid extraction and agarose gel electrophoresis

Plasmid extraction was carried out by using Alkaline lysis method as described by Birnboim and Doly midi preparation method

(Birnboim HC and Doly J, 1979). Plasmids were detected using 0.8% agarose gel stained with ethidium bromide (0.5 µg/ml). Gels were examined under ultra violet light for the presence of plasmid. Supercoiled plasmid DNA ladder (Bangalore Genei) was used as a reference marker. Gels were analysed using transilluminator (BIO RAD, Gel Doc ^{TM} XR+).

Aqueous garlic extract preparation

Aqueous garlic extract was made as previously described (Saravanan *et al.,* 2010; Mukhtar *et al.,* 2012). Fresh garlic bulbs were bought from the local market and used. Garlic bulbs were peeled, weighed (100gm) and surface sterilized using ethanol. The ethanol was allowed to evaporate in sterile conditions, in a laminar flow chamber and garlic was crushed aseptically using mortar and pestle under sterile conditions. The homogenized mixture was filtered through sterile muslin cloth and was considered as 100% concentration. The concentrations of 75%, 50% were made by diluting concentrated extract with appropriate volumes of sterile distilled water.

Antibacterial activity

Antibacterial activity was carried out by agar well diffusion method. Strains were inoculated into 10 ml of sterile Müller-Hinton broth, incubated at 37°C for 24 hours in a shaker incubator. The broth culture was incubated at 37°C until it achieved turbidity equivalent to 0.5 McFarland's standard. The turbidity of the actively, growing broth culture was adjusted with sterile broth. The cultures were swabbed on the surface of sterile Müller-Hinton agar plate using a sterile cotton swab. Wells were then bored in four quadrants of plate using a borer 5 mm (sterilized with alcohol). Aqueous extracts (100%, 75% and 50%) were added in three wells and sterile distilled water which served as control was added in fourth well using sterile pipette. All experiments were carried out in triplicates. Plates were incubated at 37°C for 24 hours. Diameter of zones of inhibition were measured in mm and results were recorded (Saravanan *et al.,* 2010).

Phytochemical analysis was carried out for the following

Phytochemical screening of garlic was carried as follows:

Flavonoids

One ml of 10% lead acetate solution was added to 1ml of aqueous garlic extract (100%) and observed for the formation of yellow precipitate, which indicated the presence of flavonoids.

Phenols

Two ml of ferric chloride solution was added to the aqueous garlic extract (100%) and observed for the formation of green or blue colour which indicated the presence of phenols.

Steroids

Two ml of the aqueous garlic extract (100%) extracts were dissolved in 2 ml of chloroform and 2 ml of concentrated sulfuric acid was added. The formation of red colour produced in the lower chloroform layer indicated the presence of steroids.

Tannins

2 ml of the aqueous garlic extract (100%) extracts was mixed with 2 ml of distilled water and few drops of ferric chloride

solution were added. The presence of green precipitate indicated the presence of tannins.

Glycosides

Glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4 was added to 2 ml aqueous garlic extract (100%) extract. Reddish brown colour at junction of the two liquid layers and upper layer appeared bluish green and indicated the presence of glycosides.

Reducing sugars

The aqueous garlic extract (100%) was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for the colour reaction. A brick red precipitate indicated the presence of reducing sugars.

Carbohydrates (Molisch's test)

The aqueous garlic extract (100%) was combined with a small amount of Molisch's reagent (□- naphthol dissolved in ethanol) in a test tube. After mixing, a small amount of concentrated sulfuric acid was slowly added down the sides of the sloping test-tube, without mixing, to form a bottom layer. A positive reaction was indicated by appearance of a purple ring at the interface between the acid and test layers.

MIC and MBC

The minimum inhibitory concentration was determined using the tube dilution technique. Varying amounts of the extract in the following concentration 0.75% to 100% were prepared. An 8 ml of the Müller Hinton broth was pipetted into the various test tubes. Later each tube was inoculated with an overnight culture of test organism, adjusted to McFarland 0.5 turbidity standard and then transferred into the test tube containing the extract. The test tubes were incubated at 37°C for 24 hours. The least concentration of the garlic extract that did not permit any visible growth or turbidity of the inoculated test organisms in broth culture were taken as the minimum inhibitory concentration. All tubes not showing visible growth were subcultured and incubated at 37°C overnight. The highest dilution showing at least 99% inhibition was taken as MBC (IAMM, 2004).

Separation of compounds

Bioactive compounds from garlic extract were separated by using TLC technique. Solvent system consisting of butanol: acetic acid: water (12:3:5) respectively was used. Garlic extract was diluted with sterilized distilled water (10%) and spotted on the TLC plates using capillary tube. Plates were run in a chamber containing the solvent system. Plates were dried at room temperature. Plates were then developed using ninhydrin. Rf value for each band was calculated.

Result and Discussion

Plasmid profiling

Plasmid isolation was carried out for twenty five clinical isolates, five of P*seudomonas* (P1 – P5), fourteen of *E. coli* (E1 - E14) and six of *Klebsiella* (K1 - K6). All six *Klebsiella* isolates (K1-K6) showed presence of atleast one plasmid band. One isolate (K1) showed presence of four plasmid bands. Plasmid was detected in four *Pseudomonas* isolates (P1-P4) and six *E.coli* isolates. Plasmids were detected in 64% (16/25) of total isolates included in this study as shown in table 1. Six different plasmids of sizes 2.7 kb, 5 kb, 8 kb, 8.7 kb, 9.4 kb and greater than 10 kb were detected

from the isolates. Two plasmids of size 2.7 kb, 5 kb were seen only in the *Klebsiella* isolates, while plasmids of size 8 kb, 8.7 kb and greater than 10 kb were seen amongst the *E. coli*, *Klebsiella* and *Pseudomonas* isolates. A 9.4 kb plasmid was seen in *E.coli* and *Klebsiella* isolates.

Antibacterial activity of Garlic extract

The antibacterial activity of 50%, 75% and 100% Garlic extract was seen against all clinical isolates of *Pseudomonas, Klebsiella* and *E. coli.* Maximum antibacterial activity was seen with 100% aqueous garlic extract in all isolates. The average inhibition zone ranged from 17 mm to 30 mm for *E. coli*, 11 mm to 35 mm for *Pseudomonas* and 14 mm to 23 mm for *Klebsiella*, respectively. The highest average zone of inhibition was seen in *Pseudomonas*, followed by K*lebsiella* and *E. coli.*

MIC and MBC

Table 3(a) and 3(b) shows the MIC and MBC value for one isolate each of *Pseudomonas* (P4), *E. coli* (E8) and *Klebsiella* (K6) respectively. The MIC values for *Pseudomonas, Klebsiella and E.coli* were 12.5, 12.5 and 3.1 % respectively. The MBC values for the *Pseudomonas, E. coli* and *Klebsiella* were 12.5%, 12.5% and 6.25%, respectively. MIC and MBC values were the least for *E. coli.*

Phytochemical screening

Phytochemical screening of 100% aqueous Garlic extract, showed presence of flavanoids, steroids, tannins, glycosides and carbohydrates.

Thin Layer Chromatography

The separation of bioactive compounds from aqueous garlic extract showed the presence of four spots on the TLC plates. Rf values were calculated and found to be 0.08, 0.24, 0.39 and 0.42.

The problem of increasing multidrug resistance is very evident from reports of various studies all over the world (WHO, 2014). In India, too different studies have reported different patterns of drug resistance. This reinstates the need for local studies, to determine if there is a pattern to this drug resistance. The isolates included in this study showed multidrug resistance with 68% isolates showing resistance to at least six drugs. Given this bleak scenario it is very necessary to look for adjuncts or alternatives to current drugs.

The problem of drug resistance is further compounded by the presence of plasmids. Plasmids play a very unique role in acquired drug resistance. Shahid *et al* have reported emergence of plasmid-mediated resistance to amikacin in *P. aeruginosa* strains. They have later reported presence of a 19.9-kb plasmid encoding cefoxitin and tetracycline resistance in isolates of *E. coli* and *K. pneumonia*. 64% of isolates in this study showed presence of plasmids. Some plasmid bands are common across the three species while some are unique. However transformation studies need to be carried out to determine the plasmid encoded drug resistance.

Garlic has a unique role to play as a potential therapeutic agent. Studies have reported antimicrobial effect of garlic against some *Enterobacteriaceae* sp (Alzowahi F. A. M. *et al.,* 2013). Others have shown the antimicrobial effect of garlic against pathogenic bacterial strains (Saravanan *et al.,* 2010). The antimicrobial activity of Allium species to bacteria has been extensively studied and attributed to Allicin, other thiosulfinates, and their products.

Table.1 Plasmid profile of multi drug resistant isolates

Serial number	Isolate	Size of plasmid					
		>10 kb	9.4 kb	8.7 kb	8 kb	5 kb	2.7 kb
1	K1	+	+	-	-	+	+
2	K2	+	-	+	-	-	-
3	K3	-	-	-	+	-	-
4	K4	-	-	-	+	-	-
5	K5	-	-	-	+	-	-
6	K6	-	+	-	-	+	-
7	P1	+	-	-	-	-	-
8	P3	+	-	-	+	-	-
9	P4	-	-	+	-	-	-
10	P5	+	-	-	+	-	-
11	E1	+	+	-	-	-	-
12	E2	-	-	-	+	-	-
13	E3	-	-	-	+	-	-
14	E7	+	-	-	-	-	-
15	E8	-	-	-	+	-	-
16	E13	+	-	+	-	-	-

Fig.1 Detection of plasmids using agarose gel electrophoresis (0.8%) Lane 1: Supercoiled plasmid DNA ladder Lane 2, 3: *Klebsiella* isolates Lane 4, 5: *E. coli* isolates Lane 6, 7: Pseudomonas isolates

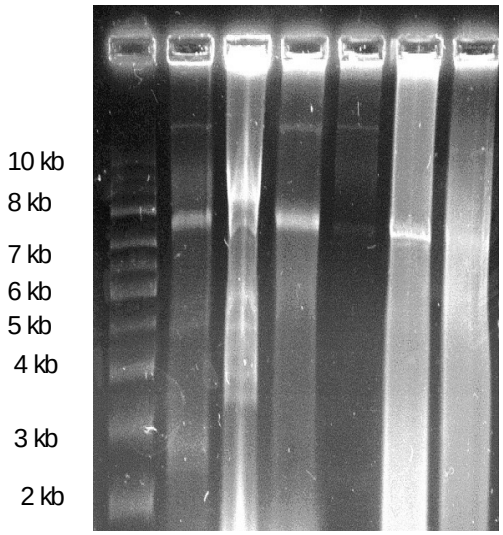


Table.2 Results for antimicrobial activity of garlic extract

Isolate no.	Zone of inhibition (mm)											
	50%				75%				100%			
	1	2	3	Av.	1	2	3	Av.	1	2	3	Av.
E1	24	24	26	27	25	25	26	25	27	27	29	28
E2	23	23	23	23	24	23	23	23	27	26	27	27
E3	25	24	24	24	26	26	25	26	27	28	29	28
E4	25	24	23	24	26	24	23	24	27	27	26	27
E5	23	22	21	22	24	22	25	24	27	27	26	27
E6	26	24	27	26	19	19	18	19	23	23	25	24
E7	21	20	22	21	24	25	26	25	29	29	28	28
E8	17	17	16	17	18	18	19	18	20	22	22	21
E9	27	27	26	27	29	28	27	28	32	29	28	30
E10	30	27	27	28	30	28	28	29	32	29	29	30
E11	25	24	24	24	25	24	24	24	27	27	25	26
E12	24	23	23	23	25	25	24	25	26	30	27	28
E13	26	26	29	27	28	29	31	29	19	20	19	19
E14	23	25	25	24	25	27	28	27	21	20	19	20
P1	24	24	24	24	21	21	23	21	25	25	25	25
P2	29	26	29	28	31	29	31	30	37	31	37	35
P3	14	15	14	14	6	15	13	11	21	23	20	21
P4	10	13	10	11	12	14	11	12	13	14	12	13
P5	14	11	13	13	14	13	13	13	15	14	14	14
K1	23	17	18	19	23	17	18	19	24	22	23	23
K2	16	16	15	16	16	17	18	17	19	20	19	19
K3	18	16	16	17	17	18	18	18	21	20	19	20
K4	14	14	14	14	14	14	14	14	20	20	19	20
K5	15	14	14	14	15	14	14	14	20	22	22	21
K6	11	18	18	16	13	20	19	17	14	21	20	18

Table.3(a) MIC values of garlic extract against tested bacteria

	MIC values (%)		
	Pseudomonas (P4)	*Klebsiella* (K6)	*E.coli* (E8)
Garlic	**12.5**	**12.5**	**3.1**

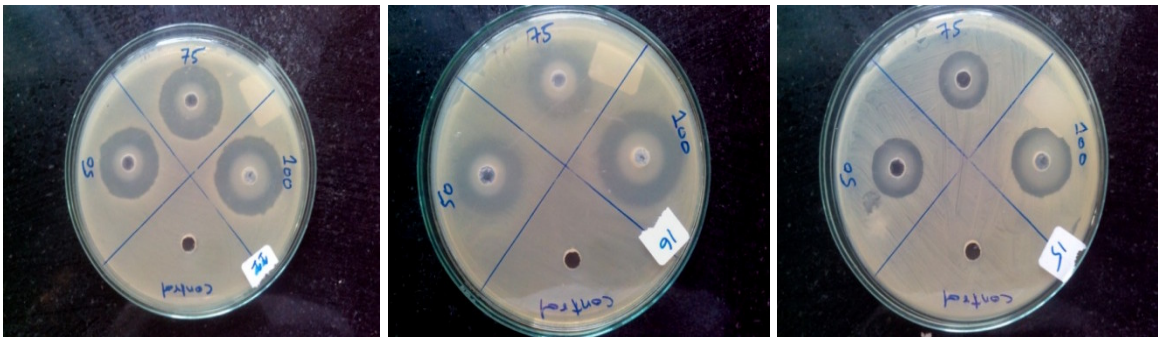
Table.3(b) MBC values of garlic extract against tested bacteria

	MBC values (%)		
	Pseudomonas (P4)	*Klebsiella* (K6)	*E.coli* (E8)
Garlic	**25**	**25**	**6.25**

Table.4 Results for phytochemical screening of garlic extract (100%)

Flavanoids	Phenols	Steroids	Tannins	Glycosides	Reducing sugars	Carbohydrates
+	-	+	+	+	-	+

Fig.2 Antimicrobial activity of garlic extract on *E. coli (E1), Klebsiella (K1), Pseudomonas (P1)*



Alliums inhibit multi-drug-resistant microorganisms and also show a synergestic action with common antimicrobials. It has been reported that allium-derived antimicrobial compounds inhibit microorganisms by reacting with the sulfhydryl (SH) groups of cellular proteins.

It used to be thought that allicin reacts only with cysteine and not with non-SH amino acids, but evidence has accumulated that allicin and other thiosulfinates also react with non-SH amino acids (Kyung KH, 2012). However in spite of these huge number of studies the mode of action and the active elements causing antimicrobial activity remains elusive.

The present study demonstrated the antimicrobial effect of a crude aqueous extract of garlic against drug resistant clinical isolates of *Pseudomonas*, *E. coli* and *Klebsiella*.

The benefit of using garlic is that, it is a part of the Indian diet, it is non toxic in moderate amounts and easy available. Further studies however are required to determine the other biologically active ingredients and explore its potential as an effective antimicrobial and plasmid curing agent.

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