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Antibiotic Resistance Patterns of Gram-Negative Isolates from Water: A Case Study of Nasik

P.P. WAGH[1]*, V. S. SHINDE [2], G.R. CHAUDHARI[3], L. L. SAYYED[4], T. U. KHAPRE [5]
**K.T.H.M College, Shivajinagar, Gangapur road, Nasik-2*

ABSTRACT: The aim of this study was to study antibiotic resistance in Gram negative isolates from various sources of ground water such as open wells, bore wells, hand pumps, water from river Godavari as well as waldevi and treated water by Municipal Corporation of Nasik. Nasik is an important city of Maharashtra state of India. Water samples were checked for presence of antibiotic resistance in gram negative bacteria using disc diffusion method. Resistance was tested against amikacin, netilmicin, cefadroxil, sparfloxacin, ceftriaxone, ciprofloxacin, gentamicin, cefotaxime, cefoperazone, lomefloxacin, ceftriaxone + tazobactam and ceftazidime. Frequent resistance was observed for ceftriaxone + tazobactam (85%) followed by up to 50 % to ceftazidime, 25-35% to cefadroxil and netilmicin, up to 20% for ceftriaxone and cefoperazone, up to 6% for sparfloxacin and lomefloxacin while all isolates show highest sensitivity to ciprofloxacin and amikacin. Isolates from hand pump water samples found to be more resistant followed by isolates from river water. Majority isolates are belonging to family Enterobacteriaceae and from *Pseudomonas* spp. As anthropogenic activities are the main cause of development of resistance among bacteria, present research work may be beneficial by raising awareness among people regarding misuse of antibiotics.

KEYWORDS: Antibiotic resistance, disc diffusion method, gram negative bacteria, Nasik city, groundwater

I. INTRODUCTION:

There is a significant increase in the infections caused by multidrug resistant gram-negative organisms in the past years. At the same time very few new antibiotics have been discovered. Therefore, it is necessary to study the pattern of antibiotic resistance among gram negative isolates present in the aquatic environment which are potential pathogens for numerous gastrointestinal tract infections.

Development of antibiotic resistance in bacteria: Anthropogenic activities might be causing evolution of antibiotic resistance among microbial population in the environment. Antibiotics received by sewage treatment plants may be the causative factor in spreading antibiotic resistance among bacteria in the aquatic environment (Reddy, P. etal., 2020). Bacteria with intrinsic resistance to antibiotics are found in nature. Such organisms may acquire additional resistance genes from bacteria introduced into soil or water, and the resident bacteria may be the reservoir or source of widespread resistant organisms found in many environments (Ash R Jet al., 2002). In the environment antibiotic resistant bacteria accumulate, arising either by accidental mutation, by mutation in humans or animals under antibiotic treatment selection or are naturally occurring environmental bacteria who, under selective pressures (an evolutionary response resulting from natural selection) have developed resistance for their own protection against competitors (Seiler C and Berendonk T U, 2012). Antimicrobial resistance also is getting passed on to several other cohabitating organisms by horizontal gene transfer and leading to emergence of newer resistant strains (Mudaliar, N. et al, 2019). Increase in fatality rate due to infections by antibiotic resistant organisms has been estimated to account for 7 lakh deaths a year and prediction of 10 lakh deaths per year by 2050, if increase in antimicrobial resistance (O'Neill, J., 2016). Outer membrane of Gram-negative organisms is one of the important barriers which provides protection against a number of antibiotics (Heijden, J. et al., 2016). Role of aquatic environment is important in the spread of antibiotic resistant bacteria including those encoding extended-spectrum beta-lactamases (ESBL) and carbapenemases. Presence of amoxicillin resistant, ciprofloxacin, rifampicin resistant heterotrophic bacteria in treated and untreated water, observed in research in the USA (Xi, C. et al., 2016)

Settings: Nasik is a city in Maharashtra, situated at a short distance from Mumbai, an important financial capital of India. It has a population of about 19-20 lakhs, thus, a high-water demand. The city is traditionally dependent on Godavari river and three dams near the city (Sawardekar, S. and Patil, S.A., 2018). In Nasik city, contamination of groundwater by leachate is already reported in previous studies (Wagh, P. and More, S., 2019). In this study, water samples from various sources of water such as open wells, bore wells, hand pumps, water from river Godavari as well as waldevi and treated water by municipal corporation of Nasik were collected. These samples were screened for presence of gram-negative bacteria. The antibiotic resistance

pattern of the isolates was then determined. A total of 14 isolates were identified using conventional biochemical methods. Antibiotic susceptibility testing of all the isolates was performed using disc diffusion method. Interpretation of the antibacterial susceptibility tests was based on the clinical and laboratory standards institute guidelines.

Antibiotic disc diffusion sensitivity tests: These tests are preferred for the work as they give a very approximate measure of the degree of sensitivity to various antibiotics.

Heatmap: A data visualization technique, cluster heatmap is used for analysis showing the response of different gram-negative bacteria under study, against the range of antibiotics. It is a technique that shows the magnitude of a phenomenon as colour in two dimensions. In a cluster heat map, magnitudes are laid out into a matrix of fixed cell size whose rows and columns are discrete phenomena and categories, and the sorting of rows and columns is intentional and somewhat arbitrary, with the goal of suggesting clusters or portraying them as discovered via statistical analysis. The size of the cell is arbitrary but large enough to be clearly visible (Wilkinson et al., 2009).

Present work will elucidate spread of antibiotic resistant gram-negative bacteria in water from different sources. Screening of antibiotic resistant gram-negative isolates from water may lead to tackle the threat posed by them, developing prophylactic measures for spread and emergence of new antibiotic resistances among them.

II. MATERIALS AND METHODS

Sample collection: Samples were collected from different divisions of Nasik city.

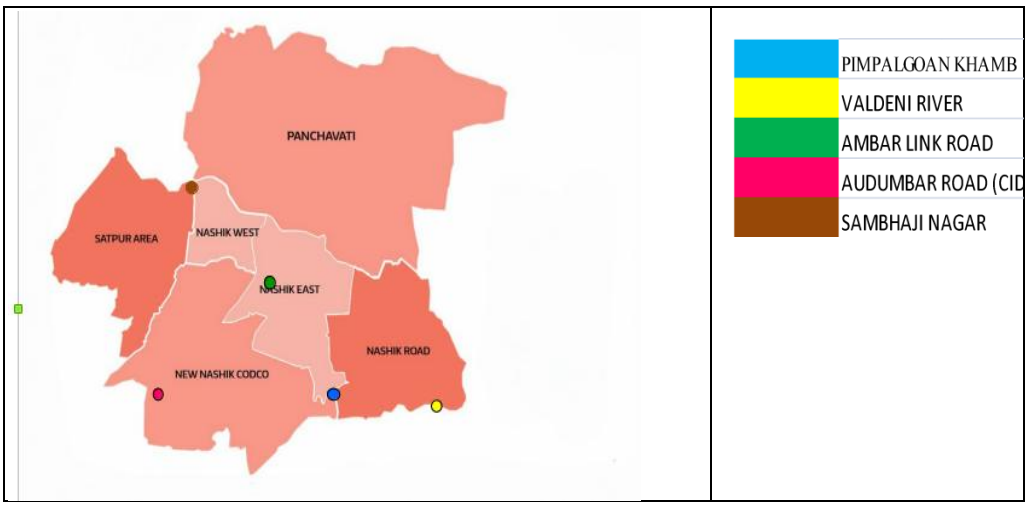


Fig.1 Nasik City map showing the sample locations

Total 53 samples were collected which includes 13 samples from tap water supply of Nasik municipal corporation, one from Godavari and Waldevi river each, 17 bore well samples, 10 from hand pumps and 11 samples from open wells. The samples were collected in sterile bottles and kept in an icebox containing ice bags during transportation and then kept under refrigeration prior to isolation of bacteria.

Isolation of organisms from water samples: After sample collection, 0.1 ml of each water sample was spread on a sterile nutrient agar and incubated at 37˚C for 24 to 48 hours. After incubation, colony characteristics were noted and colonies with different morphology were streaked on a sterile nutrient agar slant and incubated for 24 to 48 hours. These slants were labelled appropriately. Altogether 65 isolates were preserved for further testing.

Gram Staining: Gram staining of the isolated pure cultures was done with standard protocol. 32 Isolates of Gram-negative rod-shaped organisms were selected for further testing from among 65 isolates.

Screening of Antibiotic Resistant organisms by disc diffusion method: Screening of antibiotic resistant organisms by disc diffusion method using Muller Hinton Agar (M.H) (Bauer A.W. et al.,1996) as per guidelines by clinical laboratory standard institute (CLSI, 2015) which is carried out as follows:

a) Suspension of the isolate is prepared to 0.5 McFarland standards, and then spread evenly onto an M.H. agar in petri dish using standard protocol for spread plate method.
b) As a quality control stock culture of E. coli (ATCC 25922) is used.
c) Multi Discs from Dynamicro labs, impregnated with various antibiotics used for gram negative bacteria are placed onto the surface of the seeded M.H agar plates.
d) Plates were incubated at 35^0C for 18-24 hours.
e) Zones of growth of inhibition around each of the antibiotic discs were measured.
A clear circular zone of no growth in the immediate vicinity of a disc indicates susceptibility to that antimicrobial. By using reference table number 1, based on the size of the zone, results were compared and recorded as the isolate is either susceptible (S), intermediately susceptible (I), or resistant (R) to that antibiotic.

Table 1 Reference chart of inhibition zones of gram-negative bacteria by disc diffusion method by Dynamicro labs.

Antibiotics	**Concentration**	**Resistant**	**Intermediate**	**Sensitive**
Amikacin AN	30 mcg	≤14	15 – 16	≥17
Netilmicin NET	10 mcg	≤12	13 – 14	≥15
Cefadroxil CD	30 mcg	≤14	15 – 17	≥18
Sparfloxacin SF	5 mcg	≤15	16 – 20	≥21
Ceftriaxone CTX	30 mcg	≤13	14 – 20	≥21
Ciprofloxacin CIP	5 mcg	≤15	16 – 20	≥21
Gentamicin G	10 mcg	≤12	13 – 14	≥15
Cefotaxime CF	30 mcg	≤14	15 – 22	≥23
Cefoperazone CFP	75 mcg	≤15	16 – 20	≥21
Lomefloxacin LM	10 mcg	≤18	19 – 21	≥22
Cefoperazone Tazobactam CFS	30/10 mcg	≤28	29 – 35	≥36
Ceftazidime CPZ	30 mcg	≤14	15 – 17	≥18

Identification of Antibiotic resistant gram-negative rods: Total 14 isolates of gram-negative rods showing resistance to at least one or more than one antibiotic under study is selected for further identification.
Following tests for identification of gram-negative rods were carried out: Motility, oxidase test, glucose fermentation test, lactose fermentation test, urease test, H_2S production, growth at 41°C, gelatin liquefaction as well as tests for identification of members belonging to family Enterobacteriaceae as per Bergey's manual of Systematic bacteriology.

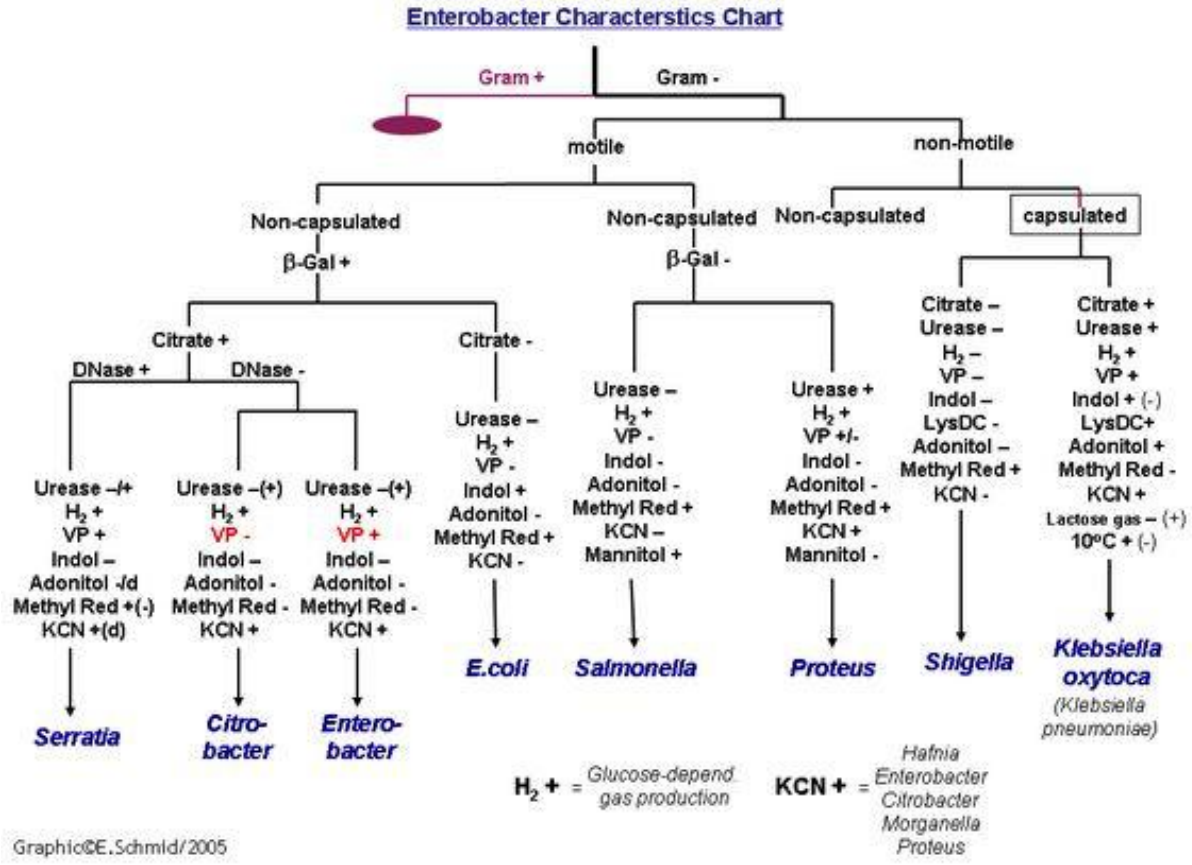


Fig.2 Chart showing tests used for identification of members belonging to family Enterobacteriaceae.

III. RESULTS

Isolation of organisms from water samples and gram staining: Total 65 isolates were screened from 53 sources of water samples. Out of which 32 isolates were identified as gram-negative rods. After antibiotic susceptibility tests,14 isolates were screened for resistance to one or more than one antibiotic.

Table 2 Gram negative isolates from different locations and sources of water samples in Nasik

Sr. No	Sample No.	Source of water Sample	Sample Location
1	**16C**	Bore well	Sambhaji Nagar
2	**2A**	Open well	Pimpalgaon khamb
3	**2B**	Open well	Pimpalgaon khamb
4	**2C**	Open well	Pimpalgaon khamb
5	**46B**	Waldevi river water	Nasik road
6	**5C**	Bore well	Ambad
7	**45A**	Tap water (Treated)	Nasik road
8	**10C**	Bore well	Cidco
9	**4C**	Hand pump	Ambad
10	**23A**	Tap water (Treated)	Kapaleshwar mandir
11	**25A**	Hand pump	Panchwati

12	**33A**	Bore well	Makhmalabad
13	**35B**	Open well	Tapovan
14	**51A**	Hand pump	Krishna nagar (Panchwati)

Sensitivity of Gram-negative rods to various antibiotics: All gram-negative rods are tested for antibiotic susceptibility. All isolates which have shown sensitivity (no growth) to all antibiotics under the testing were discarded and only 14 isolates were selected, showing resistance to one or more than one antibiotic.

Fig. 3 An example of antibiotic susceptibility test

Based on the observation of the diameter of zone of inhibition or no inhibition to different antibiotics, they were classified as resistant, intermediate or sensitive to particular antibiotics as per guidelines as in table 3.

Table 3 Susceptibility of Gram-Negative bacteria to various antibiotics

Sr no	Sample	AN	Net	CD	SF	CTX	CIP	G	CF	CFP	LM	CFS	CFZ
1	**2A**	S (20)	S (17)	R	S(20)	S (23)	S(28)	S	S(23)	S	S	R(20)	S
2	**2B**	I (18)	S (18)	R(10)	S	R (10)	S	S	I(20)	S	S	R(24)	S
3	**2C**	S (22)	R (13)	S (20)	S(24)	S (24)	S(26)	S(16)	S(29)	S(22)	S (35)	R(25)	R(10)
4	**4C**	S (21)	S (19)	R(10)	S(27)	R	S(25)	I(13)	I(15)	R(10)	S(28)	R (9)	R(10)
5	**5C**	S	S (24)	S (30)	I (19)	I (17)	I(16)	R(10)	S	S	I(20)	S	R(10)
6	**10C**	S (25)	S (21)	R (6)	R(15)	I (20)	S	S	R (4)	S	S	R (8)	S
7	**16C**	S (20)	S (18)	S (19)	S(23)	S (23)	S(28)	S(20)	I(15)	I (20)	R (10)	R (23)	R (12)
8	**23A**	S (28)	S (19)	S	S(22)	S	S	S	S	S	S	R (22)	S
9	**25A**	S (20)	R (10)	R	I (20)	I (18)	S(21)	R(10)	R(10)	R	I (20)	R (12)	R (9)
10	**33A**	S (21)	S (15)	S (27)	S(32)	S (23)	I(17)	S	S	S	S	R (24)	S
11	**35B**	S (23)	R	S (20)	I (20)	S (22)	S	S(20)	R	I (20)	S	R	S
12	**45A**	S (20)	R (11)	S (13)	I (18)	R	S (25)	S (16)	R (4)	I (20)	S (30)	R	R(20)
13	**46B**	S (20)	R (10)	S (22)	I (18)	R	S (21)	S (16)	R	R	I (21)	R	R
14	**51A**	S	S	S	S	S	S	S	S	S	S	S	S

*Note: AN= Amikasin, NET= Netilmicin, CD= Cefadroxil, SF= Sparfloxacin, CTX = Ceftriaxone, CIP= Ciprofloxacin, G= Gentamicin, CF= Cefotaxime, CFP= Cefoperazone, LM= Lomefloxacin, CFS= Ceftriaxone + Tazobactam, CPZ= Ceftazidime.

Identification of Antibiotic resistant gram-negative rods: Organisms identified are *E. coli, Salmonella* spp., *Serratia* spp., *Proteus* spp. and *Klebsiella* spp. belongs to family Enterobacteriaceae and while others are from genus *Pseudomonas.*
Based on the biochemical tests including oxidase test, oxidative fermentation of glucose and pigment production, *Pseudomonas* is identified. Tests for members belonging to members of family Enterobacteriaceae are carried out. Lactose fermenters such as *E. coli* found nearly in all samples except two treated water samples. While *Salmonella* spp, *Serratia* spp, *Proteus* spp and *Klebsiella* spp were identified mainly in open well and river water samples.

Drug resistance pattern of isolated organisms in various water samples: Isolates of gram-negative organisms from hand pump water samples found to be more resistant to various antibiotics followed by isolates from river water, borewell and open well. Organisms present in treated water in the distribution system at Panchwati (23A) by Municipal Corporation are comparatively sensitive to majority antibiotics but isolates from treated water samples of the Nashik road area (46B) have shown resistance to more than 50% antibiotics under study

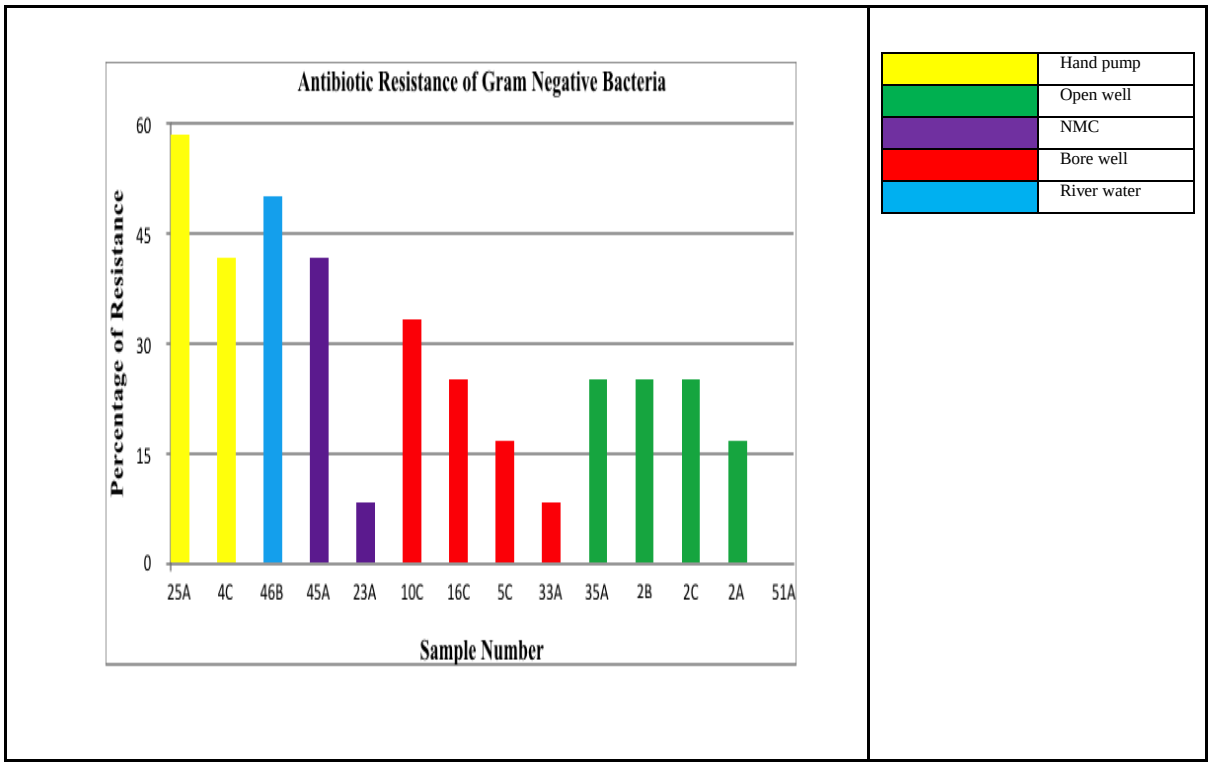
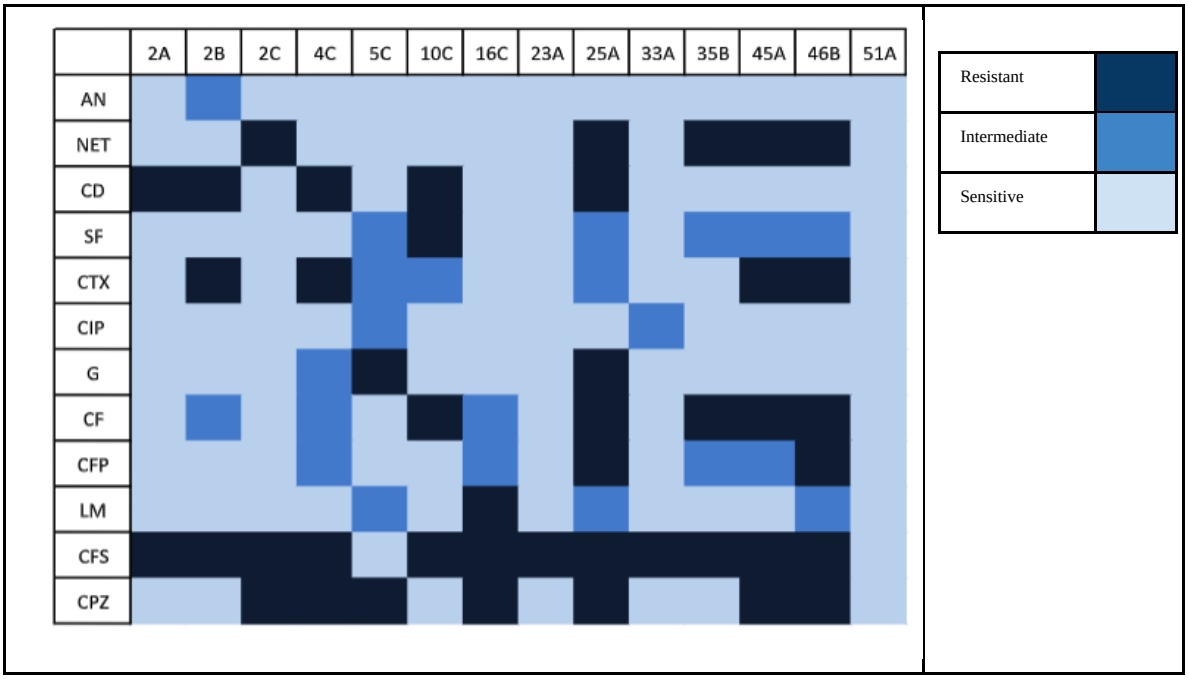


Fig. 4 Drug resistance patterns of isolated organisms in various water samples.

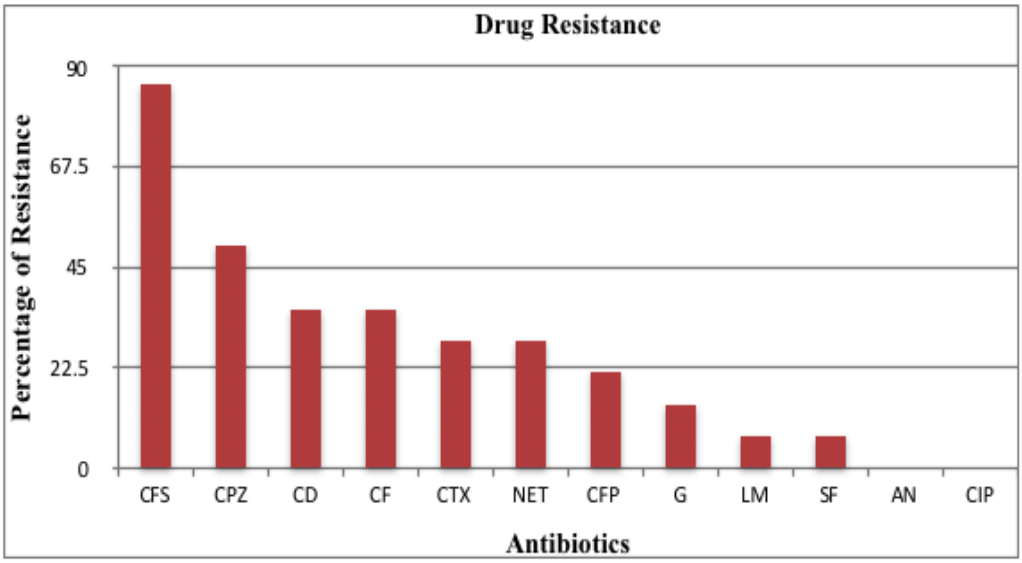
Cluster Heatmap - A data visualization technique is used for analysis showing the response of different gram-negative bacteria under study, against the range of antibiotics.



Note: AN= Amikacin, NET= Netilmicin, CD= Cefadroxil, SF= Sparfloxacin, CTX = Ceftriaxone, CIP= Ciprofloxacin, G= Gentamicin, CF= Cefotaxime, CFP= Cefoperazone, LM= Lomefloxacin, CFS= Ceftriaxone + Tazobactam, CPZ= Ceftazidime.

Fig. 5 Antibiotic Screening Heatmap represents the response of differential gram-negative bacteria under study, against the range of antibiotics.

3.6 Percentage of resistance to various antibiotics by different isolates: More than 85% resistance observed for ceftriaxone + tazobactam followed by up to 50 % to ceftazidime, 25-35% to cefadroxil and netilmicin, up to 20% for ceftriaxone and cefoperazone, up to 6% for sparfloxacin and lomefloxacin while all isolates show highest sensitivity to ciprofloxacin and amikacin.



Note: AN= Amikacin, NET= Netilmicin, CD= Cefadroxil, SF= Sparfloxacin, CTX = Ceftriaxone, CIP= Ciprofloxacin, G= Gentamicin, CF= Cefotaxime, CFP= Cefoperazone, LM= Lomefloxacin, CFS= Ceftriaxone + Tazobactam, CPZ= Ceftazidime.

Fig. 6 Percentage of resistance to various antibiotics by different isolates

IV. DISCUSSION:

Antibiotic resistance in Gram negative isolates from various sources of water such as open wells, bore wells, hand pumps, water from river Godavari as well as from river Waldevi and treated water by Municipal Corporation of Nasik was checked. Based on the biochemical test including oxidase test, oxidative fermentation of glucose and pigment production, highly resistant *Pseudomonas* is identified in treated water samples as well as in open well samples. Tests for members belonging to members of family Enterobacteriaceae are carried out. Lactose fermenters such as *E. coli* found almost in all samples. While *Salmonella* spp, *Serratia* spp, *Proteus* spp *and Klebsiella* spp were identified mainly in open well and river water samples. Resistance of these isolates was tested against Amikacin, Netilmicin, Cefadroxil, Sparfloxacin, Ceftriaxone, Ciprofloxacin, Gentamicin, Cefotaxime, Cefoperazone, Lomefloxacin, Ceftriaxone + Tazobactam and Ceftazidime. Isolates from treated water samples from different locations showed varied results ranging from presence of most sensitive to highly resistant isolates to majority of antibiotics under study. Frequent resistance was observed for ceftriaxone + tazobactam (85%) followed by up to 50 % to ceftazidime, 25-35% to cefadroxil and netilmicin, up to 20% for ceftriaxone and cefoperazone, up to 6% for sparfloxacin and lomefloxacin while all isolates show highest sensitivity to ciprofloxacin and amikacin. Isolates of gram-negative organisms from hand pump water samples found to be more resistant to various antibiotics followed by isolates from river water, borewell as well as open well. As there are chances of development of antibiotic resistance among bacteria due to anthropogenic activities, present research work may be beneficial by raising awareness among people regarding misuse of antibiotics.

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