Isolation and characterization of multidrug resistance bacteria from hospital sewage samples, Maharashtra, India

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Multiple antibiotic resistance is a major cause of clinical infections worldwide. This study determined the pattern of multidrug bacterial resistance in hospital sewage samples from the Marathwada region of India. Forty-eight isolates of bacteria were obtained from 6 locations of Aurangabad. An antibiotic sensitivity test was carried out using the disc diffusion method. Among all the antibiotics tested, the highest level of resistance was observed in the beta lactam class (85%), followed by Tetracycline (58%), Cephalosporin (58%), quinolones (52%) and gentamycin (45%). Escherichia coli and Klebsiella pneumoniae are the most prevalent bacteria, showing antibiotic resistance to all tested antibiotics with a MAR index of 1. It is concluded that hospital sewage water could be a reservoir of antibiotic resistant bacteria, which may further contaminate drinking water bodies, potentially presenting a public health risk to the general populace.

Key words: Antibiotics, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas species, Staphylococcus aureus, Streptococcus pyogenes.

INTRODUCTION

Antibiotics are compounds produced by micro-organisms and that are capable of inhibiting bacterial growth. Nowadays, the term antibiotic is broadly used for all those compounds that can be used against the bacterial infection. However, antibiotic resistance is increasing, and it is projected that by 2050, antibiotic resistant organisms will contribute to over 10 million deaths annually worldwide (De Kraker et al., 2016). This is mainly because of the slow pace of developing new antibiotics (De Kraker et al., 2011). The problem of antibiotic resistance has attracted the attention of World Health Organization (WHO) and several other stakeholders. The WHO announced in 2011 that antibiotic resistance is an urgent priority of research area (WHO, 2011) and several countries, including India (Government of India, 2017) accordingly framed their national health action plans for managing drug resistant bacteria (Smith et al., 2016).

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The problem of antibiotic resistance occurs due to the uncontrolled and excessive use of antibiotics by hospital and home care patients (Sidhu et al., 2016). This leads to the spread of resistant genes in water systems (Mukhopadhyay et al., 2012). For instance, Sidhu et al. (2016) analyzed and demonstrated the presence of E. coli in the drinking water from different schools in Northern India. Many multiple drug resistant bacteria like Pseudomonas aeruginosa, coliforms and Enterococcus species have been isolated from household water samples in Karnataka, India (Mukhopadhyay et al., 2012). Mulamattathil et al. (2014) documented the multiple antibiotic resistance profile in bacteria from sewage water samples (Mafikeng, South Africa).

In rural communities of developing countries like the India, untreated water from rivers, dams, and streams is directly used for drinking (Biyela et al., 2004). These water resources could possibly be contaminated with microbes from sewage water through rainfall runoff and other sources (Obi et al., 2002). Most of the areas are semi-arid with very low rainfall and a high evaporation rate, so water should be reused (Mckenzie et al., 2003). For obtaining good quality water, there is need for proper waste water treatment procedures. Biological waste water treatment processes may selectively increase the antibiotic resistance in water, which is a major health concern issue of reuse of water (Mulamattathil et al., 2000). Release of hospital sewage directly into the water ecosystem further contributes to the antibiotic resistant bacteria. Therefore, there is pressing need to focus on this aspect locally and globally. Hence, the current study has been undertaken to evaluate the presence of potential pathogenic bacteria in hospital sewage samples from Marathwada region of Maharashtra, India, as well as to determine the antibiotic resistance profiles of the isolated bacteria.

**MATERIALS AND METHODS**

**Collection of sewage samples**

One hundred milliliters of hospital sewage samples were collected from six different sites in Aurangabad, Maharashtra region, in sterile screw cap tubes and brought to the laboratory in a cooled condition. Serial dilutions \(10^{-5}\) of each samples were prepared, and 1 ml of each sample were spread on the nutrient agar medium. Further plates were incubated at 37°C for 24 h and different colonies were obtained. Pure bacteria were procured by streak plate method utilizing Mueller-Hinton agar (Hi-Media, India) or Nutrient Agar [Hi-Media, India]. The isolated bacteria were characterized on the basis of colony morphology study. All the pure colonies were subjected to Grams staining and distinguished bacteria in two groups, viz: Gram positive and Gram negative. These pure cultures were further subjected to biochemical tests according to Nandi and Mandal (2016), Holt (1984) and Forbes et al. (2007). Morphologically different colonies of bacteria were maintained on Nutrient Agar or Cystine tryptone agar (Hi-Media, India) stabs, at 4°C for further studies. Of the 30 isolates obtained, eight were further used for antibiotic sensitivity testing.

**Collection of samples**

A total of six hospital sewage samples were collected during the study period, from different sampling sites of Marathwada region, Maharashtra, India and labelled as S1, S2, S3, S4, S5 and S6 (Figure1) in sterile plastic bottles. After dilution and inoculation of each sample on nutrient agar plates, many colonies were obtained. Three Gram positive and 5 Gram negative bacteria were isolated from each of the 6 hospital sewage samples (Table 1). Further, their sensitivity to different classes of antibiotics namely beta lactam group, cephalosporin group, tetracycline, quinolones and aminoglycosides was analyzed. It was observed that all of the 6 samples carry antibiotic resistant bacteria for different antibiotics (Tables 2 and 3).

**Antibiotic sensitivity test**

Isolated bacteria were further subjected to the antibiotic sensitivity tests by disc diffusion method given by Kirby-Bauer. It was performed through following steps.

**Preparation of the test organisms for sensitivity test**

Three colonies from each sample were taken and subcultured in sterile nutrient broth aerobically at 37°C for 24 h. Broth cultures of the isolates were centrifuged at 3000 rpm for 10 min. The sediments were diluted with sterile phosphate buffer saline (PBS) and adjusted to the \(10^8\) CFU/ml using McFarland matching standard (mixture of 0.6 ml of 1% BaCl\(_2\), H\(_2\)O and 99.4 ml of 1% concentrated H\(_2\)SO\(_4\)) using spectrophotometer at 540 nm.

**Antibiotic sensitivity testing**

100 µl of each aliquot was spread on nutrient agar or Mueller Hinton agar medium. By using sterile forceps, antibiotic sensitivity discs were applied on the surface of the medium. The set-up was incubated aerobically at 37°C for 24 h. The inhibition zone diameters were measured using meter rule after 24 h, incubated and recorded.

The results, in terms of ZDI (zone diameter of the inhibition) values of the test antibiotics, were interpreted following the guidelines of the Clinical and Laboratory Standards (CLSI) Institute (2011). As per the CLSI guidelines, bacteria were classified into three groups viz. resistant, intermediate and sensitive to a particular antibiotic.

**Determination of antibiotic resistance pattern**

Bacteria showing antibiotic resistance to three or more antibiotics were considered as the multiple antibiotic resistant bacteria [MAR] and MAR index value for each sample using the following formula.

\[
\text{MAR index} = \frac{M}{n}
\]

Where, \(M\) is number of antibiotics to which the isolate showed resistance and \(n\) is the number of total antibiotics used in the test (Krumperman, 1983) that was calculated. Generally, MAR index value higher than 0.2 indicates the isolate is multiple antibiotic resistant (Adesiyoe and Okoh, 2017).

To know the prevalence of antibiotic resistance as per the sample collection sites, ARI [Antibacterial resistance index] was determined using the formula described by Krumperman (1983), which is mathematically expressed as
Figure 1. Hospital sewage sample location map of Aurangabad Urban Area, Maharashtra India.

ARI = y/nx

Where, y is actual number of resistance microbes in the sample, n is the number of isolates tested and x is the total antibiotics tested in sensitivity test. Generally, ARI index is directly proportional to the prevalence of antibiotic resistance as per sample collection sites.

RESULTS AND DISCUSSION

Biochemical analysis

Further, by using the biochemical tests and referring to
the Bergey’s Manual of Systematic Bacteriology, all isolates were identified (Table 1). In biochemical tests carried out for 48 isolates, we found a total of 6 different types of bacteria out of which 3 were Gram negative and 3 were Gram positive namely, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas species*. Additionally, we were able to identify 4 strains of *Escherichia coli* and 1 strain of *Klebsiella pneumoniae* through biochemical tests. Table 1 provides a summary of the biochemical tests performed on the 48 isolates, including the results for glucose, sucrose, lactose, maltose, and mannitol fermentation, as well as the bacterial identity of each strain.
Table 2. Results of staining, biochemical tests and identity of isolates selected for further study.

| Site | Sample ID | GS | Biochemical test and results | Sugar fermentation test results | Bacterial identity |
|------|-----------|----|-----------------------------|-----------------------------|-------------------|
|      |           |    | CO  | CI | IN | CT | OD | MR | VP | DN | Glucose | Sucrose | Lactose | Maltose | Mannitol |
| S1   | S16       | +  | +   | -  | -  | +  | +  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S17       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Enterococcus faecalis |
|      | S18       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Enterococcus faecalis |
| S2   | S26       | +  | +   | -  | -  | +  | +  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S27       | +  | -   | ND | -  | -  | -  | +  | -  | +  | +       | +       | +       | -       | -       | Streptococcus pyogenes |
|      | S28       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
| S3   | S36       | +  | -   | ND | -  | -  | -  | +  | -  | +  | +       | +       | +       | -       | -       | Streptococcus pyogenes |
|      | S37       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S38       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Enterococcus faecalis |
| S4   | S46       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S47       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S48       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Enterococcus faecalis |
| S5   | S56       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S57       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S58       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Staphylococcus aureus |
| S6   | S66       | +  | +   | -  | -  | -  | -  | +  | +  | +  | +       | +       | +       | +       | +       | Staphylococcus aureus |
|      | S67       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Enterococcus faecalis |
|      | S68       | +  | +   | -  | -  | -  | -  | ND | +  | ND | +       | +       | +       | +       | +       | Enterococcus faecalis |

Cl: Citrate, IN: Indole, CT: Catalase, OD: Oxidase; MR: Methyl red; VP: Voges–Proskauer; - Negative; +: Positive; CO: coagulase, DN: Dnase, ND: No data; V= variable.

Escherichia coli, Pseudomonas species, Staphylococcus aureus, Enterococcus faecalis and Streptococcus pyogenes. Among all bacteria obtained, Gram negative *E. coli* and Gram positive bacteria *S. aureus* were the most prevalent bacteria found at all sites.

Antibiotic resistance pattern

Based on the zone of inhibition (mm) obtained, antibiotic sensitivity of isolates as per the guidelines given by Clinical and Laboratory Standards Institute (CLSI, 2009) mentioned in Table 3 (Gram negative bacteria) and Table 4 (Gram positive bacteria), the isolates are divided into 2 types, viz: resistant and sensitive. Isolates with intermediate phenotypes obtained in AST were considered as antibiotic resistant bacteria. Among all the antibiotics tested, highest level of resistance was observed for beta lactam class [85%], then for Tetracycline [58%], Cephalosporin [58%], quinolones [52%] and gentamycin [45%] (Figure 2). *E. coli* was the most prevalent bacteria showing antibiotic resistance to all tested antibiotics.

Multiple antibiotic resistance pattern

Further MAR index values were calculated for all the isolates. MAR index values indicate the number of antibiotics to which the isolate is showing resistance. When the MAR index value is >0.2, the bacteria is considered as multiple antibiotic resistant bacteria. From the MAR index values we can predict that around 41 out of 48 isolates show multiple antibiotic resistance and only four are not MAR (Tables 5 and 6). Some of the isolates S11, S22, S34, S35, S38, S54, S55, S56 and S64 were found to be resistant to all the
Table 3. Results of antibiotic sensitivity test of Gram negative isolates.

| Site | Sample ID | Zone of Inhibition (mm) | Beta lactams [Amp] | Tetracycline [Tet] | Cephalosporin | Quinolones [Cip] | Aminoglycosides |
|------|-----------|-------------------------|--------------------|-------------------|---------------|-----------------|-----------------|
|      |           |                         |                    |                   |               |                 |                 |
| S1   | S11       | 10                      | 14                 | 12                | 11            | 11              |                 |
|      | S12       | 20                      | 10                 | 23                | 15            | 09              |                 |
|      | S13       | 19                      | 22                 | 13                | 14            | 10              |                 |
|      | S14       | 23                      | 12                 | 10                | 24            | 20              |                 |
|      | S15       | 29                      | 14                 | 11                | 26            | 28              |                 |
| S2   | S21       | 18                      | 22                 | 20                | 17            | 24              |                 |
|      | S22       | 14                      | 11                 | 08                | 10            | 12              |                 |
|      | S23       | 29                      | 18                 | 00                | 28            | 24              |                 |
|      | S24       | 22                      | 10                 | 10                | 26            | 18              |                 |
|      | S25       | 20                      | 25                 | 18                | 14            | 25              |                 |
| S3   | S31       | 06                      | 22                 | 23                | 27            | 26              |                 |
|      | S32       | 09                      | 24                 | 13                | 15            | 11              |                 |
|      | S33       | 29                      | 22                 | 11                | 26            | 28              |                 |
|      | S34       | 12                      | 14                 | 10                | 13            | 10              |                 |
|      | S35       | 19                      | 12                 | 08                | 10            | 09              |                 |
| S4   | S41       | 20                      | 11                 | 10                | 30            | 25              |                 |
|      | S42       | 30                      | 18                 | 17                | 18            | 14              |                 |
|      | S43       | 20                      | 18                 | 09                | 13            | 08              |                 |
|      | S44       | 21                      | 11                 | 19                | 27            | 22              |                 |
|      | S45       | 22                      | 12                 | 13                | 22            | 19              |                 |
| S5   | S51       | 12                      | 11                 | 26                | 27            | 18              |                 |
|      | S52       | 14                      | 09                 | 11                | 24            | 06              |                 |
|      | S53       | 24                      | 12                 | 24                | 24            | 22              |                 |
|      | S54       | 19                      | 14                 | 13                | 14            | 11              |                 |
|      | S55       | 10                      | 13                 | 10                | 11            | 10              |                 |
| S6   | S61       | 16                      | 09                 | 08                | 08            | 20              |                 |
|      | S62       | 12                      | 05                 | 07                | 09            | 22              |                 |
|      | S63       | 30                      | 11                 | 10                | 11            | 23              |                 |
|      | S64       | 10                      | 14                 | 13                | 10            | 10              |                 |
|      | S65       | 08                      | 25                 | 19                | 15            | 21              |                 |

antibiotics tested in the present study while the sample S48 and S58 showed sensitivity to all the antibiotics used. Mainly, *K. pneumoniae* bacteria showed resistance to most of the tested antibiotics.

**ARI index**

ARI index indicates the prevalence of antibiotic resistant and sensitive bacteria as per the locations. In the present study, it was observed from the ARI (Table 7) that beta lactam resistant bacteria are more prevalent in nature as compared to other antibiotics at all the sample collection sites. The prevalence of antibiotic resistant bacteria is shown in Graph 1. Beta lactam antibiotic resistant bacteria are the more prevalently occurring bacteria in nature. Bacteria obtained from the site 1 appear more resistant to all the antibiotics as compared to other sites.

**Conclusion**

The present study on the hospital sewage water collected from six different locations of Aurangabad, Maharashtra, India has shown *E coli* as the most prevalent antibiotic resistant bacteria. We obtained Gram negative bacteria more abundantly in hospital sewage samples. Isolates number 48 and 41 showed >0.2 MAR indices and hence, it is considered to have the potential to cause human infections. Most of the isolates showed multiple antibiotic resistance. Among tested antibiotics, beta lactam group antibiotic resistant bacteria were found to be most prevalent and comparatively less number of isolates was resistant to gentamycin. Therefore, there is need to prepare effective guidelines for judicious use of antibiotics and release of hospital sewage directly into the water bodies, in order to avoid the spread of bacterial multiple antibiotic resistances.
### Table 4. Results of antibiotic sensitivity test of Gram positive isolates.

| Site | Sample ID | Zone of Inhibition (mm)   |
|------|-----------|---------------------------|
|      |           | Beta Lactams [Amp] Cephalosporin [Cx] Quinolones [Cip] Aminoglycosides [Gen] Tetracycline [Tet] |
|      |           | 20 16 14 14 11           |
|      | S1        | 18 20 22 10 20           |
|      | S17       | 19 22 12 09 10           |
|      | S2        | 18 23 18 10 15           |
|      | S26       | 15 20 22 19 13           |
|      | S27       | 06 06 32 20 06           |
|      | S28       | 08 19 24 24 09           |
|      | S3        | 12 11 13 10 25           |
|      | S36       | 13 10 10 24 09           |
|      | S37       | 07 08 30 25 11           |
|      | S38       | 20 30 30 11 31           |
|      | S4        | 32 25 22 23 25           |
|      | S46       | 13 11 14 11 09           |
|      | S47       | 12 11 30 22 20           |
|      | S48       | 29 22 30 30 21           |
|      | S5        | 12 18 '13 16 22           |
|      | S56       | 06 20 21 23 22           |
|      | S57       | 06 20 21 23 22           |
|      | S58       | 06 20 21 23 22           |
|      | S6        | 06 20 21 23 22           |
|      | S66       | 06 20 21 23 22           |
|      | S67       | 06 20 21 23 22           |
|      | S68       | 06 20 21 23 22           |

![Figure 2. Prevalence of antibiotic resistance.](image-url)
### Table 5. MAR index values of Gram negative isolates.

| Site | Sample ID | Beta lactams [Amp] | Tetracycline [Tet] | Cephalosporin [Cx] | Quinolones [Cip] | Aminoglycoside [Gen] | MAR |
|------|-----------|--------------------|-------------------|-------------------|-----------------|-----------------------|-----|
| S1   | S11       | R                  | R                 | R                 | R               | R                     | 1   |
|      | S12       | R                  | R                 | S                 | R               | R                     | 0.8 |
|      | S13       | R                  | S                 | R                 | R               | R                     | 0.8 |
|      | S14       | R                  | R                 | R                 | S               | S                     | 0.6 |
|      | S15       | S                  | R                 | R                 | S               | S                     | 0.4 |
| S2   | S21       | R                  | S                 | S                 | R               | S                     | 0.4 |
|      | S22       | R                  | R                 | R                 | R               | R                     | 1   |
|      | S23       | S                  | S                 | R                 | S               | S                     | 0.2 |
|      | S24       | R                  | R                 | R                 | S               | R                     | 0.8 |
|      | S25       | R                  | S                 | S                 | R               | S                     | 0.4 |
| S3   | S31       | R                  | S                 | S                 | S               | S                     | 0.2 |
|      | S32       | R                  | S                 | R                 | R               | R                     | 0.8 |
|      | S33       | S                  | S                 | R                 | S               | S                     | 0.2 |
|      | S34       | R                  | R                 | R                 | R               | R                     | 1   |
|      | S35       | R                  | R                 | R                 | R               | R                     | 1   |
| S4   | S41       | R                  | R                 | R                 | S               | S                     | 0.6 |
|      | S42       | S                  | S                 | S                 | S               | S                     | 0.2 |
|      | S43       | R                  | S                 | R                 | R               | R                     | 0.8 |
|      | S44       | R                  | R                 | S                 | S               | S                     | 0.4 |
|      | S45       | R                  | R                 | R                 | S               | S                     | 0.6 |
| S5   | S51       | R                  | R                 | S                 | S               | S                     | 0.4 |
|      | S52       | R                  | R                 | R                 | S               | R                     | 0.8 |
|      | S53       | R                  | R                 | S                 | S               | S                     | 0.4 |
|      | S54       | R                  | R                 | R                 | R               | R                     | 1   |
|      | S55       | R                  | R                 | R                 | R               | R                     | 1   |
| S6   | S61       | R                  | R                 | R                 | R               | S                     | 0.8 |
|      | S62       | R                  | R                 | R                 | R               | S                     | 0.8 |
|      | S63       | S                  | R                 | R                 | R               | S                     | 0.6 |
|      | S64       | R                  | R                 | R                 | R               | R                     | 1   |
|      | S65       | R                  | S                 | S                 | R               | S                     | 0.4 |

### Table 6. MAR index values of Gram positive isolates.

| Site | Sample ID | Beta Lactams [Amp] | Cephalosporin [Cx] | Quinolones [Cip] | Aminoglycoside [Gen] | Tetracycline [Tet] | MAR |
|------|-----------|--------------------|-------------------|-----------------|----------------------|-------------------|-----|
| S1   | S16       | R                  | I                 | R               | I                   | R                 | 0.6 |
|      | S17       | R                  | S                 | S               | R                   | S                 | 0.4 |
|      | S18       | R                  | S                 | R               | R                   | R                 | 0.8 |
| S2   | S26       | R                  | S                 | I               | R                   | I                 | 0.4 |
|      | S27       | R                  | S                 | S               | S                   | R                 | 0.4 |
|      | S28       | R                  | R                 | S               | S                   | S                 | 0.6 |
| S3   | S36       | R                  | S                 | S               | S                   | R                 | 0.4 |
|      | S37       | R                  | R                 | R               | R                   | S                 | 0.8 |
|      | S38       | R                  | R                 | R               | S                   | R                 | 1    |
| S4   | S46       | R                  | R                 | S               | S                   | R                 | 0.6 |
|      | S47       | R                  | S                 | S               | R                   | S                 | 0.4 |
|      | S48       | S                  | S                 | S               | S                   | S                 | 0    |
| S5   | S56       | R                  | R                 | R               | R                   | R                 | 1    |
Table 6. Contd.

| Site | R | R | S | S | S | 0.4 |
|------|---|---|---|---|---|-----|
| S57  | R | R | S | S | S | 0.4 |
| S58  | S | S | S | S | S | 0 |
| S66  | R | S | R | I | S | 0.4 |
| S67  | R | S | S | S | S | 0.2 |
| S68  | R | S | R | S | S | 0.4 |

Table 7. ARI index.

| Site | Beta Lactams [Amp] | Cephalosporin [Cx] | Quinolones [Cip] | Aminoglycosides [Gen] | Tetracycline [Tet] |
|------|---------------------|---------------------|------------------|------------------------|-------------------|
| S1   | 0.175               | 0.125               | 0.125            | 0.15                   | 0.15              |
| S2   | 0.175               | 0.1                | 0.1              | 0.075                  | 0.125             |
| S3   | 0.175               | 0.15               | 0.125            | 0.1                    | 0.1               |
| S4   | 0.15                | 0.1                | 0.025            | 0.075                  | 0.1               |
| S5   | 0.175               | 0.125              | 0.075            | 0.1                    | 0.15              |
| S6   | 0.175               | 0.1                | 0.175            | 0.05                   | 0.1               |

Graph 1. Prevalence of antibiotic resistance as per site of sample collection.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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