

Original Article

Isolation of Multidrug Resistant Bacteria from Aspirates of Cancer Patients

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Infections due to Gram-negative bacteria are common affairs in cancer patients during aggressive therapy. The present study characterized multi-drug resistant bacteria (MDR) isolated from cancer aspirates collected from patients admitted to the National Cancer Hospital in Dhaka, Bangladesh. A total of 210 aspirate samples were collected from cancer patients. Out of 210 samples *Acinetobacter spp.* led the list of isolates (8.89%, n=45). Of these species, 50% exhibited resistance to Amoxicillin and Nitrofurantoin, each, 25% exhibited resistant to Cefotaxime, Azithromycin, Ciprofloxacin, Clindamycin, and Sulfamethoxazole. A total of 33.33% of the *Bordetella spp.* which accounted 6.67% of the total isolates exhibited resistance to Cefotaxime. All of the *Legionella pneumophila*, comprising 4.4% of the isolated species, were resistant to Cefotaxime, Azithromycin, and Clindamycin. In contrast, 50% were resistant to Cefotaxime, Azithromycin, and Ceftriaxone. Of the *Escherichia coli* (4.4%, n=45) isolated, 50% exhibited resistance to Cefotaxime, Clindamycin, Ceftriaxone, Amoxicillin and Sulfamethoxazole. The only isolate of *Klebsiella sp.* was demonstrated to be an ESBL producer. The isolation of multidrug resistant bacteria from cancer patients is of particular concern in Bangladesh where cancer and drug resistance are both common phenomena but treatment facilities are poor. To our knowledge this is the first report of the isolation of drug resistant bacteria from cancer patients from Dhaka city.

Introduction

Multidrug resistance is a common affair among the people of third world. The earliest mechanisms by which many cancers develop resistance to chemotherapy drugs is a prime factor in the dysfunction of many forms of chemotherapy. It affects patients with a variety of blood cancers and solid tumors, including breast, ovarian, lung, and lower gastrointestinal tract cancers. Most patients with head and neck cancer have dysphagia and are at great risk of having aspiration and subsequent pneumonia. It can cause prolonged hospitalization, treatment delay and/or interruption and mortality in cancer patients. The treatment of these infections often relies on empirical antibiotics based on local microbiology and antibiotic sensitivity patterns¹⁻².

Multidrug resistance, the principal mechanism by which many cancers develop resistance to chemotherapy drugs, is a major factor in the failure of many forms of chemotherapy. Tumors usually consist of mixed populations of malignant cells, some of which are drug-sensitive while others are drug-resistant. Chemotherapy kills drug-sensitive cells, but leaves behind a higher proportion of drug-resistant cells. As the tumor begins to grow again, chemotherapy may fail because the remaining tumor cells are now resistant. Resistance to therapy has been correlated to the presence of at least two molecular “pumps” in tumor-cell membranes that actively expel chemotherapy drugs from the interior. This allows tumor cells to avoid the toxic effects of the drug or molecular processes within the nucleus or the cytoplasm.

The two pumps commonly found to confer chemo resistance in cancer are P-glycoprotein and the so-called multidrug resistance-associated protein (MRP). Because of their function and importance, they are the targets of several anticancer efforts³.

In recent years, the emergence of antimicrobial resistance has become a significant problem worldwide, and cancer patients are among those affected with various species of both Gram positive and Gram negative bacteria. Treatment of infections due to multidrug-resistant (MDR) bacteria represents a clinical challenge, since the therapeutic options are often very limited. As the antibiotics active against MDR bacteria present several disadvantages (limited clinical experience, higher incidence of adverse effects, and less knowledge of the pharmacokinetics of the drug), a thorough acquaintance with the main characteristics of these drugs is mandatory in order to provide safe treatment to cancer patients with MDR bacterial infections⁴⁻⁵. The aims of the present study were to determine multi drug resistance of bacteria isolated from cancer patients undergoing treatment at National Cancer Institute, Mohakhali, Dhaka, Bangladesh.

Materials and methods

Sample collection and processing

A total of 210 blood and aspirate samples were aseptically collected by a laboratory professional from various cancer patients after taking their consent. Samples were inoculated in TSB. Samples were transported to the laboratory in ice box and

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then incubated for 24 to 48 hour at 37°C. The incubated broth was carefully transferred to a separate autoclaved clean Eppendorf tube, leaving behind the blood pellet and centrifuged for 3 min at 10,000 rpm to collect all the cells as growth was seen to be very poor. This centrifugation process was done to concentrate the cell before inoculate in the culture media. Incubated culture samples were stored by adding 95% glycerol at -20°C for further research.

Bacteriological analysis of the clinical samples

After 24 to 48 hours incubation at 37°C, on the basis of visual turbidity centrifuged culture samples 100µl were inoculated in nutrient agar plates by spread plate technique. If the culture appeared to be too turbid then streak plate method was applied. All the plates were incubated for 24 to 48 hours at 37°C to have the isolates. After incubation primary plates were screened to identify different colonies for Gram staining and subculture.

Storage of bacterial isolates

For preservation, 300 µl of fresh bacterial culture grown in TSB at 37°C for 24 to 48h was taken in a sterile Eppendorf tube and 700 µl of sterile glycerol (95%) was added to the broth culture; culture broth and glycerol was mixed by vortexing. The Eppendorf tube was stored at -20C with proper labeling.

Morphological characteristics

Colony morphology is a method that scientists use to describe the characteristics of an individual colony of bacteria growing on agar in a Petri dish. It can be used to identify them. Different types of bacteria produce different colony characteristics. Colony characteristics were observed following growth in Nutrient agar and MacConkey agar.

Gram staining

A small portion of bacterial fresh culture was taken out with a sterile inoculating loop and was placed on previously cleaned oil free slide. A thin smear was prepared and allowed to dry in air. The smear was heat fixed by slightly heating under surface of a slide over a gas burner. Crystal violet, the primary stain, was flooded and left over the smear for one minute and readily washed with slow moving tap water. The smear was then flooded with Gram's iodine as mordant to stabilize the color of the primary stain. Allowing one minute the smear was washed again with slow moving tap water thoroughly. The smear was then decolorized by the applying 95% ethanol or acetone for 5 to 10 seconds and raised well with tap water. Finally, the secondary stain Safranin was applied for one minute and subsequently washed off with distilled water. The slide was then kept for air-drying before microscopy. The dried smear was then examined under oil-immersion lens.

Biochemical characterization

Biochemical tests viz. catalase, oxidase, Indole, Methyl red, Voges-Proskauer test, Motility Indole Urease test, Nitrate Reduction test, Citrate utilization, Lysine Decarboxylase test, Coagulase test, Kligler Iron Agar test,

Biochemical identification through software

Identification of species was done by the aid of an online software, Gideon⁶.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was carried out by the standard disc diffusion test described previously⁷. A total of ten antibiotics were used to identify the antibiogram of the bacteria. These were CTX – Cefotaxime, AZM – Azithromycin, AMC – Amoxycillin, DA - Clindamycin, SXT - Sulfamethoxazole, CRO – Ceftriaxone, AML - Amoxyclav, CIP - Ciprofloxacin, TE - Tetracycline, and F – Nitrofurantoin. Isolates that exhibited resistance to the ² lactam antibiotics were tested for ESBL production by using the double disk diffusion test as described previously⁸. After overnight incubation at 37°C, any enhancement of the zone of inhibition between a beta-lactam disk and that containing the beta-lactamase inhibitor was indicative of the presence of an ESBL.

Results

Bacteria isolation

A total of 210 samples were studied. Of the samples, 53.8% were from female patients and 46.2% from males. The sites of cancer in these patients were 0.48% in ovary and lymph node each, 0.95% in kidney, 1.42% in breast and liver each and 95.24% in blood. The isolates were primarily identified on the basis of their colony morphology and cultural characteristics on the MacConkey agar (MAC), Eosin Methylene Blue (EMB) and XLD agar plates. Gram Staining was performed. The percentages of male and female cancer patients investigated together with their age group is shown in Figure 1. The culture based characteristics and Gram staining reaction of different isolates are given in Table 1.

Biochemical Tests for Identification of Isolates

After growth on MacConkey and Eosine Methylene Blue Agar plates, the isolates were further identified by biochemical tests as Triple Sugar Iron (TSI) test, Motility Indole Urea (MIU) test, IMVIC test, MIO, Ornithine decarboxylase, Lysine decarboxylase, Catalase and Oxidase tests. An online software, Gideon⁶, was used for identification of the isolates. The frequencies of bacteria identified by Gideon are given in Figure 2.

Antibiotic susceptibility of the isolates

Following the Clinical and Laboratory Standard Institute (CLSI) guideline, most of the isolates were resistant to Cefotaxime, Nitrofurantoin, Clindamycin, Ceftriaxone, and Amoxycillin. Most of the isolates were sensitive to Ciprofloxacin (Figure 3).

Extended spectrum ² lactamase (ESBL) activity of the isolates

In this study 45 isolates (55.6%, n= 45) were tested for ESBL production and only one isolate (2%, n=45) was an ESBL producer.

Table 1. *The morphological characteristic of bacterial colonies on different media in the supplied sample.*

Sample No.	MAC	EMB	XLD	Gram Staining	
				Shape	Ve+/ve-
38/s	White/gummy	-	-	rod	-
17	White/gummy	-	+	coccus	-
24	White/gummy	-	+	rod	-
52/s	Yellow/gummy	+	+	cocci	-
43	White/gummy	-	-	rod	-
41	White/gummy	-	-	cocci	-
58/L	Slightly yellow/gummy	+	+	rod	-
16/C	White/gummy	+	-	cocci	-
182	Water like	-	-	rod	+
167	White/gummy	+	-	rod	-
179	Red/gummy	+	-	rod	-
162	White/gummy	-	+	rod	-
149	Water like	+	-	rod	-
32	White/gummy	+	-	cocci	-
60	Colorless	-	-	cocci	-
57	pink/gummy	-	+	rod	-
16w/c	White/gummy	+	+	rod	-
36	Red/gummy	+	+	rod	-
40	Pink/gummy	-	+	rod	-
181	Pink/gummy	+	-	cocci	-
64	Colorless	-	-	cocci	-
96	Pink/gummy	+	-	rod	-
109	Slightly red/huge gummy	+	-	rod	-
14	Colorless	+	-	cocci	-
39	Colorless	+	-	cocci	-
81	Pink/gummy	+	+	rod	-
34/s	White/gummy	-	+	rod	-
207	Colorless	-	-	cocci	-
30	Colorless	+	-	cocci	-
94	Colorless/gummy	-	+	cocci	-
36/L	Colorless/gummy	+	-	cocci	-
19	Colorless/gummy	-	-	rod	-
12	Pink/gummy	+	+	rod	-
18	Colorless/gummy	-	-	rod	-
20	White/gummy	+	+	cocci	-
51	White/gummy	+	+	rod	-
25	White/gummy	+	+	rod	-
21	Colorless	+	+	rod	-
55	White/gummy	-	-	rod	-
99	Pink/gummy	+	+	rod	-
11	Colorless	-	-	rod	-
13	Pink	+	-	rod	-
28	Colorless	-	-	cocci	-
202	White/gummy	+	-	cocci	-
196	Colorless	-	-	rod	-

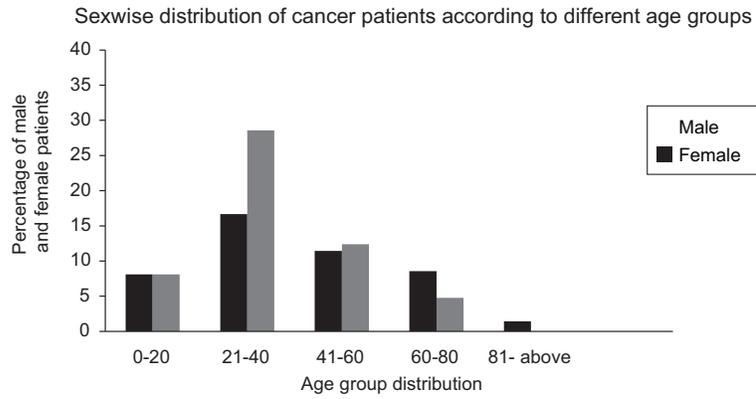


Figure 1. Percentage of female and male cancer patients in different age groups investigated

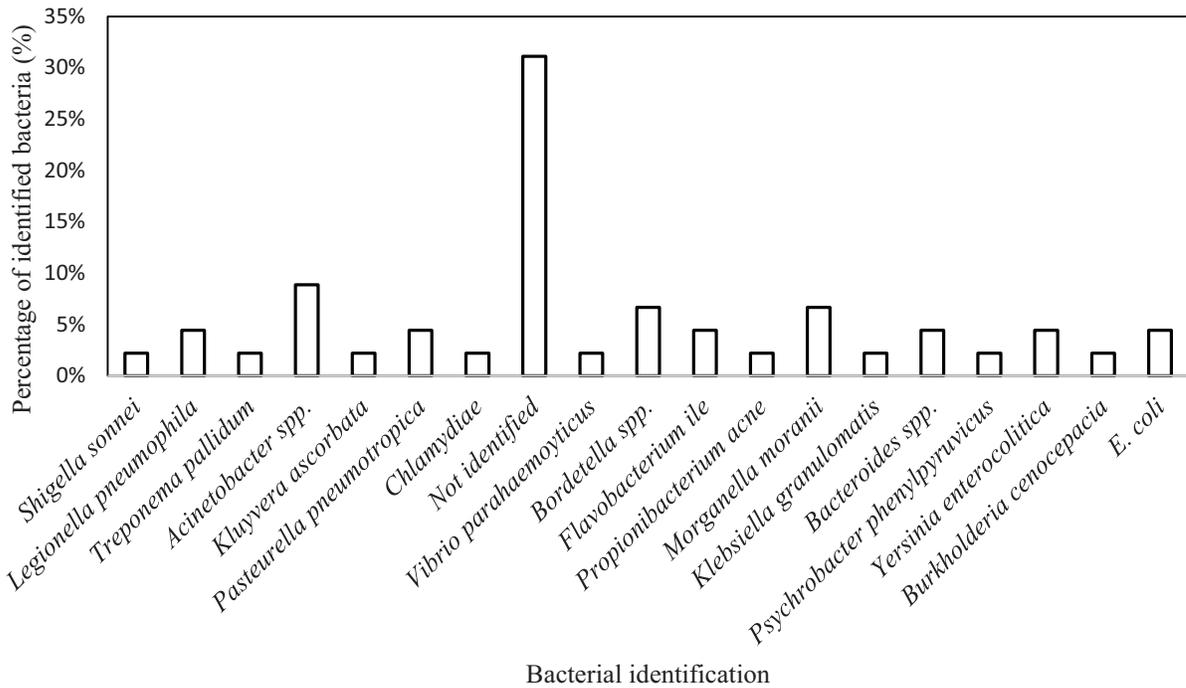


Figure 2. Relative percentages of bacteria present in isolated fluids of cancer patients. Isolate identification was carried out by Gideon (<http://web.gideononline.com>) by using the results from the biochemical tests carried out as inputs.

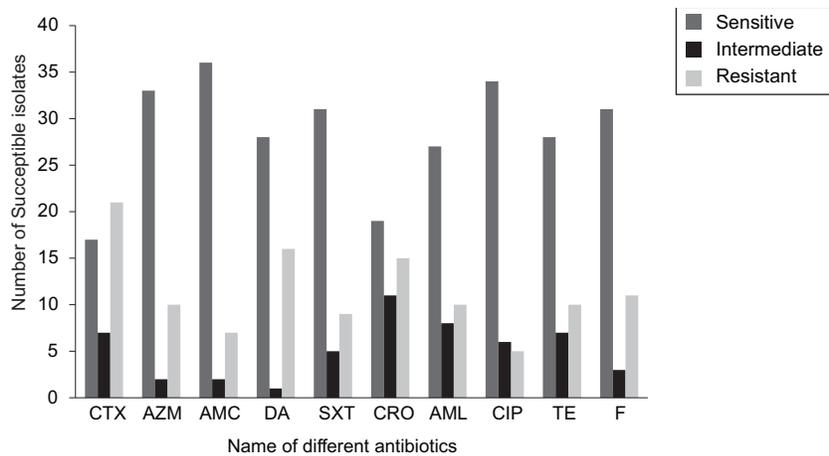


Figure 3. Antibiotic Susceptibility pattern of the isolates (CTX – Cefotaxime, AZM – Azithromycin, AMC – Amoxycillin, DA - Clindamycin, SXT - Sulfamethoxazole, CRO – Ceftriaxone, AML - Amoxyclav, CIP - Ciprofloxacin, TE - Tetracycline, and F – Nitrofurantoin)

Discussion

Recently, the emergence of antimicrobial resistance has become a health problem worldwide. Infections due to multidrug-resistant (MDR) Gram-negative bacteria are of special concern, since the therapeutic options are often very limited. Several studies have shown that cancer patients infected with resistant bacteria more often receive inadequate initial empirical antibiotic therapy, which may impair outcomes, increase mortality and prolong hospitalization.

In our study out of 210 samples collected from National Cancer Hospital in Dhaka, Bangladesh, *Acinetobacter spp.* led the list of pathogens (8.89%) followed by *Bordetella spp.* (6.67%), *Morganellamorani*(6.67%), *Legionellapneumophila*(4.4%), *Escherichia coli* (4.4%), *Yersinia enterocolitica*(4.4%), *Bacteroides spp.* (4.4%), *Shigellasonnei*(2.2%) and *Klebsiella granulomatis*(2.2%). Zinner reported that gram-negative bacteraemia remains an important cause of morbidity and mortality in neutropenic patients⁸. In his study, *E. coli* led the list of pathogens. In our study, 4.4% of the total gram-negative isolated bacteria were *E. coli* followed by *Yersinia enterocolitica*(4.4%) which is similar to a study by Saghir, 2009¹⁰. Previous studies have shown the presence of some multi drug resistant bacterial species among cancer patients. Other *Enterobacteriaceae* (*Citrobacter spp.*, *Enterobacter spp.*, *Proteus spp.* and *Serratia spp.*) are less common, although their frequency may vary according to institution¹¹. Significantly, antimicrobial resistance among *Enterobacteriaceae* has recently been described in cancer and HSCT patients, mainly due to ESBL-producing and Amp-C cephalosporinase hyper producing strains¹². In another study on 328 bloodstream infections in the pediatric oncology unit at the National Cancer Institute *Pseudomonas* species, *Acinetobacter species*, *Enterobacter species*, *Klebsiella species* and *E. coli* were isolated with the following percentages 5.5%, 6.7%, 2.7%, 1.5% and 2.1%¹². In another study on urinary tract infection in 4 intensive care units in Egypt *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas species* were the most commonly isolated bacteria¹⁴.

In vitro activity of different antibiotics was evaluated in our study, most of the isolates were resistant to Cefotaxime, Nitrofurantoin, Clindamycin, Ceftriaxone, and Amoxicillin. Most of the isolates were sensitive to Ciprofloxacin. In an earlier study, it was shown that increasing consumption of Ceftazidime was associated with decreasing susceptibility of *Acinetobacter species* and *S. maltophilia*⁹. High resistance rates against Cephalosporins in *Ps. aeruginosa* and *Enterobacteriaceae*¹⁵. In their study, *Enterobacter species* exhibited 95.8% resistance to both antibiotics whereas *Pseudomonas* species exhibited 87.6% and 66.3% resistance to Cefotaxime and Ceftazidime, respectively. Resistance was high in *Escherichia coli* and *Klebsiella species*, which was consistent with a study from Egypt that reported high resistance levels to Cefotaxime (74.4%) in Gram-negative rods¹⁴. This high resistance in

Enterobacteriaceae may be attributed to ²-lactamase activity¹⁶.

Although the prevalence of ²-lactamase producers in our study was low (%) continued surveillance to confirm the presence of ES²L enzymes among future cancer isolates may actually reveal a higher prevalence of the enzyme. This is an important future avenue specially when the rates of extended-spectrum ²-lactamase (ESBL) producing isolates among *E. coli* and *Klebsiella species* are shown to be increasing in another study¹⁷. Studies on the resistance to β -lactam antimicrobial agents, especially extended-spectrum Cephalosporins and other antimicrobial agents among clinical isolates of Gram-negative bacteria are on the rise worldwide¹⁸. In Egypt it was reported that extended spectrum ²-lactamase was detected in 78.6% and 56% of *E. coli* and *K. pneumoniae* strains, respectively¹⁴. In the present study, no extended spectrum ²-lactamase was detected in the 45 isolates. One isolate of *Klebsiella* sp. produced ²-lactamase among the 149 isolates studied.

Multidrug-resistance organisms (MDRO) such as *P. aeruginosa*, *K. pneumoniae* and the other *Enterobacteriaceae* species with emerging resistance, are important causes of morbidity and mortality in hospitalized critically ill patients and patients with underlying medical condition such as neutropenia and immunosuppressants¹⁹. The return to the pre-antibiotic era has become a reality in many parts of the world. MDR microorganisms were recently named as the 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), indicating their 'escape' from the effects of antibacterial agents or the non-existence of newer active antibiotics²⁰. In our study, MDR organisms were observed which is alarming. The lack of alternative agents that are active against gram-negative bacteria necessitates the use of measure for controlling emergence of resistance in bacterial strains particularly in cancer patients.

Multidrug resistance is a worldwide problem that does not obey international borders and can indiscriminately affect members of all socioeconomic classes. In this study, resistance to selected antibiotics was studied in 45 clinical samples isolated from a total of 149 samples. It was found that the test isolates were resistant to all antibiotics in variable extents. Extended Spectrum ²-lactamase activity was absent among the isolates. Only one isolate presented as ²-lactamase producer. The antibiotics active against MDR bacteria have several disadvantages, including limited clinical experience, a higher incidence of adverse effects and less knowledge of the pharmacokinetics of the drug. Therefore, a thorough acquaintance with the main characteristics of these drugs is essential in order to safely treat cancer patients with MDR bacterial infections. It can be concluded that, treatment of infections due to MDR bacteria has become a clinical challenge. The presence of MDR bacteria in cancer patients is of particular concern because these patients are immunocompromised. Implementation of antibiotic stewardship

programs and infection control measures is essential in order to avoid antimicrobial resistance development and dissemination.

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