

Multi-drug Resistant Bacterial Isolates Associated with Blood Stream Infection

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Abstract

Multidrug resistant (MDR) bacteria complicate therapeutic management and limit treatment options. With the increase of antibiotic resistance among bacterial isolates, monitoring of the of drug resistance pattern became critical for appropriate empiric selection of antibiotic therapy. Between June 2014 to January 2015, a prospective study was carried out in Manmohan Memorial Medical College and Teaching Hospital, Kathmandu with an objective to determine the status of Extended Spectrum Beta- Lactamase (ESBL) and Biofilm producing MDR bacterial isolates from blood samples. Identification of the isolates was done by standard microbiological techniques and antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method following Clinical and Laboratory Standard Institute (CLSI) guidelines. ESBL screening of gram negative isolates was done using Ceftriaxone, Aztreonam, Cefotaxime, Ceftazidime and Cefpodoxime followed by confirmation using MASTDISCSTM Extended Spectrum Beta- Lactamase (ESBL) Detection Discs and Biofilm detection was done by Congo-Red and Tube- adherence Method. The culture positivity of 16% and 10 different species of bacteria were isolated. The most frequently occurring isolate was *Escherichia coli* followed by *Staphylococcus aureus*. *In vitro* antibiotic susceptibility test showed that Amikacin remains the principle antibiotic of choice based on its effectiveness on both gram positive and gram negative bacteria.

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Ninety five percent of isolates were MDR with 77.19% ESBL producers and 72.5% were biofilm producers. A statistically significant relationship was found between increasing spectrum of drug resistance and ESBL production and drug resistant in biofilm production ($p < 0.05$).

Keywords: Multidrug resistant; Extended Spectrum Beta- Lactamase; Biofilm

1. Introduction

The presence of viable bacteria in the circulating blood is termed as Bacteremia. Bacteria may enter the blood stream giving rise to bacteremia from an existing focus of infection from a site with the commensally flora or by direct inoculation of contaminated materials into the vascular system. Bacteremia can occur in daily activities like tooth brushing and some minor medical procedures like dental work but also during infection that leads to bacteremia [1]. These organisms are often cleared from the blood within minutes, so the bacteremia is silent and transient, but if the immune system is overwhelmed or evaded, organisms persist in the blood and bacteremic symptoms would arise [2]. Bloodstream infections are potentially life-threatening and require rapid identification and antibiotic susceptibility testing of the causative pathogen in order to facilitate specific antimicrobial therapy [3]. Bloodstream infections remain one of the most important causes of morbidity and mortality worldwide [4]. Despite important progresses in treatment and prevention of infectious diseases, they are considered as leading causes of death and disability and worsening life quality especially for millions of people in developing countries. Bacteremia can occur in healthy individuals, new born babies, and immune compromised individuals such as patients with diabetes mellitus, solid tumor and leukemia [5]. The incidence rate of community acquired bacteremia varies according to the geographical location [6]. Nosocomial blood stream infection is leading cause of bacteremia in critically ill patients [7]. The overall incidence of bacteremia has been increasing recently [8, 9, 10]. The poor prognosis and fatal outcomes are associated with septic shock, deterioration of mental status, poly microbial bacteremias and having certain underlying conditions such as tumors [11, 12]. In particular, Gram- negative organisms are renowned for inducing septic shock, which is often followed by sepsis related death. Several groups have reported that the prevalence of Gram- negative bacteria has increased among episodes of febrile neutropenic in the hematology units [13]. The most common bacteria responsible for the bacteremia are *E.coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* [14]. Bloodstream infections are potentially life-threatening and require rapid identification and antibiotic susceptibility testing of the causative pathogen in order to facilitate specific antimicrobial therapy [3]. Bacteria isolated from bloodstream infections are numerous [15] and their associated diseases need urgent and invasive management with antimicrobial drugs. Both gram positive and gram negative bacteria causes bacteraemia and septicaemia. Gram negative septicaemia, also known as endotoxic shock, is more severe than gram positive septicaemia. Many septic episodes are nosocomial and may be due to microorganisms with increased antimicrobial resistance. *Staphylococcus aureus*, *Escherichia coli*, Coagulase-negative staphylococci (CONS), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Streptococcus* spp., *Candida albicans*, and *Enterobacter cloacae* are the most frequent etiological agents of bacteraemia and fungaemia in Europe and the United States [3]. The mortality associated with bloodstream infections may range from 20% to 50% and depends on several factors, including the pathogen and host [16]. It is estimated that 2 million patients per year in the United States acquire infections while in hospitals, approximately 350,000 (10-20%) of these infections

involve the bloodstream, and 90,000 (4.5%) are fatal. In Nepal, febrile illness is one of the most common reasons for medical consultation. The etiology of bloodstream infection in febrile patients is poorly characterized in Nepal, mainly due to limited laboratory resources, a poor recording system and an inadequate number of trained personnel [17].

1.1 *The effect of antimicrobials and the problem of drug resistance*

Multi drug resistance is defined as the resistance to at least two classes of first line agents including Ampicillin, Chloramphenicol, Trimethoprim-Sulfamethoxazole, Fluroquinolones (Ciprofloxacin and Ofloxacin), and Cephalosporins (Cefotaxime, Ceftriaxone and Ceftazidime) [18]. The overuse and misuse of the antimicrobials have led to the death of the sensitive strains leaving resistant strains to survive, multiply and infect new hosts. Multidrug resistance is now commonly described among isolates of streptococcal, staphylococcal, and enterococcal species. Increasing drug resistance has occurred among “community-acquired” isolates such as HACEK microorganisms, *Salmonella* spp and Enterobacteriaceae, as well as among nosocomial isolates such as *Pseudomonas* spp [19]. The increasing incidence and prevalence of methicillin resistant *S. aureus* (MRSA) and the emergence of community-associated MRSA (CA-MRSA) is an important challenge for clinicians when treating *S. aureus* bacteremia [20]. Although first identified just >4 decades ago, methicillin-resistant *S. aureus* (MRSA) has undergone rapid evolutionary changes and epidemiologic expansion to become a major cause of nosocomial and community-acquired infections worldwide. Increasing resistance to vancomycin among MRSA strains in conjunction with availability of new antibiotics, including daptomycin and linezolid, have increased treatment choices but made clinical treatment decisions more challenging [21]. Beta lactamases are the most common cause of bacterial resistant to β lactam antimicrobial agents which are used in the treatment of infections caused mainly by bacteria belonging to *Enterobacteriaceae*, *Haemophilus* spp and *Neisseria* spp. The ability of β - lactamases to cause resistance varies with its activity, quantity, cellular locations and permeability of the producer strain. Bacteria responded with plethora of new β - lactamases including extended spectrum β - lactamases (ESBLs), plasmid mediated AmpC β - lactamases and carbapenem hydrolyzing β - lactamases (carbapenemases) [22, 23]. Extended spectrum β - lactamases (ESBLs) is defined as the β - lactamases that are capable of conferring bacterial resistance to the penicillins, first, second and third generation Cephalosporina and Aztreonam (but not the Cephameycins or Carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β - lactamases inhibitors such as Clavulanic acid. The first of these enzymes capable of hydrolyzing the newer β - lactams, SHV-2 was found in a single strain of *Klebsiella ozaenae* isolated in Germany. These ESBLs enzymes are produced frequently by *E. coli*, and *Klebsiella* spp but other bacteria like *Enterobacter* spp, *Salmonella* spp, *Citrobacter* spp and *Pseudomonas* also produce ESBLs [26, 25]. Thus, rapid and reliable detection of bloodstream infections, including characterization of the pathogen to the species level and determination of its antibiotic susceptibility pattern, is crucial for several reasons:

- Appropriate antimicrobial agents can be selected, and thus, unnecessary treatment with ineffective antibiotics can be avoided
- The prognosis of the patients can be improved
- The acquisition of resistance in pathogens may be decelerated
- Expenditure on antimicrobials and overall hospital costs can be reduced [26, 27].

This study could be a guideline for physicians in order to administer proper antibiotics prior to obtaining blood culture reports. This study may also help as a guideline for the treatment of patients in the developing countries where the most of the health intuitions do not have good culture equipments and sensitive facilities.

1.2 Biofilm

Biofilm is defined as microbial sessile aggregation characterized by cells that are irreversibly attached to a substratum that may be living or non-living surfaces, enclosed within a self-produced extracellular matrix [28]. The matrix is composed of polysaccharides, proteins, and extracellular microbial DNA, and the biofilm may consist of one or more microbial (bacterial or fungal) species. The matrix is responsible for providing structural stability and protection to the biofilm against adverse environmental conditions, such as, host immunological system and antimicrobial agents[29]. It is a survival strategy that most microorganisms in the environment employed for the survival in the hostile environment that might be natural, industrial or even within living hosts. Its formation is triggered by specific environmental factors, such as nutrient and oxygen availability [30, 31]. Biofilm is estimated to have contribution to more than 80% of human infections [32]. and are source of persistent infections caused by various pathogenic microbes [33]. All microbes including Gram positive and Gram negative bacteria are capable of synthesizing biofilm. Bacteria commonly involved include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Enterococcus faecalis* *Klebsiella pneumoniae*, *Acinetobacter spp*, *Enterobacter spp* *Proteus mirabilis* and *Pseudomonas aeruginosa* [31].

In biofilm involved infections, bacteria adhere to biotic or abiotic surface, whichever is accessible and form bacterial communities, initially microcolonies which later on mature to resistant biofilms [30]. The biofilms are resistant to host immune responses as well as antibiotic treatment. As a result, the infections are difficult to resolve and tend to be chronic [33]. Biofilm has proved to be nuisance in the management of diseases especially those which are device related and thus has gained utmost importance in the medical field [29].

1.2.1 Biofilm and Antimicrobial Resistance

Biofilms are highly resistant to antimicrobial agents (antibiotics, disinfectants or germicides) [28]. Antimicrobial resistance exhibited by biofilm is conferred to delay penetration of antimicrobial agents across the biofilm matrix i.e. EPS, altered growth rate and other physiological changes due to the biofilm mode of life [30]. Biofilm matrix acts as an efficient barrier to antimicrobial agents either by decreasing the transport of these agents to interior of biofilm or by reacting with the agents itself [34].

Biofilms impose a serious threat in clinical settings due to their involvement in chronic and recurrent infections and their higher antimicrobial resistance [33, 35]. Biofilm-involved infections impose a great problem, because biofilm-associated bacteria are able to withstand host immune defenses, antibiotics, biocides, and hydrodynamic shear forces better as compared to the corresponding planktonic bacteria, thus, making biofilm-associated infections particularly recalcitrant to standard antimicrobial treatment as well as host immune counterattacks, and even after the treatment, the surviving biofilm-associated bacteria will carry on the infection [32]. Biofilms

may also be responsible for spread of antibiotic resistance via horizontal gene transfer or quorum sensing [33].

2. Methodology

2.1 Study site, study duration and study population

The study was conducted in Manmohan Memorial Medical College and Teaching Hospita (MMMCTH), Kathmandu, Nepal from June 2014 to January 2015.

2.2 Study Population

Study was conducted on patients visiting MMMCTH for requesting of blood culture and antibiotic susceptibility test by the physicians.

2.2.1 Sample size and sample type

A total of 500 blood samples were included in the study

2.2.2 Inclusion and Exclusion Criteria

Labeled blood cultures with correct patient's information sheet were included in the study. The blood culture bottle were rejected if it is cracked or broken or the media was turbid prior to sample inoculation.

2.3 Laboratory analysis

2.3.1 Blood specimen collection, transportation and analysis

The blood samples were collected by trained health professional using standard aseptic techniques. About 5ml of blood from adult and 3ml of blood from children were collected aseptically and inoculated into brain heart infusion (BHI) broth. Inoculation of blood sample into the culture broth was done immediately after collection. Immediately, after the blood culture bottles were received in the laboratory, they were provided with laboratory identification numbers and further processed. The culture bottles were incubated at 37⁰c for 7 days and sub-cultured at 42 hours, 48 hours or unless the visible growth was obtained. Details on clinical history, age, sex, etc. of the patients were recorded after taking informed consent (Appendix I).

2.3.2 Subculture from broth culture

The broth cultures were sub-cultured on Nutrient Agar (NA), Mac Conkey Agar (MA) and Blood Agar(BA) plates. The plates were then incubated at 37°C for 24 hours and readings were being done at 48 hours of incubation. For cultures that showed positive bacterial growth, identification of culture isolates was done by colonial characteristics, gram stain and biochemical tests according to the standard methods [38].

2.3.3 Identification of the isolates

The gram positive organism was identified primarily on the basis of their response to gram's staining, catalase, oxidase and coagulase tests. The gram negative isolates were identified by standard diagnostic procedure as: For lactose fermenters, media inoculation for motility, indole production and citrate utilization tests were carried out and incubated overnight. Individual colonies of clinically significant, lactose fermenters were inoculated into 2 ml of urea broth and incubated for 4 hours at 37°C. Any urease positive was then check for purity. The 4 hours suspension would serve as the inoculums for biochemical tests for strains of other genera, and purity check on Mac Conkey agar. The Gram-staining procedure is mentioned in the Appendix-II. The procedure for performing biochemical tests are mentioned in Appendix-III.

2.3.4 Antimicrobial susceptibility testing

2.3.4.1 Antibiotic susceptibility test of bacterial isolates

Antibiotic susceptibility testing of bacterial isolates was done by Kirby Bauer disc diffusion method [40] using Muller Hinton Agar. The inoculum was prepared by direct colony suspension method by transferring several isolated pure colonies from fresh non-selective agar plate to normal saline. The mixture was vortexed well and turbidity was adjusted visually with sterile normal saline to match a 0.5 Macfarland standard. The suspension was then swabbed uniformly over entire surface of a sterile MHA plate. With a sterile forcep, selected antibiotics discs were placed on inoculated plate no closer than 15 mm from the edge and 24 mm from center of discs. The plates were then left at room temperature for 15 minutes for prediffusion and then incubated at (35-37)°C. On the basis of zone-size compared with that of control strains, the result was interpreted [39] based on standard interpretative chart provided by manufacturer was done.

2.3.4.2 Methicillin resistant *Staphylococcus aureus* (MRSA) and *S. epidermidis* (MRSE) Screening

S. aureus and *S. epidermidis* were screened for methicillin resistance by modified Kirby Bauer disc diffusion method using Oxacillin disc (1 mcg) as per the standard guidelines [40].

2.3.4.3 Determination of multidrug resistance in bacterial isolates

The isolates were defined as multidrug resistant if they were resistant to two or more class of antibiotics tested [41].

2.3.4.5 List of antibiotics used for susceptibility test

For Gram Positive cocci: Azithromycin(30µg), Amikacin(30µg), Clindamycin(10µg), Cefixime(5 mcg) , Ciprofloxacin(5µg), Cloxacillin(5µg), Ceftriaxone(30µg),Cefotaxime(30mcg), Ceftazidime(30µg), Cotrimoxazole(25µg),Chloramphenicol(30 mcg),Erythromycin(15mcg), Gentamicin(10µg),Mupirocin(5 mcg), Penicillin G(10µg),Tegecyclin(10mcg), Oxacillin(5µg), Vancomycin(30µg) ,

For Gram Negative bacilli: Amikacin(30µg), Amoxicillin(30 mcg), Ciprofloxacin(5µg), Ceftriaxone(30µg), Ceftazidime(30µg), Cotrimoxazole(25µg), , Cefepime(30 mcg), Cefpodoxime(10mcg), Chloramphenicol(30

mcg), Cefixime(5mcg), Tigecycline(10 mcg) , Imipenem (10 mcg) Nalidixic Acid(30 mcg), Nitrofurantoin(100 mcg), Norfloxacin(10 mcg), Gentamycin(10 mcg), Ofloxacin(5 mcg), Piperacillin/Tazobactam(100/10 mcg), Gentamicin(10µg), Meropenem(10µg), Ofloxacin(5µg),

2.3.5 Preservation of the MDR isolates

After performing the antimicrobial susceptibility testing, MDR isolates in pure culture were preserved in 20% glycerol containing Tryptic Soya Broth and kept at -4°C for the detection of ESBL and Bio film production test.

2.3.6 Screening and confirmation for ESBL producers

Screening of the suspected ESBL strains was performed according to the guidelines for screening issued by the CLSI in 2005. According to this guidelines MDR isolates were screened for possible ESBL production using Ceftriaxone (30µg), Cefotaxime (30 µg), Cefpodoxime (10µg), Ceftazidime (30µg) and Aztreonam(30µg) and the isolates showing zone of inhibition to Ceftriaxone≤25mm , Cefotaxime≤27mm, Cefpodoxime≤17mm, Ceftazidime≤22mm,and Aztreonam≤27mm are possible ESBL producers. The suspected ESBL producers were tested for confirmatory ESBL production using MASTDICTM Extended Spectrum Beta- Lactamase Detection Discs. The kit consists of:

Set 1: Ceftazidime(30µg) and Ceftazidime(30µg) plus Clavulanic acid(10µg)

Set2: Cefotaxime (30 µg) and Cefotaxime (30 µg) plus Clavulanic acid(10µg)

Set 3: Cefpodoxime (10µg) and Cefpodoxime (10µg) plus Clavulanic acid(10µg)

Set 4: Aztreonam(30µg) and Aztreonam(30µg) plus Clavulanic acid(10µg)

The zone of inhibition for the Ceftazidime, Cefotaxime, Cefpodoxime was compared to that of these discs combined with Clavulanic acid and an increase in zone diameter of ≥ 5mm in the presence of Clavulanic acid from any or all of the kit was concluded as confirmed ESBL producers. The detailed working protocol is described in Appendix IV.

2.3.7 Detection of Biofilm Production

Biofilm production was estimated qualitatively for all the isolates by tube adherence method [42] and Congo-red Method [37].

Tube Adherence Method (TA)

Suspension of tested strains was incubated in the glass tubes containing Brain Heart Infusion Broth (BHI) aerobically at the temperature of 35°C for the period of 2 days. Then the supernatant was discarded and the glass tube was stained by 0.1% safranin solution, washed with distilled water 3 times and dried. A positive result is defined by the presence of a layer of stained material adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid-air interface should be considered negative.

Congo Red Agar Method (CRA)

The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5gm/l), agar number 1 (10 gm/l) and Congo red dye (0.8 gm/l). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121 C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55 C. Plates were inoculated with test organism and incubated at 37 C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; weak producers usually remained pink, though occasional darkening at the centre of colonies was observed.

Table 1: Pattern of bacterial isolates according to the gram stain

Bacteria	Frequency	Percentage
Gram Negative rods	61	76.25%
<i>E.coli</i>	30	37.5%
<i>Salmonella Paratyphi A</i>	14	17.5%
<i>Salmonella typhi</i>	4	5%
<i>Proteus mirabilis</i>	1	1.25%
<i>Proteus vulgaris</i>	3	3.75%
<i>Klebsiella pneumonia</i>	1	1.25%
<i>Klebsiella oxytoca</i>	1	1.25%
<i>Pseudomonas aeruginosa</i>	3	3.75%
<i>Citrobacter freundii</i>	4	5%
Gram positive cocci	19	23.75%
<i>Staphylococcus aureus</i>	15	18.75%
CONS	4	5%
Total	80	100%

2.4 Quality control

To obtain reliable microbiological result, it is necessary to maintain quality control. Quality of each test was maintained by using standard procedures. Control strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for the standardization of the Kirby-Bauer test and also for correct interpretation of zone of diameter. Quality of sensitivity tests was maintained by maintaining the thickness of MHA at 4 mm and the pH at 7.2-7.4. Similarly antibiotics discs containing the correct amount as indicated were used. Strict aseptic conditions were maintained while carrying out all the procedures.

2.5 Statistical analysis

The results were expressed as percentages for the analysis of various epidemiological details and for analysing the distribution of different bacterial isolates and their sensitivity pattern. Microsoft excel and Chi square test was used for the interpretation of these results.

3. Results

3.1 Rate of bacteremia

All together 500 blood samples were collected during the study period and subjected to bacterial cultures in which growth was seen only in 80 cases (16%). Four hundred twenty cases were found to be no growth on 72 hours of incubation at 37^oC.

3.2 Pattern of bacterial isolates

Out of 80 isolated bacteria, 61(76.25%) were gram negative and 19(23.75%) were gram positive. The most frequently occurring gram negative isolate was *E.coli* with (37.5%) and gram positive isolate was *S. aureus* with (18.75%). Results are shown in table 1.

3.3 Antibiotic susceptibility pattern of gram positive isolates.

Table 2: Antibiotic susceptibility pattern of gram positive isolates.

Antibiotics used	Sensitivity pattern of <i>S.aureus</i>				Sensitivity pattern of CONS			
	Sensitive		Resistant		Sensitive		Resistant	
	No.	%	No.	%	No.	%	No.	%
Clindamycin	3	20	12	80	3	75	1	25
Cefotaxime	1	6.67	14	93.33	3	75	1	25
Cloxacillin	0	-	15	100	0	-	4	100
Vancomycin	12	80	3	20	3	75	1	25
Erythromycin	3	20	12	80	4	100	0	-
Gentamycin	12	80	3	20	4	100	0	-
Penicillin	1	1.67	14	93.33	0	-	4	100
Co-Trimoxazole	1	1.67	14	93.33	3	75	1	25
Mupirocin	3	20	12	80	0	-	4	100
Ceftriaxon	2	13.33	13	86.66	3	75	1	25
Chloramphenicol	9	60	6	40	4	100	0	-
Oxacillin	1	6.67	14	93.33	0	-	4	100
Amikacin	15	100	0	-	4	100	0	-

Tigecyclin	6	40	9	60	4	100	0	-
Cefixime	0	-	15	100	0	-	4	100
Ciprofloxacin	10	66.67	5	33.33	4	100	0	-
Azithromycin	15	100	0	-	4	100	0	-
Novobiocin	*	*	*	*	3	75	1	25
Polymyxin B	*	*	*	*	4	100	0	-
Fusidic acid	*	*	*	*	0	-	4	100

* denotes antibiotics not used.

3.4 Methicillin resistance in staphylococci

Pattern of methicillin resistance in staphylococci was studied. Out of 19 isolates 18 were resistant to methicillin as determined by Oxacillin (1mcg) disk by Kirby Bauer disk diffusion method. Among 15 *S. aureus*, 14 (93.33%) were resistance to methicillin (MRSA) and among 4 CONS 4(100%) were resistant to methicillin (MRSE).

Table 3: Methicillin resistance pattern among staphylococci

S.N.	<i>Staphylococcus</i> spp. (N=19)	Methicillin Resistant n (%)
1	<i>S. aureus</i> (15)	14(93.33)
2	CONS(4)	4(100.0)

3.5 Antibiotic Susceptibility pattern of the gram negative isolates

Table 4: Antibiotic Susceptibility pattern of the gram negative isolates

Resistance to antibiotics used(%)	Isolates Identified					
	<i>E.coli</i>	<i>Salmonella</i> Sps	<i>Klebisella</i> Sps	<i>Pseudomonas</i> <i>aeruginosa</i>	<i>Citrobacter</i> Sps	<i>Proteus</i> Sps
AMK	0	0	0	0	0	2(50)
AMX	28(93.33)	18(100)	2(100)	3(100)	3(75)	3(75)
CPM	18(60)	9(50)	0	2(66.67)	2(50)	3(75)
CTR	14(46.67)	12(66.67)	2(100)	2(66.67)	2(50)	3(75)

CPD	16(53.33)	9(50)	0	3(100)	3(75)	3(75)
C	0	6(33.33)	2(100)	2(66.67)	1(25)	2(50)
CFM	22(73.33)	13(72.22)	2(100)	2(66.67)	4(100)	3(75)
TGC	2(6.67)	6(33.33)	0	2(66.67)	1(25)	2(50)
IPM	1(3.33)	0	0	0	0	2(50)
NA	6(20)	7(38.89)	2(100)	3(100)	0	2(50)
NIT	7(23.33)	0	0	1(33.33)	1(25)	1(25)
NX	0	0	0	2(66.67)	0	1(25)
GEN	3(10)	0	0	2(66.67)	0	2(50)
OF	2(6.67)	0	0	2(66.67)	0	2(50)
PIT	3(10)	0	0	0	0	3(75)

Note: The antibiotics written in short forms: AMK-Amikacin, AMX–Amoxicillin, CPM-Cefepime, CTR-Ceftriaxone, CPD- Cefpodoxime, C-Chloramphenicol, CFM-Cefixime, TGC - Tigecycline ,IPM- Imipenem NA-Nalidixic Acid, NIT-Nitrofurantoin, NX-Norfloracin, GEN-Gentamycin, OF-Ofloxacin, PIT-Piperacillin/Tazobactam

3.6 MDR and ESBL Production profile among isolates

Of 80 isolates, 76(95%) were MDR. All the gram positive isolates and 57 out of 61(93.44%) of gram negative isolates were found to be MDR. The gram negative isolates are tested for ESBL production among which 44 out of 61(72.13%) are ESBL producers.

Table 5: MDR and ESBL Production profile among isolates

Organisms	Total Isolates	No.of MDR	ESBL Screening	ESBL Confirmed
<i>Escherichia coli</i>	30	29	30	20
<i>Salmonella</i> spp.	18	15	18	14
<i>Klebsiella</i> spp.	2	2	2	1
<i>Pseudomonas aeruginosa</i>	3	3	3	3
<i>Citrobacter freundii</i>	4	4	4	3
<i>Proteus</i> spp	4	4	4	3
Total	61	57(93.44%)	61	44

3.7 Bio film production by Congo red method and Tube adherence Method

A total of 58 isolates were biofilm producers. Congo red method results 20 weak and 38 strong biofilm producers whereas Tube adherence method results 24 weak and 34 strong biofilm producers.

Table 6: Bio film production by Congo red method and Tube adherence Method

Organisms	Congo-Red Method		Tube adherence Method		Total biofilm Producer
	Weak	Strong	Weak	Strong	
<i>S.aureus</i>	9	1	7	3	10
CONS	1	2	1	2	3
<i>E.coli</i>	5	18	10	13	23
<i>Salmonella</i> spp	2	12	3	11	14
<i>Klebsiella</i> spp	0	1	0	1	1
<i>Citrobacter</i> spp	1	1	1	1	2
<i>Proteus</i> spp	1	2	1	2	3
<i>Pseudomonas aeruginosa</i>	1	1	1	1	2
Total	20	38	24	34	58

3.8 Distribution and biofilm production of isolates

Among 80 isolates, 58 (72.5%) were biofilm producers by Tube adherence and congo-red method. The majority of organisms were associated with the biofilm production. Results are shown in table 7.

Table 7: Distribution and biofilm production of isolates

Isolates	Total (%)	Biofilm production
<i>Staphylococcus aureus</i>	15 (18.75%)	10(66.67%)
CONS	4 (5%)	3 (75%)
<i>Escherichia coli</i>	30 (37.5%)	23 (76.67%)
<i>Salmonella</i> sps	18 (22.5%)	14 (77.78%)
<i>Klebsiella</i> sps	2 (2.5%)	1(50%)
<i>Citrobacter freundii</i>	4 (5%)	2 (50%)
<i>Proteus</i> sps.	4 (5%)	3 (75%)
<i>Pseudomonas aeruginosa</i>	3 (3.75%)	2 (66.67%)
Total	80 (100%)	58 (72.5%)

3.9 Biofilm production in ESBL producers

Among 61 gram positive isolates, 44(72.13%) isolates were ESBL producers. Of 44 ESBL producers

37(84.09%) were biofilm producers. Detail results are shown in table 8.

Table 8: Biofilm production in ESBL producers

Isolates	ESBL producer	Biofilm producer(%)
<i>Escherichia coli</i>	20	18 (90%)
<i>Salmonella</i> sps	14	12 (85.71%)
<i>Klebsiella</i> sps	1	1 (100%)
<i>Citrobacter freundii</i>	3	2 (66.67%)
<i>Proteus</i> sps.	3	1 (33.33%)
<i>Pseudomonas aeruginosa</i>	3	3 (100%)
Total	44	37 (84.09%)

4. Discussion

The rate of Bacteremia reported in the present study is similar to the findings of other investigators elsewhere in the world [43, 44, 45]. However the marked difference in the rate has been reported by many investigators in compare to the present study [46, 47, 48, 49, 50]. Different investigators reported different evidences for the variation of the results. The prior use of antibiotics has markedly decrease the rate of bacteremia [58]. In addition, the technique of blood culture, anaerobic infection and effective control in spread of nosocomial infection [45]. The low isolation rate is common in developing countries mainly because of prior and haphazard use of antibiotics before blood collection for culture [51, 52] as availability and misuse of antibiotics even for mild cases of fever in Nepal [53, 54]. The isolation rate of pathogens is as high as 30-70% in well equipped laboratories in developed countries where diagnosis system is well established and medication is proper [55]. During our study period, only monomicrobial infection, not the polymicrobial infection was found. Mono microbial bacteremia most prevalent (97.5%) than polymicrobial bacteremia[56]. Our study showed that the gram negative isolates were predominant cause of blood stream infection over gram positive isolates which was similar as reported in [57].

Our study showed that *E.coli* to be the causative agent of bacteremia. [43, 58] also reported high percentage of isolation of *E.coli* from blood. The high prevalence of *Salmonella* spp in bloodstream isolates in febrile patients in our study corresponds to the findings of previous studies performed in Nepal [59, 60, 61]. Similarly, high prevalence of *S.aureus* in this study is similar to the studies done on blood stream infection [62]. CONS had been reported as the most common organism from blood culture isolates from different studies [63]. The present study isolated 5% of the CONS. The clinical significance of CONS when isolated from blood cultures should always be evaluated. Some studies have reported that upto 85% of CONS represent contamination rather than true bacteremia [64]. However, in recent years, CONS have become an important nosocomial pathogen partly because of the increasing use of medical devices such as long term indwelling catheters, vascular grafts, and

prosthetic heart valves and joints [65]. Present study excluded *Bacillus* spp. and *Micrococcus* spp. as a contaminant but finding of this microorganism must be reported if patients had suspected endocarditis or in immunocompromised [66].

Antibiotic susceptibility testing has become a very essential step for the proper treatment of the infectious diseases. The result of present study suggest Amikacin remains the principle antibiotic of choice based on its effectiveness on both gram positive and gram negative bacteria which is according to [46] who also reported Amikacin effective against both gram negative and gram positive isolates. Our results on antibiotics susceptibility pattern of gram negative isolates is comparable to the study conducted in [50, 17, 57].

Our study finds, Amikacin and Azithromycin to be the most effective for *S. aureus* but Cloxacillin, Clindamycin, Penicillin, Oxacillin, Cefixime, Co- trimoxazole were ineffective. Resistance of *S. aureus* to most of the commonly used antibiotics ranged from 60% to 90% [67]. On similar type of studies conducted in [66] Vancomycin, Amoxicillin, Clindamycin and Ciprofloxacin have optimum activities against Staphylococci whereas Cloxacillin was found least active. Similarly, High rates of antibiotic resistance was seen among CONS to commonly-used antibiotics and majority of them were methicillin-resistant [68]. Our study also reports CONS resistant towards Oxacillin, Fusidic acid, Penicillin and Mupirocin. [69] also reports 100% resistant to penicillin and ampicillin but [69] reported the highest number were resistant to oxacillin (94.5%) followed by Gentamycin(40%).

Already a major cause both of nosocomial and community infections, *S. aureus* has reached endemic proportions with the worldwide emergence of antibiotic-resistant strains. The introduction of MRSA has increased the morbidity and mortality associated with bacteremia and endocarditis and has significantly reduced the standard therapeutic options [21]. In present study a large proportion of staphylococcal isolates were resistant to methicillin which is consistent to the study of [70] who reported high prevalence of Methicillin and Vancomycin resistant *S. aureus* in Sikkim. Various other studies have also reported high prevalence of MRSA in hospital and community acquired infections [67].

The increasing incidence of MRSA infection is a serious problem in patient management as therapeutic options are limited for such resistant strains. Vancomycin is a drug of choice for the treatment of MRSA infection [71] [72]. However, tolerance to vancomycin among MRSA is on rise. Therapy with vancomycin is associated with a long duration of bacteremia on therapy and relapses [73]. An elevated vancomycin MICs for MRSA isolates was reported elsewhere [74]. In our study, 80% of isolates were sensitive to Vancomycin. Daptomycin as an alternative to vancomycin in the therapy of *S. aureus* bacteremia and endocarditis for those with either methicillin-resistant organisms or intolerance to beta-lactams has been suggested by studies [21, 73]. But treatment failure even with vancomycin and daptomycin has also been reported [75]. Therapy with linezolid and fusidic acid was successful in clearing MRSA bacteremia [75].

Among the isolates of *E. coli*, Amikacin, Chloramphenicol, Imipenem, Tigecycline, Ofloxacin Nalidixic acid, Gentamycin and Piperacillin/Tazobactam were found to be the most effective drug where as Amoxicillin is least effective. This result is comparable to the study conducted in [76]. They found Amikacin, Chloramphenicol,

Imipenem, Nitrofurantoin to be most effective drug against E.coli isolates and also reported the presence of high resistant rates for different classes of antibiotics. In Salmonella species, Amikacin, Imipenem, Nitrofurantoin, Gentamycin, Ofloxacin, Piperacillin/Tazobactam were found to be effective with 100% sensitivity and Amoxicillin is ineffective. [77, 78] also reported 100% susceptible of *Salmonella* towards Ofloxacin.

It is neither possible nor desirable to specify drug of choice by seeing only the resistant pattern because specification of drug of choice depends upon the several factors like differences in local prescribing habits, resistant pattern of local pathogens, cost, toxicity, pharmacokinetics and spectrum of activity of antibiotics [79].

Antimicrobial resistance is a global problem. It is now generally accepted as major public health issue and has significant implication on health and patient care. Resistance to antimicrobial drugs is associated with high morbidity and mortality, high health-care cost and prolonged hospitalization. The problem of the drug resistance to antimicrobial drug is more troublesome to developing countries. Our Ninety five percent isolates are MDR and these pathogens are more common in hospital setting and are mainly accountable for nosocomial infection. Our result is slightly higher than that reported in [44]. However, the incidence of bacteremia caused by MDR strain is increasing [80]. The emergence and increasing trend of MDR among E.coli has been also reported [43].

The emergence of MDR is clearly related to the quantity of antibiotics and how they are being used [81]. Resistant strains are now reported against all available classes of antibiotics [81, 82]. The morbidity and mortality rates because of MDR strains among the very young, elderly population and among immunocompromised patients are very high [82]. The recent increase of MDR strains in hospital has started to pose great difficult in selecting antimicrobial agents for the management of the infection they caused and obviously the cost in the management of infection caused by MDR strains will be definitely high because of need of acquiring new drug which is of course will be high in cost as well as the cost of prolong staying in the hospital. Some factors responsible for the emergence of resistant strains in hospital include the indiscriminate use of antibiotics, the prolonged hospitalization, the increase in uses of insertion devices etc [16].

Our study reported 77.19% ESBL producers among MDR isolates. [43, 87] reported 69.23% and 62% ESBL producers among MDR isolates respectively. Therefore the study highlights the emergence of ESBL producing strains endowed with extremely wide spectrum of antibiotic resistant. ESBL production is coded by genes that are prevalently located on large conjugative plasmid of 80-160 kb in size. Since these plasmids are easily transmitted among different members of the *Enterobacteriaceae*, accumulation of resistant genes results in strains that contain multi-resistant plasmids. So, ESBL producing isolates are resistant to variety of classes of antibiotics [84]. Currently, ESBL and AmpC β -lactamase producers are becoming a major threat for patients in the hospital, long-term care facilities, and community. These bacteria have not only caused outbreaks, but have become endemic in many hospitals throughout the world. In addition, antibiotic resistance of bacteria in the biofilm mode of growth contributes to the chronicity of infections such as those associated with implanted medical devices [22].

A biofilm is a structured consortium of bacteria adhered to a substratum and embedded in a self-produced extracellular polymer substance (EPS) consisting of polysaccharide, protein and DNA. Bacterial biofilms are of

clinical relevance since they confer resistance to antibiotics and disinfectants, as well as resistance to phagocytosis and the host immune system generally, all factors promoting chronic infections. Bio film production test in our study is conducted by two methods i.e Tube adherence method (TAM) and Congo red method (CRA), which can be indicated for the routine detection of biofilm production. Tube adherence method to be easily applicable, cost effective and it also guarantees reliable results with excellent sensitivity and specificity [85]. The CRA method is fast, reproducible, and presents an advantage: the colonies remain viable in the medium for further analysis. The method is easy to carry out and the results are usually based on the colony color produced, which ranges from red for non-biofilm-producing strains to black for biofilm-producing strains [86]. The CRA method presented 89% sensitivity and 100% specificity when compared to PCR as a standard [87]. These phenotypic methods are simple and cost-effective for screening method of biofilm formation and do not require technical expertise, which makes it appropriate for laboratory use in a developing country like Nepal. Tissue culture plate technique is considered the most reliable method for screening of biofilm. However, TCP method was also not used due to the requirement of technical expertise for the use of sophisticated ELISA reader to measure density of stained bacterial biofilms adherent to plastic surfaces spectrophotometrically[88].

Using these methods, 72.5% of total blood isolates were found to be biofilm producers. It has been reported that more than 50% of total human infections are associated with biofilm production [89]. Similar type of studies [90] on *Enterobacteriaceae* showed 59.2% of the tested organisms have shown the potential to make biofilms in-vitro. Similarly, 66.67 % of *S.sureus* isolates in our study have potential to form biofilm in vitro which is slightly lower than that reported on [91] and among the CONS isolated 75% were biofilm producers which is comparable to [92]. They reported 82% CONS to be biofilm producers.

In this study, we found that ESBL producing isolates had a higher ability to form biofilm in comparison with non-ESBL producing isolates. Our study reported 84.09% of ESBL producers showed biofilm producers which is in comparison to [90]. It has been suggested that a number of chromosomal gene rearrangement occurs upon acquisition of the ESBL plasmid and higher mortality and severity of infection caused by ESBL producing isolates is due to the expression of several virulence genes simultaneously.

There was significant association of ESBL production and level of multidrug resistance among MDR ESBL isolates ($p>0.05$) and similar results was found on [83, 93]. Similarly, the association between Multi-drug resistant and Biofilm producers was found to be statistically significant ($p>0.05$).

5. Conclusion

Blood culture still remains as one of the most important microbiological tests available to the clinician for the diagnosis of bacteraemia. The blood culture positivity rate in our study was 16%. The study revealed that the gram-negative bacilli predominates the culture positive cases and *E.coli* are the leading etiological agents followed by *S.aureus*. The isolates showed higher rate of in-vitro susceptibility towards Amikacin for both gram-negative and gram- positive isolates. The most of the isolates are MDR and biofilm producers. Increased rate of multi drug resistance (including methicillin resistance in *staphylococci*) is challenging conventional therapeutic regimen complicating the management of bacteremia. Resistance to recommended drugs like

cephalosporins, aminoglycosides and fluroquinolones are of major concern and aware the clinicians and the health care workers to seek for alternative antimicrobial agents. Our data underscore the need for periodic survey of etiological agents and their resistance surveillance reports which can provide valuable insight into resistance trends to assist in guidance in the appropriate choice of empiric therapy. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments.

6. Recommendations

- Resistance to recommended drugs like cephalosporins, aminoglycosides and fluroquinolones are of major concern and aware the clinicians and the health care workers to seek for alternative antimicrobial agents. Our data underscore the need for periodic survey of etiological agents and their resistance surveillance reports which can provide valuable insight into resistance trends to assist in guidance in the appropriate choice of empiric therapy.
- Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments.

7. Limitations

As the study conducted in Manmohan Memorial Medical College and Teaching Hospital does not represent whole scenario of country, the surveillance should be carried out through out the year covering wide geographical region in order to obtain information regarding variation of pathogen and their antibiotic sensitivity profile.

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APPENDIX-I

Questionnaire

1. Patient's ID:

Date:

2. Patient's name:

3. Age:

4. Sex:

5. Address:

6. Patient type: OPD/ Emergency/Ward

7. Clinical signs and symptoms:

Clinical Sign and symptoms	Yes	No
Fever		
Abdominal pain		
Headache		
Vomiting		
Cough		
Constipation		
Diarrhea		
Back pain		
Chills and rigor		
Red spots		

8. Duration of illness: less than 1 week / 1 to 2 weeks / More than 2 weeks

9. Temperature record:

10. Prior consultant:

11. Prior treatment of antibiotics: Yes/No

If yes, name of antibiotic:

APPENDIX-II

A. GRAM-STAINING PROCEDURE

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to be washed out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

A thin film of the material to be examined was prepared and dried.

The material on the slide was heat fixed and allowed to cool before staining.

1. The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.
2. The slide was rinsed with tap water, shaking off excess.
3. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
6. The slide was flooded with counter-stain (safranin) for 1 minute and washed off with tap water.
7. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

APPENDIX-III

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test: This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H₂O₂ was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test: This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria*, *Alcaligenes*, *Aeromonas*, *Vibrio*, *Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-*p*-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test: This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red test: This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) test: The principle of this test is to determine the ability of some organisms to produce an acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanediol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges-Proskauer negative or methyl red negative and Voges-Proskauer positive. The Voges-Proskauer test for acetoin is used primarily to separate *E. coli* from *Klebsiella* and *Enterobacter* species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for

maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

F. Citrate Utilization test: This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon's Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility test: This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile. Motile bacteria may contain a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37°C for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test: The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

- a. Yellow (Acid)/ Yellow (Acid), Gas, H₂S → Lactose/ Sucrose fermenter, H₂S producer.
- b. Red (Alkaline) / Yellow (Acid), No Gas, No H₂S → Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H₂S production.
- c. Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.

d. Yellow (Acid)/ No Change → Glucose- Oxidiser.

e. No Change / No Change → Non-fermenter.

I. Urea Hydrolysis test: This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. Positive organism shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

J. Coagulase test: This test is used specifically to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. saprophyticus*, *S. epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *S. aureus*. Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

Slide Coagulase Test: Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

Procedure: For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

Tube Coagulase Test: This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

Procedure: In the tube coagulase test, plasma was diluted 1:10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*S. aureus* culture), and 0.5 ml negative control (*S. epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37°C on a water bath for 6 hours and observed for gel formation in every 30 minutes. The clotting is observed by gently tilting the tube for positive coagulase test.

APPENDIX IV

Inhibitor Potentiated Disk diffusion (IPDD) Test/ Combined Disk Assay for ESBL confirmation using MASTDISCS™ ID Extended Spectrum beta- lactamase (ESBL) Detection Discs

ESBL production was confirmed among the suspected bacterial strain according to the guidelines of CLSI (Clinical and Laboratory Standard Institute) for phenotypic confirmatory testing. According to these guidelines, when confirming ESBL production among the suspects using Combined Disk (CD) assay, an increase in zone size of ≥ 5 mm from either of the combination disk i.e. clavulanate containing disk indicates the presence of ESBL in the test organism. The suspected organism was inoculated into Muller hinton broth and incubated at 37°C until the turbidity matched 0.5 Mc farland standard. Using a sterile cotton swab the test organism was aseptically cultured on a MHA plate.

- 1) With the help of sterile forceps, the ESBL detection disc were placed onto the inoculated medium ensuring that they are evenly placed.
- 2) The plate was incubated at $35-37^{\circ}\text{C}$ for 18-24 hours and the results interpreted.
- 3) Interpretation of the results: we compare the zone of inhibition for the ceftazidime and cefepime disc to that of the ceftazidime, and cefepime plus clavulanic acid combination discs. An increase in zone diameter of ≥ 5 mm in the presence of clavulanic acid from any or all of the discs indicates the presence of ESBL in the test organism.