MICROBIOLOGICAL STUDY OF ANAEROBIC AND AEROBIC ISOLATES FROM DEEP SEATED INFECTIONS IN PATIENTS ATTENDING A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN

THESIS

SUBMITTED TO

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF MEDICINE (MD)

(MICROBIOLOGY)

JUNE, 2022 **DR. ADITYA KUNDU**

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DECLARATION

I, hereby declare that the thesis entitled "**MICROBIOLOGICAL STUDY OF ANAEROBIC AND AEROBIC ISOLATES FROM DEEP SEATED INFECTIONS IN PATIENTS ATTENDING A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN**" embodies the original work carried out by me in the Department of Microbiology at All India Institute of Medical Sciences, Jodhpur.

I further state that no part of the thesis has been submitted either in part or in full for any other degree of All India Institute of Medical Sciences or any other Institute/ University.

Aditya Kundu

Dr Aditya Kundu

Department of Microbiology

All India Institute of Medical Sciences, Jodhpur

CERTIFICATE

This is to certify that the thesis entitled "**MICROBIOLOGICAL STUDY OF ANAEROBIC AND AEROBIC ISOLATES FROM DEEP SEATED INFECTIONS IN PATIENTS ATTENDING A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN**" is the bonafide work of **DR. ADITYA KUNDU** carried out under our guidance and supervision in the Department of Microbiology, All India Institute of Medical Sciences, Jodhpur.

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It is further certified that the candidate has fulfilled the pre-requisites necessary for the submission of this thesis work.

Dr. Sneha Ambwani

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Dr. Aditya Kundu

INDEX

LIST OF ABBREVIATIONS

- AST : Antimicrobial Susceptibility Testing
- CDC : Centre for Disease Control and Prevention
- CIM : Carbapenem Inactivation Method
- CLSI : Clinical Laboratory Standards Institute
- CRE : Carbapenem Resistant Enterobacteriaceae
- DSPI : Deep seated pyogenic infection
- ECA : Enterobacterial Common Antigen
- ESBL : Extended Spectrum Beta-Lactamases
- eCIM : EDTA-modified Carbapenem Inactivation Method
- GNB : Gram-negative bacilli
- GPC : Gram-positive cocci
- GPB : Gram-positive bacilli
- HLAR : High level aminoglycoside resistance
- IMP : Imipenem-resistant Pseudomonas Carbapenemases
- IPD : In Patient Door
- IUCDs : Intra-uterine contraceptive devices
- KPC : Klebsiella pneumoniae Carbapenemase
- LPS : Lipopolysaccharide
- MacF : MacFarland
- MBL : Metallo-beta-lactamases
- mCIM : Modified Carbapenem Inactivation Method
- MDR : Multi Drug Resistant
- MHT : Modified Hodge Test

- MIC : Minimum Inhibitory Concentration
- MR : Methyl Red
- MRSA : Methicillin Resistant *Staphylococcus aureus*
- NDM : New Delhi Metallo Beta-lactamase
- ONPG : Ortho-Nitrophenyl-Beta-Galactopyranoside
- OXA : Oxacillinase-type β-lactamases
- OPD : Out Patient Door
- PAD : Phenyl-Alanine Deaminase
- PBP : Penicillin Binding Proteins
- PCR : Polymerase Chain Reaction
- SCV : Small Colony Variant
- SHV : Sulph-Hydryl reagent Variable
- SPS : Sodium Polyanethol Sulphonate
- TEM : Temoniera
- VIM : Verona integron-encoded metallo-β-lactamase
- VP : Voges Proskauer
- VRE : Vancomycin Resistant *Enterococci*

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SUMMARY

Deep seated pyogenic infections (DSPI) are one of the most common causes of hospitalization resulting in high mortality & morbidity due to Multidrug resistant (MDR) superbugs. DSPI are majorly polymicrobial in nature caused by anaerobic and aerobic bacteria. Very few studies have reported anaerobic isolates in DSPI since most set ups do not routinely perform anaerobic culture. Thus, this study was formulated to determine both aerobic & anaerobic bacteria from deep seated infections in patients presenting to this Institution.

AIM & OBJECTIVES: To study the anaerobic and aerobic bacterial isolates from deep seated pyogenic infections in patients attending a tertiary care hospital and to study the antimicrobial susceptibility pattern of the isolates.

MATERIAL AND METHODS: This was a prospective observational study conducted in the Bacteriology section of Department of Microbiology of a tertiary care hospital of Jodhpur from Jan 2020 – Dec 2021. Patients of all age group presenting in the OPD, IPD and OT with DSPI were included in this study. The sample from deep seated infections like aspirated pus, tissue, biopsy material, etc., were collected and processed in both anaerobic and aerobic conditions and the isolates obtained were identified by both conventional and automated methods. Their antibiotic susceptibility was done according to CLSI 2020.

RESULT: Out of the 107 samples collected during the study period from Jan 2020-Dec 2021, culture positive was seen in 74.8% (n=80) samples, out of which 85% (n=68) were monomicrobial and 15% (n=12) were polymicrobial. Among these 80 culture positive samples, 92 isolates were isolated; in which aerobic isolates were 92.3% (n=85) and anaerobic were 7.7% (n=7). Among aerobes, most common organism was *Klebsiella pneumoniae* 35% (n=30) followed by *Escherichia coli* 26% (n=22). *Staphylococcus aureus* were isolated in 18% (n=15). Among the anaerobes, *Bacteroides fragilis* 71% (n=5) was the predominant isolate followed by *Peptostreptococcus anaerobius* and *Clostridium perfringes*. Among the aerobes, MDR was detected in *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii*. MRSA was detected in 60% cases. ESBL and MBL was detected in 15.7% and 43.7% of total GNBs respectively. ESBL was seen in 16.6% and 22.72% in *Klebsiella*

pneumoniae and *Escherichia coli* respectively. MBL was also detected in 60% *Klebsiella pneumoniae*.

Conclusion: This cross-sectional study gives an idea about prevalence of the common aetiology causing deep seated pyogenic infection and also the antibiotic resistance pattern of the isolates in the western part of Rajasthan. This study will help the clinicians to select the antibiogram against the common isolates isolated from DSPI as a part of good antimicrobial stewardship programme which will help further in controlling the MDR superbugs among DSPI.

INTRODUCTION

Deep-seated infections are generally pyogenic in nature. Pyogenic infections are infections that are caused by microorganisms and are characterized by the formation of exudates [1]. Exudates or pus is composed of cellular debris, dead leukocytes and necrotic debris. Deep-seated infections may be localized as an abscess or inflammatory necrosed tissue caused by suppuration in a tissue, an organ, or a confined space, which are lined by a pyogenic membrane [1]. In DSPI, there may be necrosis of the underlying tissues which ultimately leads to generalized disease through toxic metabolites associated with the invasion of the bacteria. Deep-seated infections can occur in any part of the body like an infection involving fascia or muscle mass or deep involving internal organs. These infections are generally associated with significant morbidity and mortality [2,3].

Pyogenic infections are generally non-fatal if present in superficial surfaces of skin and dermis. Cases of liver abscess, brain abscess can lead to the death of the patient if not treated properly [4]. Pyogenic infections are predominantly caused by Gram-positive organisms mostly *Staphylococcus* spp. [1]. Nowadays many Gram-negative bacteria also lead to pyogenic infections. Data from the National Nosocomial Infections Surveillance system suggest that isolation of *Staphylococcus aureus* has been increased from 35.9% to 64.4% from pyogenic samples [2].

The diversity and pathogenicity of the pyogenic bacteria basically depend upon the anatomical site of the infection, any previous predisposing factor, type of the infection, the level of perfusion in the tissue by the organisms, and the immune response of the host to the infection [6,7].

Anaerobic bacteria cause deep pyogenic infections which are mostly polymicrobial or mixed with aerobic bacteria [3,6]. Any injury in mucosal barriers due to trauma, surgeries lead to infections by these microbes as these microbes are generally present as commensal flora. The Infections caused by *Clostridium spp.* are mainly of exogenous origin [1]. The most commonly seen anaerobes in clinical specimens include the following bacteria- *Bacteroides fragilis* group, pigmented *Prevotella* spp., *Peptostreptococcus* spp., *Fusobacterium* spp., *Porphyromonas* spp., *Clostridium* spp., *Actinomyces* spp [3,6,8]. Anaerobic bacteria have been isolated from a wide variety of

infections in patients ranging from abscesses, diabetic foot, peritonitis, dental carries, various head and neck infections to life-threatening infections such as gas gangrene. Anaerobic bacteria are the predominant indigenous microflora of humans and plays an important role in infections, some of which are serious and carry a high mortality rate.[2]

Aerobic bacteria like *Staphylococcus, Streptococcus, Pseudomonas* spp*, Escherichia coli, Klebsiella* are associated with pyogenic infections [3]. Nowadays overuse of antibiotics results in a serious condition of antibiotic resistance among the bacteria. It not only makes the treatment difficult but also makes the treatment expensive. Thus, it is necessary to know the antibiotic susceptibility for proper treatment of the patient [4].

Because the majority of these infections are dangerous and can result in high death rates, it is critical to correctly identify the organisms in deep-seated infections. However, as deep-seated infections are frequently polymicrobial, this study aims to identify both aerobic and anaerobic bacteria from deep-seated infections in patients attending this tertiary care hospital in Western Rajasthan. The research will also allow the development of a rapid antibiogram, which will be used as part of a good antimicrobial stewardship programme to tackle MDR superbugs in deep pyogenic illnesses. In order to comprehend drug resistance mechanisms and detect emerging drug resistant organisms, more study is needed in this area.

REVIEW OF LITERATURE

One of the significant subgroups of infections which are encountered by physicians in health care settings worldwide are the pyogenic infections. These infections are associated with high morbidity. Appropriate and prompt antibiotic therapy is needed to combat long-term complications associated with it. Along with this it also sometimes requires surgical drainage of the pus. Challenges are being faced in the treatment of pyogenic infections due to the emergence of the Multi drug resistance (MDR) superbugs [1]. In particular the resistance developing in the Enterobacterales and *Staphylococcus aureus* are of high concern [5].

Based on the Global Burden of Disease and the World Bank data the crude mortality due to infectious disease in India is around 416 per 100000 population [1].

Anaerobic bacteria are one of the major parts of indigenous microflora of humans and plays a crucial role in infections. Anaerobic bacteria were first described by Louis Pasteur in the year 1862.The first anaerobic bacteria to be isolated in a clinical laboratory was *Bacteroides fragilis* in the year 1893 [8]. In the subsequent decades, anaerobes were found to be one of the most important causes of puerperal sepsis, intra-abdominal abscesses, lung abscess. Over the next few decades not so much studies have been done on the non-spore forming anaerobes and the research focused on spore forming anaerobes. The reason may be due to increase in incidences of fatal diseases like gas gangrene and tetanus as these cases were more observed among the soldiers in world war. Another reason may be that there was no standardization in the nomenclature of the anaerobes [3,8].

Then in the year 1965 there was a marked increase in the studies of anaerobic bacteria by Sidney Finegold, who is also known as the father of the anaerobic microbiology [7]. By 1980 anaerobic culture for brain abscesses, dental infections, otitis media, lung abscesses, cutaneous abscesses, pelvic infections, intra-abdominal abscesses were started in India. Anaerobic infections were treated mostly with metronidazole and clindamycin. In India, metronidazole is the treatment of choice. Intravenous metronidazole is also used in critical patients [3].

DEFINITION

Deep seated infections are the infections involving the deeper layers of skin and the internal organs. The causative agents and the pathogenic mechanisms involved in both the types are different in many aspects. The localized collection of abscesses also can spread to the deeper layers or it can disseminate depending on the various host factors to cause more detrimental conditions [1,2].

An abscess is a localized collection of pus in a cavity formed by the disintegration or necrosis of tissue, resulting in a firm, tender, erythematous nodule that becomes fluctuant. [1,2]

TYPES OF DEEP-SEATED INFECTIONS

There are two different types of deep-seated infections based on the anatomy of the site involved [1,2].

They are: -

1. **Infections involving deeper layers of skin**
2. **Deep seated infections involving the internal organs**

Infections involving deeper layers of skin-

Skin or tissue infections, skin structure deep seated infection, soft tissue infections are the general terms for infection of entire skin layer which also comprises the subcutaneous, muscle layers and the respective fascia. Following anatomical structures can be involved:

- Skin layer (bacteria, yeasts, viruses, dermatophytes, parasites)
- Subcutaneous tissue layer
- Deep connective tissue also called fasciitis
- Muscle, e.g., myositis; myonecrosis

Among this, Infection involving deep connective tissue and muscles are considered as the deep-seated infections. Cellulitis is a spreading infection of the epidermis and subcutaneous tissues. Staphylococcal and streptococcal species are the most frequent isolates recovered and are also the most common organisms implicated

in recurrent cellulitis [3]. Common physical findings in cellulitis include erythema, oedema, warmth, and tenderness of the affected area [1]. Patients may also experience fever, tender lymphadenopathy, and abscess formation, especially if *Staphylococcus aureus* is implicated as the causative agent.

Necrotizing fasciitis may be caused by a single organism (e.g., *Streptococcus pyogenes, Staphylococcus aureus*) but is more commonly polymicrobial (mixed aerobic and anaerobic species). Based on the causative agents necrotizing fasciitis bare classified as Type1, Type 2 and Type 3. Necrotizing infections typically progress more rapidly (within 24–48 hr) than superficial cellulitis and have more devastating consequences, namely the destruction of fat, fascia, and underlying muscle [1,2,8].

Deep seated infection in the internal organs

1. Brain Abscess

Brain abscesses are one of the serious and life-threatening infections. Sources of abscess formation include [9]:

a) Direct spread to the brain.
b) Hematogenous spread following any chronic infections or sepsis.
c) Cryptogenic

Site of the infection also plays an important role in the prognosis; Brain stem abscesses are mostly fatal. Microorganisms isolated from abscesses are mostly bacterial which includes aerobic and anaerobic bacteria [10,11]. Mixture of aerobic and obligate anaerobes are common [12].

Among aerobes Klebsiella pneumoniae, Escherichia coli, Streptococcus pneumoniae, β-haemolytic streptococci, S. aureus and anaerobic *Streptococc*i, anaerobic Gram-negative bacilli like *Bacteroides, Porphyromonas* are most commonly isolated organisms [4,8].

According to the part of the brain involved organisms isolated also varies. Any microorganisms isolated from the abscess should be considered of clinical significance. Organisms such as skin commensals or which are environmental like *Peptostreptocci,* Clostridium species can cause brain abscess following direct trauma.

Brain abscess usually occurs if the organisms infect the cerebral tissue; most of them spreads either by hematogenous or via direct injury. In 10-37 percentage cases of brain abscess the cause remains cryptogenic. Mortality in these infections is very high even treated properly [8]. Treatment includes draining of the pus from the abscess along with appropriate antimicrobial therapy.

2. Liver Abscess

Liver abscess can be of pyogenic, parasitic, fungal (in case of immunocompromised patients). It is an exudate filled structure in the liver [1,4]. It could be single or can be of multiple numbers. Symptoms comprise of abdominal pain, fever but in general it is nonspecific without any localized features. Pyogenic abscesses are usually multiple in nature and they are fatal [13,14]. They require to be treated with aspiration of the exudate along with antibiotic therapy [14]. They generally occur in the older population and are usually secondary to a sepsis which is of portal venous distribution. Examples for cause of the liver abscess are: biliary tract infections, extrahepatic locus of any metastatic infections, trauma, surgery [13].

Different microorganisms can be isolated from it. Most commonly isolated species are [1,5]: Enterobacterales including *Klebsiella pneumoniae, Escherichia coli, Clostridium* species, *Bacteroides* species, anaerobic *Streptococci, Enterococcus*

3. Dental abscesses

Microorganisms that colonize the teeth and gums are usually responsible for dental and oral infections ultimately causing dentoalveolar abscesses [6,15]. It may also occur following trauma or after a surgery. The infection may lead to periodontitis or gingivitis. Organisms causing this infection are strictly anaerobic or facultative anaerobes. Most commonly isolated are GNBs [15,16]; other organisms may be: *α-hemolytic Streptococci,* Anaerobic Gram-negative bacilli, Anaerobic *streptococci "S. anginosus*" group, *Actinobacillus actinomycetemcomitans, Spirochaetes, Actinomyces* species. Aspiration is required for the treatment as well as diagnosis. Antibiotic therapy is essential for the treatment.

4. Pancreatic Abscess

Pancreatic abscesses are due to complications of acute pancreatitis. Mostly they are polymicrobial which includes *Klebsiella pneumoniae*, *Escherichia coli*, other Enterobacterales, *Enterococci* and anaerobes. Can also be infected by *Staphylococcus aureus* [5,7,9].

5. Anal abscesses

Anal abscesses are usually classified on their location in relation to structures which comprise and surrounds the anus and rectum region: ischioanal, perianal, supralevetor and intersphincter. Most common is the perianal abscess and the least common is the supralevator. If there is spread of the abscess partially or circumferentially around the rectum or anus region it is called horseshoe abscess. Anal abscesses are usually caused by the Enterobacterales, *Staphylococcus, Enterococci*, anaerobes.[13].

6. Intra-abdominal abscess

Intra-abdominal abscess is any infections involving the normally sterile peritoneal cavity [1,3,5]. It can be a loculated collection or diffuse. The term covers primary and secondary peritonitis.

Primary peritonitis is infection of the peritoneal fluid in which no perforation of a viscus has occurred. Infection usually arises via haematogenous spread from an extra abdominal source and is often caused by a single pathogen [1]. It is common in patients with ascites following hepatic failure [6]. In females it may also result from organisms ascending the genital tract, for example *N. gonorrhoeae* and *Chlamydia trachomatis*, *Pneumococci*, *Actinomycetes*, Enterobacterales and *Streptococci* have been associated with peritonitis in women with IUCDs but can cause primary peritonitis in any patient group at any age [17,18].

Secondary peritonitis is acute, suppurative inflammation of the peritoneal cavity usually resulting from bowel perforation or postoperative gastrointestinal leakage. Secondary peritonitis is most often treated with a combination of surgery and antibiotics. The most frequent isolates encountered in intra-abdominal sepsis with secondary peritonitis are derived from the normal gastrointestinal flora [1,2]. Anaerobic bacteria are isolated from the majority of cases with *Bacteroides* species being isolated

[8]. However, infections are usually polymicrobial and organisms that have been isolated include: *Staphylococcus aureus*, *Enterococcus* species, *Bacteroides* species, *Pseudomonads, Peptostreptococcus* species, *β-haemolytic streptococci, Clostridium* species, Enterobacterales [13,19,20].

EPIDEMIOLOGY

PREVALENCE AND DISTRIBUTION:

Aerobic pathogen	**Itzhak Brook *et al.* [17]**	**Pramodhini S *et al.* [14]**	**Saini *et al* [21]**	**Manmeet kaur Gill *et al.* [22]**	**Basireddy *et. al.* [5]**	**PoonamVerma *et al.* [23]**	**JM Kalita *et al.* [24]**
Klebsiella pneumoniae	2%	9.7%	9%	12.3%	12%	33%	14.4%
Escherichia coli	15%	17.9%	13%	29.2%	24%	16%	24.7%
Enterobacter aerogenes	-	-	-	4.9%	-	-	1.7%
Klebsiella oxytoca	-	-	-	-	-	-	-
Citrobacter freundii	-	-	-	1.3%	4%	-	1.4%
Proteus vulgaris	9%	4.4%	4%	3.1%	4%	7%	1.5%
Pseudomonas aeruginosa	3%	-	11%	11.1%	9%	18%	16.6%
Acinetobacter baumannii	-	-	-	8%	-	-	8.3%
Staphylococcus aureus	26%	38%	30%	20%	29%	40%	30%
Enterococcus faecalis	7%	-	-	1%	6%	-	4.6%
Streptococcus pyogenes	-	16%	6%	0.2%	6%	-	0.5%

Table 1: Prevalence of aerobic bacteria causing pyogenic infections

Various studies have shown the prevalence of aerobic and anaerobic bacteria in deep seated infections shown in Table 1 and Table 2 respectively.

Anaerobic pathogen	**Itzhak Brook *et al*. [21]**	**Pramodhini S *et al.* [22]**	**Saini *et al*. [21]**	**Basireddy *et. al.* [5]**	**Kedar Mohan *et al*. [25]**
Bacteroides fragilis	16%	59%	60%	23%	-
Peptostreptococcus species	35%	41%	-	41%	38%
Clostridium species	10%	-	-	9%	50%
Fusobacterium species	10%	-	20%	6%	18%
Prevotella species	10%	-	-	9%	12%

Table 2: Prevalence of anaerobic bacteria in causing pyogenic infections

In a study conducted in Europe in 2017, [26] out of the total 2227 specimens, a total number of 336 anaerobes were isolated. Anaerobes as a polymicrobial flora with aerobes were isolated from 159 (57.2%) samples.

In 2016 a study was conducted in Karnataka [3] in which 261 samples were included of which 91 (24.5%) anaerobes were isolated. Anaerobes as a monomicrobial infection were found in 21.9% cases and as a polymicrobial in association with aerobic bacteria were seen in 71.9% cases. Most predominant were *B. fragilis* (20.9%) followed by *Prevotella* species.

A study that took place in Telangana [5] in the year 2015 which included 103 samples of which 72 (70%) were culture positive. Out of these 72 samples obligate anaerobes were isolated from 14(19%) samples while facultative anaerobic or aerobic were isolated from 54(75%) samples.

In a study conducted in a Tertiary care hospital in South India [14] in the year 2017, out of 150 pyogenic specimens 28(18.66%) anaerobes were isolated. Pure

anaerobes were isolated from 51% cases and however as a polymicrobial in association with aerobes were seen in 42.8%. Predominant were *Bacteroides fragilis* (32)

In a study conducted in Western Rajasthan [24] in the year 2017, a total of 1851 pyogenic samples were processed of which, culture positivity was seen in 61.54%. Out of the culture isolates 70.59% were Gram negative organisms, 45.48% Gram positive cocci. *Staphylococcus aureus* (30.9%) was the predominant organism isolated. *Klebsiella* spp. (74.79%) and *Acinetobacter* spp. (74.32%) were mostly found to be multidrug resistant.

In a study conducted at Nepal in 2020 [27], 200 pyogenic samples were processed, in which Gram-negative bacteria were predominant. Predominant pathogens were *Escherichia coli* (35%) followed by *S. aureus* (15.21%) Most of the GNBs were susceptible to Amikacin and GPCs were susceptible to Linezolid and Vancomycin.

In a study conducted in a Tertiary care hospital in India [28] in the year 2018, a total of 1428 patients were enrolled, the total number of isolates from these patients were 1525 and in this monomicrobial infection was seen in 93.2% (1331/1428) patients whereas combined infections with growth of two pathogens in 6.8% (97/1428). *Escherichia coli* was the major pathogen isolated (38.6%) and 31.6% of cases Gram positive organisms were isolated and *Staphylococcus aureus* was the predominant organism.

In a study conducted in a Tertiary care hospital in South India [29] in the year 2017, a total of 1575 pus samples received for culture and sensitivity, 1126 (71.49%) samples were culture positive. Among the culture positive cases the male: female ratio is 1.44. *Klebsiella* species was predominantly isolated 253 (22.5 %). In this 54% of the isolates were ESBL producers, seen mostly in *Escherichia coli* (60%)

A study conducted in Rohtak, India [21] in 2003 included 117 cases. The number of microorganisms isolated per lesion was highest in secondary peritonitis (2.32). The aerobe/anaerobe ratio was 0.81 in secondary peritonitis and 1.8 in necrotizing fasciitis. The most susceptible antibiotics were cefotaxime, ceftizoxime, amikacin and ciprofloxacin

A study conducted in Chennai [25] in 2017 a total of 50 samples were collected most of it being from breast and liver. Of this growth was seen in 78% (39) samples.

Only aerobic growth was seen in 79.48% (31) samples, Mixed (Aerobic + Anaerobic) growth was Seen in 20.52% (8) samples. *E. Coli* and *Streptococcus* were the most commonly found aerobes and *Clostridium* was the most common anaerobe.

HOST FACTORS:

There are multiple risk factors for the development of pyogenic infections. Poor health hygiene, comorbidities, crowding, close contact of an infected person are some of the risk factors [19,20]. Damage in the epidermal layer of the skin leads to the entry of infective agents. The damage could be due to trauma, ulcers, peripheral vascular diseases, or some pre-existing skin diseases like eczema, psoriasis (all this causes fissures in the skin). Poor oral hygiene also leads to deep abscesses in the oral cavity. Lymphoedema and venous congestions can also lead to serious deep-seated infections as the filtration of microbes on that site reduces and microbial count increases [1].

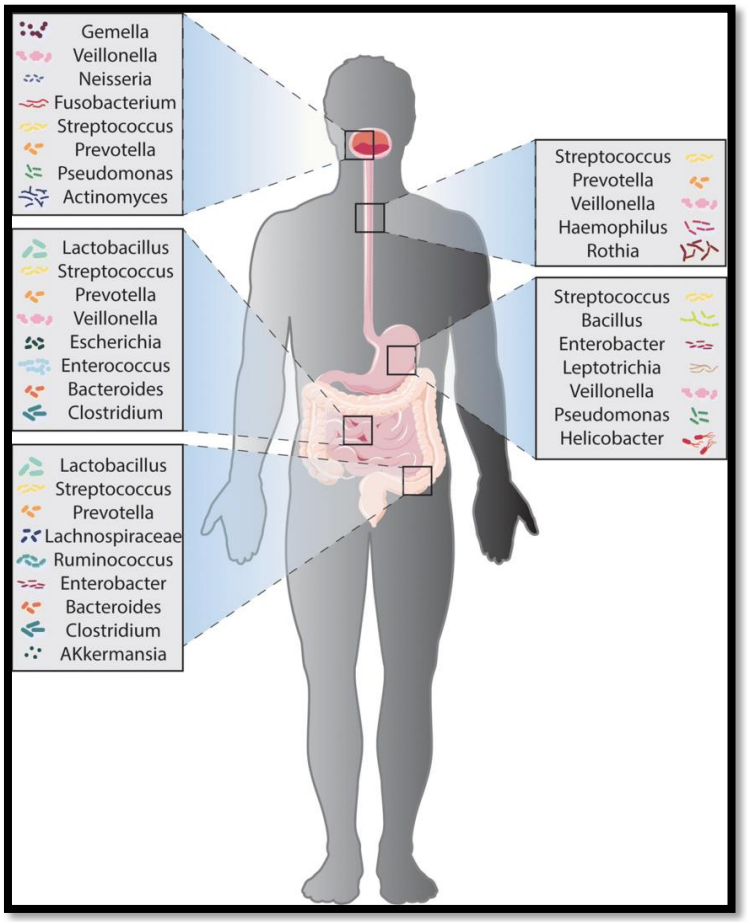


Fig 1: Commensal flora of the human body

A diverse community of trillions of commensal bacteria inhabits mucosal and epidermal surfaces in humans as shown in Fig 1. They have a major role in defence against pathogens. They complete this function by inducing protective responses by the immune to prevent colonization and invasion by pathogens [1]. Any breach in the mucus membrane will lead to the deep-seated pyogenic infections.

PATHOGENESIS AND PATHOLOGY:

The pathogenesis of deep-seated infections is a complex process. Four important events occur during an inflammatory response to promote this aim; vasodilation, activation of endothelial cells, increased vascular permeability and production of chemotactic factors [1]. Vasodilation of the blood vessels increases the blood flow at the inflammatory site, increasing the supply of cells and other factors to the area. Activation of endothelial cells leads to increased expression of cell adhesion molecules, promoting the migration of leucocytes from blood to tissue [7]. Increased vascular permeability makes it easier for cells and proteins to pass through the blood vessel wall and enter the tissue. Chemotactic factors are produced that attract cells into the tissue from the blood stream as in Fig 2. During inflammation, mast cells release chemical factors such as histamine, bradykinin, serotonin, leukotrienes, and prostaglandins [1]. These factors are responsible for sensitizing pain receptors, cause prolonged vasodilation of the blood vessels, and attract phagocytes, especially neutrophils [1,7]. Neutrophils will then trigger other parts of the immune system by releasing factors that recruit other leukocytes. Cytokines are produced by macrophages and other cells of the innate immune system and mediate the inflammatory response.

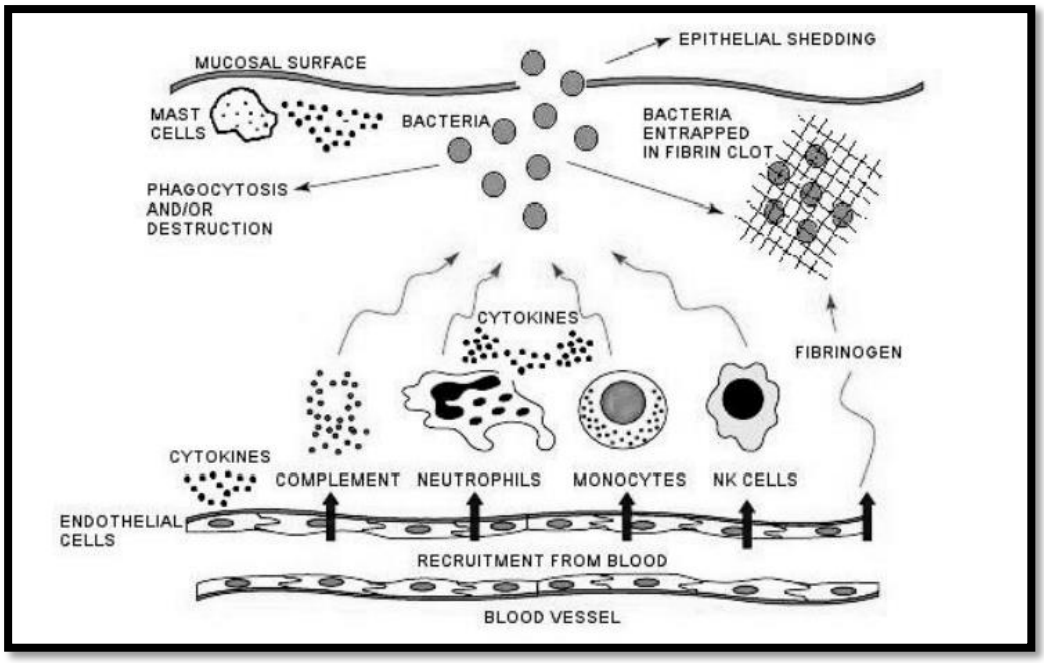


Fig 2: Schematic diagram of pathogenesis of pyogenic infections

Biofilm production: [1,5,7,9]

Biofilm formation is due to the primary attachment of the organism in the specific surface and accumulation in multiple layers over it. Bacteria in the biofilms can resist hundred to thousand-fold of higher concentration of antibiotic as compared to the genetically equivalent bacteria. It causes high mortality and morbidity even after removal of the biofilm and treating with the antibiotics as there is biomatrix associated infections. In biofilm there is also formation of small colony variants (SCV's). Host immune system eliminates the bacteria from the body but the bacteria in the biofilm are not affected as biofilm acts as a shield. As said by Levin and Rozen this persister in the biofilm acts as potential source for emergence of antimicrobial resistance. Many bacteria forms biofilm and causes infections

Causative agents:

1) <u>Aerobic organisms</u> [1]: As shown in Table 3.

SL NO.	TYPE	Causative organisms
1	Gram positive organisms	*Staphylococcus aureus*, Coagulase-negative S*taphylococci*, Beta-haemolytic *Streptococcus, Enterococci, Streptococcus* spp, *Corynebacterium* spp., *Bacillus* spp.
2	Gram negative organisms	*Escherichia coli, Serratia liquefaciens, Klebsiella pneumoniae, Klebsiella oxytoca,* *Enterobacter cloacae, Enterobacter aerogenes,* *Citrobacter freundii, Proteus mirabilis, Proteus vulgaris,* *Providencia stuartii, Morganella morganii, Acinetobacter* spp*, Pseudomonas aeruginosa, Stenotrophomonas maltophilia*

Table 3: List of aerobic organisms causing DSPI

2) <u>Anaerobic organisms</u>: As shown in Table 4.

Non-sporing anaerobes:[8]

Sl. No.	Type		Causative agents
1	Cocci	Gram positive cocci	*Peptostreptococcus, Ruminococcus, Coprococcus, Finegoldia*
		Gram negative cocci	Veilonella
2	Bacilli	Gram positive bacilli	*Eubacterium, Propionibacterium, Actinomyces, Mobiluncus, Lactobacillus.*
		Gram negative bacilli	*Bacteroides, Prevotella, Fusobacterium, Bilophilia, Leptotrichia*

Table 4: List of anaerobic organisms causing DSPI

Sporing anaerobes:[8]

Gram positive bacilli: *Cl. tetani, Cl. Botulinum, Cl. perfringes.*

Most common bacterial pathogens causing deep seated infections are from wound infections are *Escherichia coli*, *Klebsiella* spp., *Staphylococcus aureus, Enterobacter* species, *Citrobacter* species *Pseudomonas aeruginosa, Acinetobacter species* and anaerobes like *Bacteroides* species, *Peptostreptococcus* spp. Deep seated infections can be both polymicrobial or monomicrobial [30,31].

Staphylococcus aureus is a member of the genus *Staphylococcus* belonging to the family Micrococcaceae. Genus *Staphylococcus* consists of more than 30 species out of which *Staphylococcus aureus* is the most pathogenic to human due to its various virulence factors [7]. Other members of this species are *Staphylococcus epidermidis, Staphylococcus saprophyticus*. *Staphylococcus aureus* is a Gram positive 1μm size cocci which in the laboratory can be observed as single, in pairs and in grape like clusters [32]. Characteristic features of this organism are being catalase positive, coagulase positive, non-sporing, non-motile non fastidious and facultative anaerobe. In nutrient agar it gives characteristic golden yellow colour pigment and thus also called yellow staphylococci [7]. *Staphylococcus argentus, Staphylococcus schweitzeri and Staphylococcus aureus* forms *Staphylococcus aureus* complex as all of them are coagulase positive. [33]

S. aureus can adapt in different environmental conditions and mostly being a colonizer, colonize in various parts of the human body like nares, nails, skin, mucus membranes. *Staphylococcus aureus* colonizes about 30% of the population [2]. One-third of the population have colonization in the anterior nares. Asymptomatic carriers are more prone to develop subsequent infection and they play an important role in the spread of the infective strain to their contacts. Through aerosol and physical contact, the organism can disseminate among the recipient host [24]. For subsequent staphylococcal infection colonization of the bacteria is one of the important risk factors [1,2]. It can cause variety of infections ranging from skin and soft tissue infections, superficial wound infections to severe life-threatening infections like septicaemia, endocarditis, pneumoniae, septic arthritis [34]. One of the important causes of hospital acquired nosocomial infections [35]. Besides this can also cause food poisoning, toxic

shock syndrome, staphylococcal scalded skin syndrome through production of various toxins [1].

Staphylococcus aureus produces various toxins, enzymes and cell wall associated factors which play an important role in the virulence of the organism as mentioned in Table 5. [1,7,32,34].

Virulence factor	**Enzymatic function**	**Virulence effect on host**
Coagulase	Binds with prothrombin in the blood	It catalyses conversion of fibrinogen to fibrin.
Catalase	Deactivates hydrogen peroxide into water and oxygen	Protects the bacterial cell from oxidative stress
Hyaluronidase	Breaks down the hyaluronic acid in the connecting tissue	Helps in the growth of the bacteria.
Nuclease	Endonuclease and exonuclease activity	Helps to escape the neutrophil traps.
Staphylokinase	Acts as an activator of plasminogen	Bactericidal effect of the host is lost.
Exfoliative Toxins (ET)	Functions as a 'molecular scissor' which helps in the invasion of the skin.	Responsible for Staphylococcal scalded skin syndrome and impetigo.
Hemolysins	This toxins form pore with having cytolytic activity on the RBCs and the monocytes	All these cause haemolysis
Leucocidins	Leukotoxin which forms pore. PVL combines with δ – haemolysin called synergohymenotropic toxins	Damages leukocytes. PVL breaks the macrophages and neutrophils.
Toxic shock syndrome toxin	Cytokine mediated direct injury to the endothelium.	Causes 'toxic shock syndrome' (TSS)

Table 5: Virulence factors of *Staphylococcus aureus*

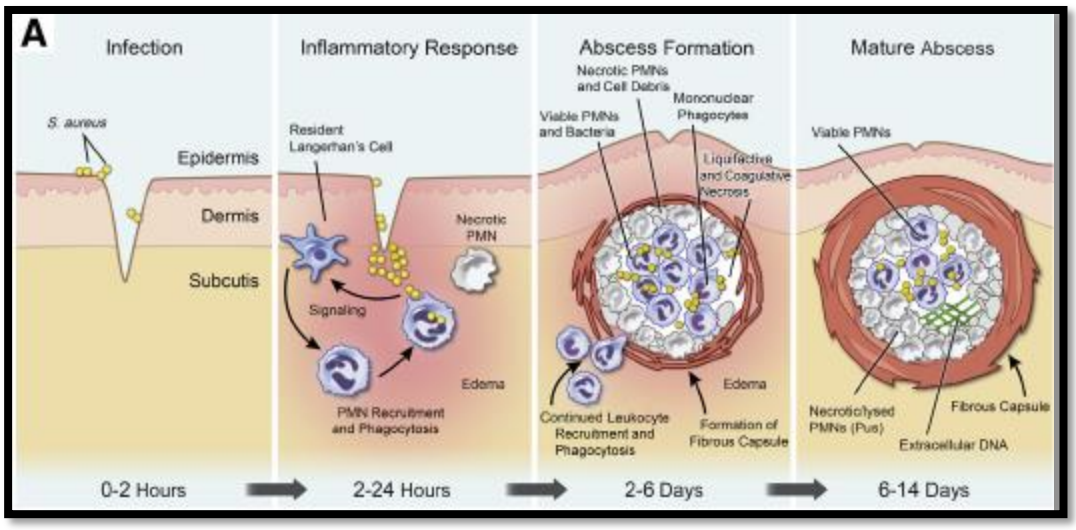


Fig 3: Schematic representation of abscess formation in *S. aureus*

Other Gram-positive cocci like Streptococci and Enterococci also cause DSPI. *S. pyogenes* normally colonizes the pharynx, genital mucosa and anus. Highly contagious infections are caused by *Streptococci*. Usually transmitted through air droplets, hand contact, skin contact with the lesions. Access to skin occurs via abrasion and may lead to cellulitis. Can also infect muscles or fascia causing necrotizing fasciitis or myositis [1,2,7]

Enterobacterales may account for 80 percent of clinically significant isolates of Gram-negative bacilli in clinical microbiology laboratories. They account for nearly 50 percent cases of pyogenic infections. Virulence factors of Enterobacterales are shown in Table 6 [1,2,7].

Organism	**Virulence Factors**
Escherichia coli (As a cause of extraintestinal infections)	Several, including endotoxin, capsule production pili that mediate attachment to host cells

Citrobacter spp., *Enterobacter* spp., *Klebsiella* spp., *Morganella* spp., *Proteus* spp., *Providencia* spp., and *Serratia* spp.	Several factors, including endotoxins, capsules, adhesion proteins, and resistance to multiple antimicrobial agents

Table 6: Virulence factors for clinically relevant Enterobacterales

Other than Enterobacterales, Gram-negative non-fermenters like *Pseudomonas aeruginosa, Acinetobacter species, Stenotrophomonas maltophilia* can also cause DSPI. There virulence factors including endotoxin, exotoxin, fimbriae, resistance to antimicrobials helps to cause the infection. Transmission can be both hospital acquired or community acquired [1,2,7].

DRUG RESISTANCE

Aerobic organisms

Now a days, there is huge increase in the number of multidrug resistance bacteria causing deep pyogenic infections. Various studies show that there is increased prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA), Extended spectrum beta-lactamase (ESBL) producing bacilli, Vancomycin resistant *Enterococci* (VRE), Multi drug resistant *Pseudomonas* etc. causing deep seated pyogenic infections. MRSA are also seen to be resistant to drugs like ciprofloxacin, amikacin. Among the *enterococci*, most of them being High level aminoglycoside resistance (HLAR) the combination of aminoglycoside plus beta lactamase are not effective for their treatment. ESBL producing Gram negative bacteria are showing resistance not only to aminoglycosides and quinolones but also to higher antibiotics like Piperacillin tazobactam and carbapenems [7,36].

In a study it is shown that >70% *Escherichia coli* from the community is resistant to ampicillin, cotrimoxazole and nalidixic acid. Aminoglycosides are

becoming resistant to nearly a third Enterobacterales isolated from clinical specimens. Over last few years third generation cephalosporins resistance increased from 78% to 85% [7]. Similarly, carbapenems are also becoming resistant. Recent studies showed *Klebsiella pneumoniae* to be resistant to carbapenems in >60% cases. Resistance to fluoroquinolones also raised to around 85% cases. High-rate of MRSA isolation has also been documented in various Indian studies, ranging from 32% to 80% [27]. In a study conducted by INSAR there was a steep increase from 29% in 2009 to 47% on 2014 [7].

Antibiotics which are used against *S. aureus* usually target the cell wall synthesis, nucleic acid synthesis, protein synthesis and metabolic pathways [1,6,37]. Antibiotic selective pressure applied by antibiotics which are used in various clinical, agricultural settings have resulted in the development and spread of many genes that are responsible for various resistance mechanisms in the bacteria. Resistance in the bacteria can be acquired due to horizontal gene transfer with the help of mobile genetic factors like transposons, plasmid, integrons etc. or due to internal mechanisms like gene targets mutational modification, efflux pump overexpression. In acquired resistance there is inactivation of the drug by enzymatic activity or by bypassing the target. Antibiotic exposure leads to production of small colony variants (SCVs), persister cells and biofilm formation. Deterministic and stochastic events in the microbial population leads to formation of persister cells which are dormant and multi drug tolerant variant. They cause overexpression of genes; for example, overexpression of toxin-antitoxin module gene in the cell which stops cellular function are altered and there is formation of more lethal and tolerant form of persister [7].

Development of beta lactams

To overcome the problem of development of penicillin resistant, penicillinase resistant penicillin came into picture. They were Beta lactams or azetidin-2, an important structural motif of penicillin, cephalosporin, carbapenem and carbapenem classes of antibiotics. Shortly afterward, the broad-spectrum penicillins and first generation cephalosporins were introduced [36,37,38,39]. They remained a first line of defence against microbes for over 20 years, before resistance due to beta lactamases produced by Gram negative bacilli became a serious problem. To counter this threat, the pharmaceutical industry marketed six novel classes of Beta lactam antibiotics

(cephamycins, oxyimino cephalosporins, carbapenems, monobactams and clavam and penicillanic acid sulfone inhibitors) within a relatively short span of 7-8 years [38,40]. Although, novel beta-lactamases had emerged gradually after the introduction of new beta lactam agents, their number and variety accelerated at an alarming rate.

β-lactamase classification [39,40,41,42]:

Bush-Jacoby system classification	Major subgroup	Ambler system classification	Main attributes
Group 1 cephalosporinases		C (cephalosporinases)	Usually chromosomal; resistance to all β-lactams except carbapenems; not inhibited by clavulanate
Group 2 penicillinases (Clavulanic acid susceptible)	2a 2b 2be 2br 2c 2e 2f 2d	A (serine β-lactamases) A A A A A A D (oxacillin hydrolysing)	Staphylococcal penicillinases Broad spectrum; TEM-1, TEM-2, and SHV-1 Extended spectrum; TEM and SHV variants, predominantly IRT b-lactamases Carbenicillin hydrolysing Cephalosporinases inhibited by clavulanate Carbapenemases inhibited by clavulanate Oxacillin-hydrolysing (OXA)

Group 3 metallo-β-lactamase	3a 3b 3c	B (metalloenzymes) B B	Zinc-dependent Carbapenemases
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

Table 7: Classification of β-lactamase

Ambler class A, C and D are serine β-lactamases which have an active serine site to hydrolyse the β-lactams. On the other Ambler class B β-lactamases are metallo-β-lactamases which require 1-2 zinc for their functions.

Extended spectrum β-lactamases (ESBLs)- Class A β-lactamases

ESBLs are one of the major groups of β-lactamases; which are plasmid coded and belongs to the Ambler class A and Bush-Jacoby class 2be [43,44]. In general, can be defined as ESBLs are resistant to penicillins, first, second and third generation cephalosporins and aztreonams but not to cephamycins or carbapenems and the resistance can be inhibited by β-lactamases inhibitors like clavulanic acid, sulbactam and tazobactam. ESBL producing organisms were first detected in Europe [44,45,]. Although the initial reports were from Germany and England, the vast majority of reports in the first decade after the discovery of ESBL's were from France. The first large outbreak in France to be reported occurred in 1986 [46]. The proliferation of ESBLs in France was quite dramatic. Phenotypic method can be divided into screening test and confirmatory test. ESBLs are most common in *E. coli* and *Klebsiella pneumonia* but do occur in other Enterobacterales especially *Enterobacter, Proteus, Pseudomonas aeruginosa*, *Morganella morganii*.[47]

Carbapenemases [36,48]

The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infections caused by β-lactam resistant bacteria. However, resistance to extended spectrum beta-lactams has been frequently observed among different types of bacteria like the non-fermenting ones. The common form of resistance is mediated by lack of drug penetration (i.e., porin mutations and

efflux pumps) and/or hydrolysing β-lactamases [1]. Phenotypic grouping of theses enzymes is a heterogeneous mixture of beta-lactamases belonging to molecular Ambler class A (penicillinases), class B (metalloenzymes) and class D (oxacillinases). These enzymes have the common property of hydrolysing, at least partially, imipenem or meropenem together with other penicillin or cephalosporin antibiotics

The Ambler class A carbapenemases

A few Amblers class A carbapenemases have been reported in rare enterobacterial isolates. They belong to the group 2f and may form part of the so-called clavulanic acid-inhibited penicillinase group [36].

Ambler Class B Carbapenemases/ Metallo-beta-lactamase [36,44,48]

Acquired carbapenemases are increasingly reported worldwide among nosocomial and community-acquired Gram-negative aerobes. Metallo beta lactamase (MBL) belongs to a group beta lactamase B, which requires divalent cations of zinc as cofactors for enzyme activity. These have potent hydrolysing activity not only against carbapenem but also against other beta lactam antibiotics. The IMP and VIM genes responsible for MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria. The genes responsible for MBL production may be chromosomally or plasmid mediated and hence pose a threat of spread of resistance by gene transfer among the Gram-negative bacteria.

The Ambler Class D β-lactamases

Class D β-lactamases were named oxacillinases (OXA) because they cleave oxacillin in addition to penicillin, distinguishing them from class A β-lactamases and were initially characterized as ESBL's.OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, and OXA-48-like family members have been found in the Enterobacterales [39,40].

AmpC β-lactamases/Cephalosporinases Class C β-lactamases [33]

Plasmid mediated AmpC β-lactamases are resistant to all the β-lactams except carbapenems. AmpC β-lactamases confers resistance to the penicillins, first, second and third generation cephalosporins and aztreonams but are not affected by fourth

generation cephalosporins or β-lactamases inhibitors [33,40,44]. Mostly constitutive but inducible AmpC (DHA-1, ACT-1, DHA-2, CFE-1, CMY-13) have also been reported.

Anaerobic organisms

Anaerobes are organisms which require very less oxygen tension for their growth. After causing infection in the primary site can disseminate to distant part hematogenously. Mostly are mixed with aerobic bacteria causing infections so appropriate methods should be followed to isolate the anaerobic organisms [1,7,8].

Characterized by the following features [8,42]:

- Occurs as local collection of pus forming abscesses
- Reduced oxidation-reduction potential in necrotic and avascular tissues are essential for their growth.
- In case of bacteraemia does not cause disseminated intravascular coagulation (DIC).

Anaerobic infections are mostly due to the breakage of the mucosal barrier and ultimately entry of the commensal into the body spaces. Pathogenesis is mostly due to various virulence factor produced by them which are discussed in Table 8 [1,2,8,42].

Virulence factor	**Effect on the host**
Capsule	Polysaccharide in nature Seen in *Prevotella*, *Porphyromonas, Bacteroides* species Prevents phagocytosis and destruction by neutrophils. Helps in adhesion to mucosal and peritoneal mesoepithelium.
Fimbriae (pili)	Adherence to mucosa
Enzymes	Lecithinase, Phospholipase, Lipase, N-acetyl glycosaminidase, neuroamidase, glucosidase, elastase, heparinase, collagenase, elastase Play dual role in providing nutrients and causing tissue damage.
Metabolic products	Short chain fatty acids (SCFA) produced as by products acts as a leukotoxins

Lipopolysaccharide	Demonstrated by *Bacteroides, Porphyromonas, Fusobacterium* Cause endotoxic damage and reduce opsonization.
Metabolic synergy	One type of bacterial species can increase the activity of another bacterial spp. in a case of mixed infection. Usually by four pathways 1. Inhibits function of leukocytes 2. Improve the surrounding micro environment 3. Provision of nutrients 4. Enhancement of virulence factors
Toxins	Such as leukotoxin, haemolysin and endotoxin Lethal, dermonecrotic and haemolytic effect on host.

Table 8: Virulence factor of anaerobic bacteria causing pyogenic infections

In the present era, anaerobic infection is increasing, and commonly used drugs for the treatment of anaerobic infections also developing resistance day by day [50]. Anaerobic organisms have high rate of virulence and their incidence are also increasing day by day, not properly responding to metronidazole therapy and thus have bad outcomes [6]. Most of these infections are serious and sometimes lead to high mortality rate and thus it is necessary to properly identify the organisms in deep seated infections [48]. However, these deep-seated infections are often polymicrobial in nature.

Now a days the commonly used drugs for anaerobic infections are carbapenems, chloramphenicol, nitroimidazole [51]. Clindamycin, penicillin, cefoxitin are less efficacious against anaerobes. Though anaerobes show good antimicrobial susceptibility in vitro study but, in some studies, it was found *B. fragilis* which is resistant to Metronidazole, Clindamycin, Vancomycin also [48,49,50,51,52].

DIAGNOSIS

Clinical and Radiological

Cardinal signs of deep-seated infections are oedema, erythema, tenderness on palpation, increased warmth. Signs of these infections are crepitus, fluctuations, blisters, indurations, or bullae help the physician to determine the grade of infection or presence of the abscess [8,53,54,55]. Symptoms like fever with chills, redness, hypotension are often present in deep infections [56,57,58].

A vigilant travelling history and history of environmental exposure should be taken, as some pathogens are connected with certain geographic locality. Examples like Pseudomonas aeruginosa infections are common from hot tubs, *Vibrio vulnificus* and *Mycobacterium marinum* infections from water source, and *Pasteurella multocida* and *Capnocytophaga canimorsus*are seen in animal bites [59]. A proper history should be elucidated to determine whether the patient have any recently hospitalization history, as this might put the patient at risk of acquiring multidrug-resistant organisms like infection (ie, HA-MRSA, *klebsiella pneumoniae, Escherichia coli*) [60].

While examining the patient of a deep infection, it is important to look for any necrotizing tissue complications. Rapid spread, induration, crepitus in the tissues, fever, hypotension, and severe pain are the physical findings suggest necrotizing fasciitis, which require prompt surgical evaluation [61,62]. Laboratory findings in patients could include leucocytosis more than 15,000 cells/μL, haemoglobin less than 11 g/dL, elevated C-reactive protein, renal failure, hyponatremia [2].

Image studies should be done when deep infections are suspected. Plain films might be helpful to see the presence of air as dark area in the tissues, and ultrasonography might be used to confirm abscess formation and deep infections [63,64]. Computed tomography scans (CT scan) and magnetic resonance imaging (MRI) usually shows air in the affected tissues or enhancement in the presence of intravenous contrast, but these are not specific to necrotizing infections or diabetic foot [65,66,67]. Early surgical analysis is essential when there are possible signs of infections appear on imaging modalities. This allows the appropriate physician to get involved early in patient's treatment. MRI can also help to determine depth of infection by visualizing increased thickness or enhancement of fascia; nonetheless, other medical scenarios like polymyositis can also cause enhancement of fascia and may lead to confusion with infectious causes. MRI has been seen to overestimate the depth of the infection in some cases [64,65]

Microscopy and doing culture are the easy method for identification of the organism. Additionally, ordering cultures are usually cost effective. Samples are collected first and then transported quickly in the laboratory. After collection the samples are stained properly for presumptive identification and then culture done. Observation of the culture plate is done after 48 hours [32]. Growth on the culture plate is examined

properly as all organisms have different growth pattern. Staining and biochemical properties of the organisms give the proper identification of the organisms. Conventional techniques are laborious and time consuming for which now a days automated techniques like VITEK 2 automated technique, MALDI-TOF are also used for identification. Skin and soft tissue infections are clinically diagnosed.

Getting antibody serology for suspected infections is usually not so helpful in diagnosing deep infections, as systemic antibodies are not produced [13,14,62]. Cultures obtained of secretions from abscesses and other deep infections (e.g., liver abscess, pancreatic abscesses) are usually helpful in identifying the causative organism [63]. Although culture of the lesion or purulent drainage yields the offending organism, empiric therapy should be initiated as early before the culture results become available [64].

Laboratory diagnosis

Aerobic isolates are to be processed by standard bacteriological methods which consists of conventional biochemicals and automated methods [7].

Anaerobic bacteriology is generally not given so much of importance as it is difficult to isolate, culture. To make anaerobic culture cost effective we have to follow some strict principles which include culturing of selective appropriate specimens that are not contaminated, reducing the time of oxygen exposure immediate transport of the specimen to the laboratories and rejecting the inappropriate sample [68,69,70]. Anaerobes are the group of organisms which require very less or no oxygen to grow. They are generally present in humans as normal commensal flora of GI tract, uro-genital tract [1]. Anaerobes are classified based on Gram staining, spore formation, tolerance to oxygen and cellular morphology. Biochemical reactions, serology testing helps in the isolation of the anaerobes [8].

VITEK Automated Identification System: [1,2,7]

First automated identification system: -VITEK system (BioMérieux, Inc., Durham, NC) (developed by NASA). Detects bacterial growth and metabolic changes in microwells using fluorescence-based technology.

Integrated modular system: Filling-sealer unit, reader, incubator, computer control module, data terminal and a multicopy printer.

The VITEK system has cards for the identification of Anaerobes and coryneform, yeast, *Neisseria* species, *Haemophilus* species, Other fastidious organisms, Gram-positive organisms, Gram-negative pathogens (Enterobacterales, non-Enterobacterales, and highly pathogenic organisms such as *Brucella* and *Francisella* species)

This helps in the identification of the species of anaerobes. It has a database of 63 taxa of anaerobes and cornybacterium (ANC). It includes the following genera *Actinomyces, Arcanobacterium, Bacteroides, Bifidobacterium, Clostridium, Cornybacterium, Collinsella, Eubacterium, Eggerthella, Fusobacterium, Finegoldia, Lactobacillus, Micromonas, Microbacterium, Peptostreptococcus, Prevotella, Propionibacterium, Peptoniphilus,Staphylo coccus, Veillonella.*

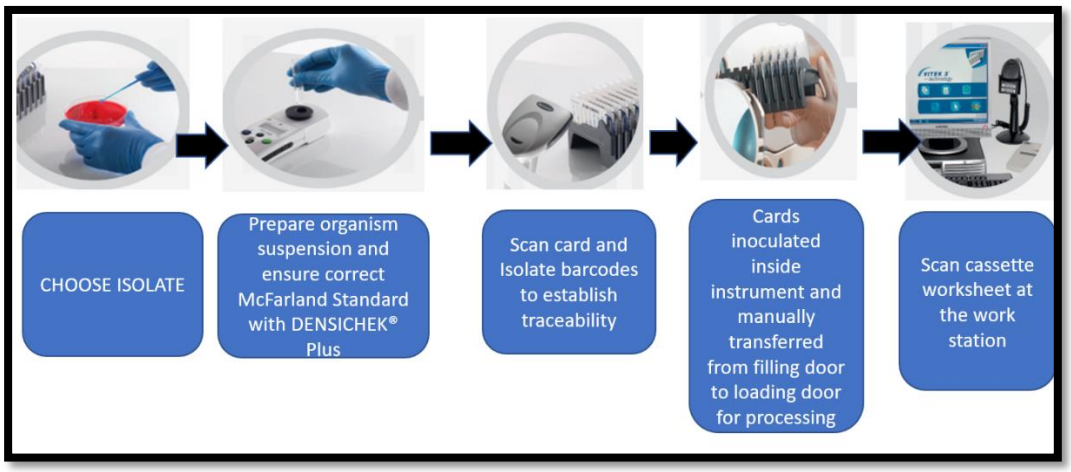


Fig 4: Schematic representation of procedure of VITEK 2.

MALDI-TOF MS: [7]

MALDI stands for matrix which assists in desorption and ionization of highly abundant bacterial and fungal proteins through energy from a laser. The matrix (e.g., α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid) isolates bacterial or fungal molecules from one another, protecting them from fragmentation and enabling their desorption by laser energy; the majority of the laser energy is absorbed by the matrix, converting it to an ionized state.

As a result of random collision in the gas phase, charge is transferred from matrix to microbial molecules. Ionized microbial molecules are then accelerated through a positively charged electrostatic field into a time of flight, or TOF, tube. Inside the tube, which is under vacuum, the ions travel toward an ion detector, with small analytes traveling the fastest, followed by increasingly larger analytes; a mass spectrum is produced, representing the number of ions of a given mass impacting the detector over time. It is highly abundant (predominantly ribosomal) proteins which generate the mass spectrum. Computer software compares the generated mass spectrum to a database of reference spectra, generating a list of the most closely related organisms with numeric rankings. Turnaround time for MALDI-TOF MS is 3 minutes or very less as per standard methods.

Fig 5: MALDI TOF MS

Detection of Drug resistance:

<u>Screening of ESBL:</u> [30,33,]

Disc diffusion test:

Screening test is done by checking the presence of resistance to five drugs ceftriaxone, cefotaxime, ceftazidime, cefpodoxime and aztreonam by disc diffusion method in a MHA plate.

Agar dilution method:

Screening test of the isolate is done using ceftriaxone, cefotaxime, ceftazidime, aztreonam 1µg/ml and cefpodoxime 4 µg/ml in MHA by Agar dilution method.

Confirmatory test for ESBl: [33,41,43]

Combined disc diffusion test:

This test is carried out against cefotaxime 30 µg and ceftazidime 30 µg disc with and without clavulanic acid 10 µg in MHA plate.

≥ 5mm increase in the zone diameter of the combination disc as compared to the individual disc.

MIC reduction test: By agar dilution MIC is performed with cefotaxime and ceftazidime with and without clavulanic acid at 4µg/ml

A ≥ 3 two fold concentration decrease in MIC of the combined drug as compared to individual drug confirms ESBL

Molecular method [28]

PCR is the method for the detection. It is gold standard, specific test. The genes like bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1\text{-}2\text{-}9}$, bla_{OXA2} can be detected for the confirmation.

Screening and Amp C detection: [33]

Screening is done using cefoxitin 30 µg disc on MHA agar

Confirmatory test for AmpC β-lactamases:

For inducible AmpC β-lactamases

Disc antagonism test:

It is done using cefotaxime and cefoxitin disc. Blunting of cefotaxime zone in presence of cefoxitin disc is suggestive of inducible AmpC β-lactamases

Boronic acid Inhibition test:

It is carried out to detect both inducible and non-inducibleβ-lactamases using cefoxitin 30 µg, cefotaxime 30µg, ceftazidime 30 µg discs with and without boronic acid 400 µg. A ≥ 5 mm increase in zone diameter for either microbial disc with boronic acid as compared to individual disc is suggestive of AmpC β-lactamases production

Detection of Carbapenemases: [33,41]

First of all, carbapenem resistance is identified through standard susceptibility testing (disc diffusion test and E test), following which additional phenotypic tests are done to identify CRE. These methods include the modified Hodge test (MHT), the Carba NP test and its variants, carbapenem inactivation method (CIM), and its modifications (modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM). All of them target Carbapenemases production but do not specify the type of Carbapenemases.

MHT [33]

It is simple and inexpensive to perform based on its ability to detect KPC producers. The MHT also demonstrates good sensitivity for many other carbapenemases, including VIM, IMP, and OXA-48-like enzymes. In case of detection of NDM, the test sensitivity is much lower; in one study, only 7 of 14 NDM isolates were MHT positive. Since, NDM producing bacteria are rapidly spreading, this is an important limitation. Also, the MHT has poor specificity because bacteria producing AmpC enzymes combined with porin mutations can give a false-positive result.

Carba NP test [33]

It detects Carbapenemases by measuring the in vitro hydrolysis of imipenem by a bacterial extract. Imipenem hydrolysis will change the pH resulting in colour change of the pH indicator. Other similar tests based on the same principle include the Blue-Carba test, the Rosco Rapid Carb screen, and the Rapidec Carba NP test.

CIM [33]

In this, a suspension of the bacterial isolate of interest and water is made, and a meropenem disc is incubated with this suspension. The meropenem disc is then removed and placed on a Mueller-Hinton agar plate that is streaked with a susceptible laboratory strain of *E. coli*. The absence of an inhibition zone indicates hydrolysis of meropenem in the first step and the presence of a Carbapenemases. The presence of a clearing zone indicates lack of meropenem hydrolysis (no Carbapenemases present. Sensitivity of 98 to100% has been shown. mCIM and eCIM follow the same principle as CIM but instead of water, Trypticase soy broth is used for suspension of the bacterial isolate. Additionally, in eCIM, EDTA is added to trypticase soy broth before suspending the bacterial isolate. eCIM is interpreted only after mCIM comes positive for Carbapenemases. eCIM helps to differentiate between serine Carbapenemases (eCIM negative) and metallo-beta lactamases (eCIM positive). mCIM and eCIM are currently recommended in CLSI guidelines for Carbapenemases detection.

TREATMENT

There are 3 treatment modalities for deep seated infections: [69,70,71,72]

1) medical- involving treatment using antibiotics;
2) Aspiration (freehand, stereo tactically or endoscopy guided)- involving removal of abscess by aspiration.
3) Total excision- involving complete removal of tissue from body

Medical Treatment:

In general, most of this infection can be easily managed on an outdoor basis, although patients having evidence of severe rapidly developing infection, high grade fevers, or other significant signs of systemic inflammation should be treated in hospital setting [48]. Superficial infections usually respond well with the topical agents not need any systemic treatment do not require systemic treatment and usually respond to topical agents. Mild superficial infections can be easily treated with heat packs. In case of deep abscesses, incision and drainage of the pus from the abscesses is required. These procedures are followed by proper application of heat compression as it is needed to solve the infection, especially which are caused by community acquired MRSA. Proper identification of causative microorganisms and its antimicrobial susceptibility is

essential for proper treatment of the patient [73,74]. In few cases, the infections improve even if the initial antibiotic choice is not appropriate, demonstrates that some infections resolve on its own. The reasons of this are not clear but could involve anti-inflammatory effects of these medications [75].

Antibiotic resistance among the pyogenic organisms is on a gradual rise, so it is much important to have knowledge about the resistance pattern and antimicrobial susceptibility for appropriate treatment regimen. This includes resistance and susceptible property of all the Gram-positive and Gram-negative bacterial isolates towards the antibiotics like amoxicillin, penicillin, ofloxacin, cefuroxime, cefazolin, erythromycin, chloramphenicol, ciprofloxacin, azithromycin, tetracycline, norfloxacin, ofloxacin, gentamycin, cefixime, cefuroxime, nalidixic acid, cefotaxime ciprofloxacin, and ceftriaxone [73,74,75,76,77].

Superimposed secondary infection with fungal isolates like dermatophyte, Pittosporum species can also occur in these types of infections, requiring combination therapy of antimicrobials. Systemic Antibiotics in addition to surgical procedures like incision and drainage is preferred in case of abscesses [54,58]

Surgical:

In case of deep infection surgical treatment is often required. It involves the removal of the dead and necrosed tissue of body. If not treated properly this can lead to severe metastatic spread of the infection in the whole body and to the vital organs. [70,77].

AIM AND OBJECTIVES

AIM:

To study the anaerobic and aerobic bacterial isolates from deep seated infections in patients attending a tertiary care hospital and to study the antimicrobial susceptibility pattern of the isolates.

OBJECTIVES:

1. To identify the anaerobic and aerobic bacteria from specimens of deep-seated pyogenic infections by conventional and automated methods.
2. To study the antimicrobial susceptibility of the isolates.

MATERIAL AND METHODS

Study was carried out in Department of Microbiology in All India Institute of Medical Sciences, Jodhpur between Jan 2020-Dec 2021.

STUDY DESIGN:

It was a prospective observational study.

STUDY POPULATION:

All age groups patient with deep seated infections from OPD, IPD, OT of the Institution were included in this study.

INCLUSION AND EXCLUSION CRITERIA:

INCLUSION:

- Patients of all age groups, having deep seated pyogenic infections including brain abscesses, diabetic foot infections, necrotizing fasciitis, deep unhealing ulcers, gas gangrene, intraabdominal abscesses, dental infections, head and neck infections, etc.
- Patients who were willing to give consent for the study.
- Patients with or without history of antibiotic treatment will also be included in the study.

EXCLUSION:

- Patients who refused to give consent for the study.
- Patients with inappropriate and inadequate samples.

SAMPLE SIZE: 107 samples were taken during the study period.

COLLECTION AND TRANSPORT OF SPECIMEN

Sample Collection:

Two samples were collected for processing of aerobic and anaerobic pathogens as per the standard bacteriological method (32). One was collected for Aerobic culture and sensitivity. The other was for Anaerobic Culture and Sensitivity. The samples like pus, aspirated fluid, tissue biopsy, necrosed tissue material, drain fluid etc. were collected.

The samples for aerobic culture and sensitivity were collected in a Universal Sterile container. For Anaerobic culture and Sensitivity, the samples like tissue, pus aspirate etc. were collected in a Robertson Cooked Meat medium (RCM) and sterile fluid like CSF, body fluids etc. were put in Thioglycolate broth [8].

Samples were collected by the following procedures

Wound swab is not an ideal collection method for anaerobic culture but can be done for aerobic culture and sensitivity. So, in the present study wound swab was not used for above culture [8].

Abscesses: As for any loculated collection; the exudate was aspirated with a needle and syringe after proper disinfection procedure. The area was cleaned with normal saline followed by 70% alcohol and then with tincture iodine or povidone iodine and allowed to dry. Both disinfectants were applied in a circumferential manner from inside out. Then the sample was aspirated out and collected in both sterile container and Robertson Cooked Meat medium or Thioglycolate broth. In case the amount of material is very less (0.2ml), pus was aspirated through a flexible plastic catheter or directly through a syringe without a needle [7].

Sinus tract or deep draining wound: The skin surface surrounding the infection was cleaned properly firstly with normal saline, 70% alcohol, then with iodine solution which was allowed to remain wet in the skin for about a minute [7]. The disinfectants were applied in a concentric manner to a radius of 2 cm beyond the sinus tract. A small curettage of the sample or pus is aspirated out with the help of a syringe and collected in both sterile container and Robertson cooked meat medium.

Oral or Gingival abscess: Normally the oral commensals interfere with the processing if the sample are not collected properly. So thorough cleaning of the surrounding structures was done before collecting sample. Following proper cleaning the sample was aspirated out.

Specimens collected at Time of Surgery: Necrosed, black, gangrenous tissue as shown in fig 6. was collected during the procedure of surgery as the chance of getting contaminated was less. The collected tissue biopsy was immediately placed in a sterile container and anaerobic transport medium and sent to lab.

Transport [2]:

The sample was transported at room temperature within half an hour and processed immediately, where the delay of more than 4 hours was anticipated, in such cases the samples was stored in low temperature of 4 ˚Celsius and processed within 4 hours.

The different approaches which were taken for the processing of the pyogenic sample in the laboratory are enlisted below:

IDENTIFICATION AND CULTURE SUSCEPTIBILITY TESTING OF AEROBIC BACTERIAL ISOLATES [7].

a) Gram Staining: After receiving the sample first Gram stain was done. As it gives a preliminary idea of the organism causing the pathogenesis. It was done according to the standard bacteriological procedure.

Principle of Gram-Stain:

1. Gram-positive bacteria have a thick peptidoglycan layer and these cells have more acidic protoplasm. So, they will retain the primary dye and appear blue in colour.
2. Gram negative bacteria contain lipid layers and these lipid layers make the primary dye to permeable and will take the counterstain. These will appear pink in colour.

Procedure:

Part 1: The slide was prepared by making it grease free dust free oil free by rubbing with a dry tissue paper or passing through flame. After cleaning, the slides are allowed for air drying until further use.

Part 2: Next step labelling of the slides are done. A circle on the slide was made using a glassware marking pen to clearly designate area for the smear.

Part 3: Preparation of the smear

From sample: With a sterile loop, one loopful of direct sample was taken and a smear was made on the slide and allowed to air dry. The sample was spread by means of circular motion 1 cm in diameter and allowed to dry.

Bacterial plate cultures: A drop of saline was put on the slide. The isolated colony was picked up by sterilized loop and a smear of 1cm x 1cm was made

Part 4: Heat Fixing: Slide was fixed by heat fixation by passing over flame 2-3 times.

Gram Stain Procedure:

1. Placed slide with heat fixed smear on staining tray.
2. Gently flooded smear with crystal violet and let stand for 1 minute.
3. Tilted the slide slightly and gently rinses with tap water or distilled water using a wash bottle.
4. Gently flooded the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinses with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorization was done using acetone for 4-5 seconds. Then the slide was rinsed with water.
7. Immediately rinses with water.
8. Saffranine was added for counterstaining of slide and kept for 1 minute.
9. Then slide was rinsed with tap water or distilled water using a wash bottle.
10. After air drying slide was focused under oil immersion lens of microscope as seen in Fig 7.

b) Culture: Aerobic culture was done by standard bacteriological method [7]

With the help of sterile loop sample was inoculated on Blood Agar, MacConkey Agar and Chocolate Agar.

The culture plates were incubated for 48 hours at 35 ± 2˚C and then the colony morphology on the plates were described based on their characteristic features as shown in Fig 8.

On next step, Gram staining was done to identify if it has Gram-positive or Gram-negative organisms and further identification was done by biochemical reaction and automated methods like VITEK 2.

c) Biochemical test for Gram positive bacteria: [7]

1) <u>Catalase test</u>

Principle: Catalase decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Catalase converts hydrogen peroxide into oxygen and water

$2H_2O_2 \rightarrow 2H_2O + O_2$ (gas bubbles)

Procedure

1. With an inoculating needle or a wooden applicator stick, transferred growth from the center of a colony to the surface of a glass slide.

2. Added one drop of 3% hydrogen peroxide and observe for bubble formation.

Interpretation

The rapid and sustained appearance of bubbles or effervescence constitutes a positive test.

Positive control: *Staphylococcus aureus*

Negative control: *Streptococcus species*

2. <u>Coagulase Test</u>

Principle: Coagulase can convert fibrinogen into fibrin. Resulting in a visible clot. In the laboratory, the coagulase test is used to identify *Staphylococcus aureus* and differentiate it from most other *Staphylococci* species. Coagulase is present in two forms, bound and free. Bound coagulase is the clumping factor attaches with the fibrinogen in the plasma when suspended. Free coagulase binds with the coagulase reacting factor to form the clot.

Procedure

Slide test (bound coagulase): Two drops of sterile water or saline was put on a glass slide. Gently emulsify colony material from the organism to be identified. Placed a drop of coagulase plasma in the suspension in one of the circles and mixed with a wooden applicator stick. Placed another drop of water or saline in the other circle as a control. Rocked the slide back and forth, observing for agglutination of the test suspension.

Tube test (free coagulase): Emulsified a small amount of the colony growth of the organism in a tube containing 0.5 mL of coagulase plasma. Incubated the tube at 35°C for 4 hours and observed for clot formation by gently tilting the tube. If no clot is observed at that time, re-incubated the tube at room temperature and read again after 18 hours

Interpretation

Slide test: A positive reaction will be detected within 10–15 seconds of mixing by the formation of a white precipitate and agglutination of the organisms in the suspension.

Tube test: The tube coagulase test is considered positive if any degree of clotting is noted.

Positive control: *Staphylococcus aureus* strain

Negative control: *Staphylococcus epidermidis* strain

3.Bile Esculin Test

Principle: The bile esculin test is based on the ability of certain bacteria to hydrolyze esculin in the presence of bile. Bacteria that are bile esculin–positive are able to grow in the presence of bile salts. Subsequent hydrolysis of the esculin in the medium results in the formation of glucose and a compound called aesculetin. Aesculetin, in turn, reacts with ferric ions to form a black diffusible complex.

Procedure

1. With an inoculating wire or loop inoculated the slant of the bile esculin medium.

2. Incubated the tube or plate at 35°C for 24–48 hours in an ambient air incubator.

Interpretation

Diffuse blackening of more than half of the slant within 24–48 hours indicate esculin hydrolysis. All group D streptococci will be bile esculin–positive within 48 hours.

Positive control: *Enterococcus* species (e.g., *E. faecalis*)

Negative control: Viridians group *streptococci,* not group D

d) Biochemical for Gram negative bacteria: [7]

1. Oxidase Test

Principle: The cytochromes are iron-containing haemoproteins that act as the last link in the chain of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome oxidase test uses certain reagent dyes, such as p-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptors. In the reduced state, the dye is colourless; in the presence of cytochrome oxidase and atmospheric oxygen, p-phenylenediamine is oxidized, forming indophenol blue.

Procedure

Commercial disk impregnated with dried p-phenylenediamine dihydrochloride are used. Suspected colony is smeared into the Disc.

Interpretation

Bacterial colonies having cytochrome oxidase activity develop a deep blue colour at the inoculation site within 10 seconds.

Positive control: *Pseudomonas aeruginosa*

Negative control: *Escherichia coli*

2. Urease Test

Principle: Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea, forming ammonia and carbon dioxide. Presence of ammonia increases the pH (>8.1) of media, thus converting colourless phenolphthalein to pink-red coloured phenolphthalein

Procedure

1.The surface of the agar slant is streaked with the test organism.

2.Incubated at 35°C for 18–24 hours.

Interpretation

Organisms that hydrolyse urea rapidly may produce positive reactions i.e conversion o the colour of media to red within 1 or 2 hours.

Positive control: *Proteus* species

Positive control (weak): *Klebsiella* species

Negative control: *Escherichia coli*

4. TSI (Triple sugar iron)

Principle: The media contains three sugars namely Glucose (0.1%), Sucrose and lactose (1%). If the test organism is one which is capable of fermenting these sugars, then there will be production of acid thus resulting in a pH fall. This fall in pH is detected by phenol red indicator in the medium. Sodium thiosulfate and ferrous ammonium sulphate present in the medium detects production of Hydrogen sulfide.

Procedure

The test organism is inoculated into the media first by stabbing through the centre with a straight wire followed by streaking the surface of the agar

Interpretation

Alkaline/Acid slant (red slant/yellow butt): Dextrose fermentation

Acid/Acid (yellow slant/yellow butt): Fermentation of all three

Alkaline/alkaline (red slant/red butt): Absence of fermentation

Gas production

Blackening of medium: H2S production.

5. Indole Test

Principle: Indole, a benzyl pyrrole, is one of the metabolic degradation products of the amino acid tryptophan. Bacteria with tryptophanase are capable of hydrolysing and deaminating tryptophan producing indole, pyruvic acid, and ammonia. Indole

reacts with the aldehyde group of p-dimethylaminobenzaldehyde. This is the active chemical in Kovac's and Ehrlich's reagents.

Procedure

Colony is inoculated into peptone water and incubated overnight and 15 drops of Kovac's reagent is added down the inner wall of the tube.

Interpretation

The development of a bright fuchsia red colour at the interface of the reagent and the broth within seconds after adding the reagent is indicative of a positive test

Positive control: *Escherichia coli*

Negative control: *Klebsiella pneumoniae*

6. Methyl Red

Principle: Methyl red is a pH indicator, with a range between 6.0 (yellow) and 4.4 (red).Test organism producing large quantities of acid from the carbohydrate substrate changes the pH.

Procedure

1.Inoculate the glucose phosphate broth with a pure culture of the test organism. Incubate the broth at 35°C for 48–72 hours.

2. Add 5 drops of the methyl red reagent directly to the broth.

Interpretation

The development of a stable red colour in the surface of the medium indicates a positive test

Positive control: *Escherichia coli*

Negative control: *Enterobacter aerogenes*

7. Voges-Proskauer Test

Principle: Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose, is metabolized through various metabolic pathways. One such pathway results in the production of acetoin (acetyl methyl carbinol). In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and α-naphthol serves as a catalyst to bring out a red complex.

Procedure

1.Inoculate a tube of glucose phosphate broth with a pure culture of the test organism.

2.Incubate for 24 hours at 35°C. Add 0.6 mL of 5% α-naphthol followed by 0.2 mL of 40% KOH.

3.Shake the tube gently to expose the medium to atmospheric oxygen.

Interpretation

A positive test is represented by the development of a red colour 15 minutes or more after addition of the reagents.

Positive control: *Enterobacter aerogenes*

Negative control: *Escherichia coli*

8. Citrate Utilization Test

Principle: Certain bacteria can obtain energy by using citrate as the sole source of carbon. The utilization of citrate is detected in citrate medium by the production of alkaline by-products.

Sodium Citrate→ alkaline metabolic products and ↑pH

bromothymols blue (Green pH:6.9) →bromothymol blue (Blue ph.: 7.6)

Procedure

1. A well-isolated colony is picked and inoculated as a single streak on the slant surface of the citrate agar tube.
2. The tube is incubated at 35°C for 24–48 hours.

Interpretation

A positive test is represented by the development of a deep blue color within 24–48 hours.

Positive control: *Enterobacter aerogenes*

Negative control: *Escherichia coli*

9. Mannitol Motility test medium

Principle: The motile bacteria can traverse through a semisolid media.

Procedure

Inoculate the tube by stabbing into the centre of the media with a straight wire and incubate it overnight at 37°C.

Interpretation

Motile: The medium will be turbid and the stab wont be visible

Non motile: The medium will be clear

To differentiate between motile and non-motile organisms. This test also gives additional information regarding mannitol fermentation which changes the colour of the medium into yellow colour.

10. Phenylalanine Deaminase test

Principle: Phenylalanine in presence of the enzyme phenylalanine deaminase gets converted to phenyl pyruvic acid and ammonia. This phenyl pyruvic acid combines with ferric ions to give a green complex.

Procedure

1.The agar slant of the medium is inoculated with a single colony of the test organism

2.After incubation at 35°C for 18–24 hours, 4 or 5 drops of the ferric chloride reagent are added directly to the surface of the agar.

Interpretation

The immediate appearance of an intense green color indicates the presence of phenyl pyruvic acid and a positive test

Positive control: *Proteus* species

Negative control: *Escherichia coli*

11. Nitrate Reduction Test

Principle: The capability of an organism to reduce nitrates to nitrites.

$$NO_3^- + 2e^- + 2H \rightarrow NO_2 + H_2O$$

Procedure

1. Inoculate the nitrate medium with a loopful of the test organism isolated in pure culture on agar medium, and incubate at 35°C for 18–24 hours.

2. After incubation, add 1 mL each of reagents to the test medium.

Interpretation

The development of a red colour within 30 seconds after adding the test reagents indicates a positive reaction for nitrate reduction

Positive control: *Escherichia coli*

Negative control: *Acinetobacter baumannii*

e) Antibiotic Susceptibility Testing [7]

After the organism is isolated and identified the antimicrobial susceptibility of the isolate is done using both conventional and automated method.

1. Kirby-bauer disc diffusion:

In this method 0.5 MacFarland turbidity of the isolate is made and then lawn culture was done on the Mueller-Hinton agar plate of 90 mm diameter. Total number of 6 drugs were put on a plate at a distance of 15 mm from each other. Different drugs were selected for Gram positive and Gram-negative bacteria based on CLSI guidelines.

The plates were incubated for overnight incubation at 35 ± 2˚C. The zone of inhibition of the drugs were measured and interpreted as Susceptible, Resistant and Intermediate according to CLSI guidelines M100 2020 document as shown in Table 9 & 10. [33]

Gram negative bacteria (GNB)

Drug	***Enterobacterales***			***Acinetobacter baumannii***			***Pseudomonas aeruginosa***		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Ceftriaxone 30µg	≥23	20-22	≤19	≥21	14-20	≤13			
Cefepime 30µg	≥25	19-24	≤18	≥18	15-17	≤14	≥18	15-17	≤14
Piperacillin- Tazobactam 100/10µg	≥21	18-20	≤17	≥21	18-20	≤17	≥21	15-20	≤14
Cotrimoxazole 1.25/23.75 µg	≥16	11-15 .	≤10	≥16	11-15.	≤10	-	-	-
Amikacin 30µg	≥17	15-16	≤14	≥17	15-16	≤14	≥17	15-16	≤14
Gentamicin 10µg	≥15	13-14	≤12	≥15	13-14	≤12	≥15	13-14	≤12
Ciprofloxacin 5µg	≥26	22-25	≤21	≥21	16-20	≤15	≥25	19-24	≤18
Meropenem 10 µg	≥23	20-22	≤19	≥18	15-17	≤14	≥19	16-18	≤15
Imipenem 10µg	≥23	20-22	≤19	≥22	19-21	≤18	≥19	16-18	≤15
Ertapenem 10 µg	≥22	19-21	≤18	-	-	-	-	-	-
Aztreonam 30 µg	≥21	18-20	≤17	-	-	-	≥22	16-21	≤15
Minocycline 30 µg	≥16	13-15	≤12	≥16	13-15	≤12	-	-	-

Table 9- Antibiotics used for screening of resistance against GNB

Gram positive cocci (GPC)

Drug	***Staphylococcus***			***Enterococcus***			***Streptococcus pyogenes***		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Cefoxitin 30 µg	≥22	-	≤21	-	-	-	-	-	-
Erythromycin 15µg	≥23	14-22	≤13	≥23	14-22	≤13	≥21	16-20	≤15
Clindamycin 2µg	≥21	15-20	≤14	-	-	-	≥19	16-18	≤15
Vancomycin 30 µg	≥2µg/ml	4-8µg/ml	16µg/ml	≥17	15-16	≤14	≥17	-	-
Linezolid 30µg	≥21	-	≤16	≥23	21-22	≤20	≥21	-	-
Teicoplanin 30µg	-	-	-	≥14	11-13.	≤10	-	-	-
Ciprofloxacin 5µg	≥21	16-20	≤15	≥21	16-20	≤15	-	-	-
Gentamicin 10µg	≥15	13-14	≤12	-	-	-	-	-	-

Table 10- Antibiotics used for screening against GPC

2. MIC testing of Vancomycin by E-strip: [33]

For some drugs like Vancomycin, Epsilometer test was done in which MIC of the drug was detected by E-strip on Mueller-Hinton Agar plate and interpreted according to CLSI as shown in Fig 10 [33].

3. Disc Elution testing for colistin in Gram negative bacilli: [33]

As per CLSI guidelines M100 2021 document, MIC testing for Colistin is recommended either by microbroth dilution or colistin disc elution method. In present study disc elution method for Colistin susceptibility was done as shown in Fig 11 [33].

Steps for Disc Elution:

1) Four tubes of 10 ml Cation adjusted Mueller-Hinton broth taken and labelled

2) Colistin discs with strength of 10 µg taken

3) Colistin discs were put in the tubes in the following manner as shown in Table 11 and vortexed slowly and kept undisturbed for 30 mins

	No. of colistin discs	Final concentration
Tube 1	1 colistin 10 µg disc	1 µg/ml
Tube 2	2 colistin 10 µg disc	2 µg/ml
Tube 3	4 colistin 10 µg disc	4 µg/ml
Tube 4	No disc	Control

Table 11- Colistin broth disc elution

4) 50 µl of 0.5 MacF turbid solution of the isolate is taken and added to the tubes and kept for incubation at 35 ± 2˚C for 16-20 hours

5) Turbidity in the tubes were interpreted based on CLSI guidelines.

4. Screening and Detection of resistance:[33]

DETECTION AND SCREENING FOR RESISTANCE IN GRAM NEGATIVE BACILLI: [33]

All the Gram negative multidrug resistant isolates were screened for Extended spectrum Beta lactamase (ESBL), AmpC, and Metallo-Beta lactamase production and confirmed by phenotypic methods.

Detection and screening method for Gram negative bacilli

For Screening of ESBL detection:

4 drugs discs were used for screening of ESBL production. Drugs used are Ceftazidime 30 µg, Ceftriaxone 30 µg, Cefotaxime 30 µg and Aztreonam 30 µg. The zone of inhibition was interpreted according to CLSI guidelines.

Isolates resistant to the above-mentioned drugs were tested for confirmation of ESBL production by Combined disc diffusion test.

Combined disc diffusion test:

This test was done using drug combination of Ceftazidime 30 µg and ceftazidime-clavulanic acid (30/10 µg) as shown in fig12.

0.5 MacF of the isolate is taken and lawn culture was done on MHA agar plate and both the discs were put at a distance of 20 mm. The plates were incubated overnight at a temperature of 37˚C.

A zone of diameter ≥5 mm in combination disc as compared to the individual drug was confirmed for production of ESBL.

AmpC beta lactamase production:

Screening for production of AmpC Beta lactamases were done using Cefoxitin disc 30 µg. Isolates resistant to the drug were tested for inducible AmpC detection by Disc antagonism test.

Disc Antagonism test:

This test was done using two antibiotic discs Cefoxitin 30 µg and Cefotaxime 30 µg as shown in Fig 13.

A 0.5 MacF of the test isolate was taken and lawn culture was put on a MHA plate and both antibiotic discs were put at a distance of 20 mm on the plate; incubated overnight at 35 ± 2˚C.

Isolates having blunting of Cefotaxime disc zone of inhibition adjacent to cefoxitin disc was confirmed as inducible AmpC production.

MBL/CRE detection in Gram negative bacilli:

It was done with discs of Imipenem 10 µg, Meropenem 10 µg, Ertapenem 10 µg as shown in Fig 14. Isolates resistant to carbapenems were confirmed by various tests.

Confirmatory test for all Carbapenemases

Modified Hodge test:

An indicator strain ATCC Escherichia coli 25922 was used in the assay.

A lawn culture was made with 0.s MacF ATCC Escherichia coli 25922 on a MHA plate and Meropenem 10 µg was put in the centre and with a inoculating loop 3-4 colonies of test isolates were taken and streaked from the edge of the plate to the edge of the disc. 3-4 isolates were tested in a single plate. The plate was incubated overnight at 35 ± 2˚C.

After incubation the test plate was interpreted for production of Carbapenemases by formation of clover leaf indentation at the intersection point of the test isolate and the ATCC strain as in Fig 15.

Confirmatory test for detection of Class B Metallo- Beta lactamases

Disc Potentiation Test:

It was done by using two discs Imipenem 10 µg and Imipenem + EDTA (10/750 µg) disc. A 0.5 MacF of the test isolate was taken and lawn culture was put

on a MHA plate and both antibiotic discs were put at a distance of 20 mm on the plate as shown in fig 16; incubated overnight at 35 ± 2˚C.

Preparation of Imipenem+EDTA (10/750 µg) disc

Imipenem 10 µg disc is taken and on it 5 µl of EDTA stock solution was added on it

Preparation of EDTA stock solution:

A 0.5 M EDTA solution was prepared by dissolving 186.1 gm of disodium EDTA.$2H_2O$ in 1000 ml of distilled water and adjusting its pH to 8 by adding NAOH. Then it is sterilized by autoclave.

Interpretation was done by ≥ 5mm diameter increase in the zone of inhibition of Imipenem+EDTA (10/750 µg) disc as compared to Imipenem 10 µg was confirmed for production of MBL production as EDTA chelates the MBL enzyme.

DETECTION AND SCREENING FOR RESISTANCE IN GRAM POSITIVE COCCI: [33]

Methicillin Resistance *Staphylococcus aureus*:

This test was done to detect MRSA strain from the *Staphylococcus aureus* isolates. It was done with Cefoxitin 30 µg disc on a MHA plate as in fig-17. The sensitivity pattern was detected according to the CLSI guidelines 2020 as shown in Table 12.

Drug	**Organism**	**Disk**	**Zone diameter(mm)**		**MIC breakpoints**	
			Sensitive	**Resistant**	**Sensitive**	**Resistant**
Oxacillin	*Staphylococcus aureus*	30µg Cefoxitin (Surrogate test for oxacillin)	≥22	≤21	≤4	≥8

Table 12: Breakpoints for detection of Methicillin Resistance *S. aureus*.

Inducible Clindamycin Resistance:

It was done to detect the MLS_B resistance in the *Staphylococcus aureus* isolates. It was done in MHA plate with Erythromycin 15 µg and Clindamycin 2 µg discs put at a 20 mm distance from each other as shown in Fig- 17. It was constitutive type of MLS_B resistance when both the drugs were resistant and inducible MLS_B resistance when there was a 'D' shape zone of inhibition around clindamycin disc adjacent to the erythromycin disc as shown in Table 13.

Organism	**Drug**	**Disk content**	**Distance**	**Incubation time**	**Result**
Staphylococcus aureus	Erythromycin	30µg	15-26 mm apart	16-18 hours	Flattening of zone of inhibition adjacent to Erythromycin disc=ICD
	Clindamycin	2µg			

Table 13: Breakpoint detection for Inducible Clindamycin resistance in *S. aureus*

<u>IDENTIFICATION AND CULTURE SUSCEPTIBILITY TESTING OF ANAEROBIC BACTERIAL ISOLATES</u> 8]

The samples collected in RCM or in Thioglycolate broth were kept for 48 hours in the incubator at 35 ± 2˚C. Then after 48 hours Kopeloff modification of Gram staining and Schaeffer and Fulton spore staining was done.

a) Kopeloff modification of Gram staining:

1. Slide is placed with heat fixed smear on staining tray.
2. Gently flooded the smear with methyl violet and let stand for 1 minute.
3. Tilted the slide slightly and gently rinsed with tap water or distilled water using a wash bottle.
4. Gently flooded the smear with Kopeloff iodine (Iodine+ 4% NAOH) as mordent and let stand for 1 minute.
5. Tile the slide slightly and gently rinsed with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorized using 95% ethyl alcohol or acetone. Tilted the slide slightly and applied the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear.

7. Immediately rinsed with water.
8. Gently flooded with 0.1% Basic fuschia to counter-stain and let stand for 45 seconds.
9. Tilted the slide slightly and gently rinsed with tap water or distilled water using a wash bottle.
10. Dried the slide with bibulous paper.
11. Seen the smear using a light-microscope under oil-immersion as in Fig 18

b) Schaeffer and Fulton spore staining:

1. Placed slide with heat fixed smear on staining tray.
2. Gently flooded smear with malachite green/Schaeffer and Fulton spore stain A and let stand for 3-5 minutes with intermitted heating.
3. Tilted the slide slightly and gently rinsed with tap water or distilled water using a wash bottle.
4. Gently flooded with 0.5% aqueous Safranine/ Schaeffer and Fulton spore stain B and let stand for 1 minute.
5. Tilted the slide slightly and gently rinsed with tap water and seen in light microscope under oil immersion field.

c) Anaerobic Culture:

Culture were inoculated on anaerobic medium like

Non-selective Media-

- Egg Yolk Agar Base Medium (Willis &Hobbs Medium) – For GPBs

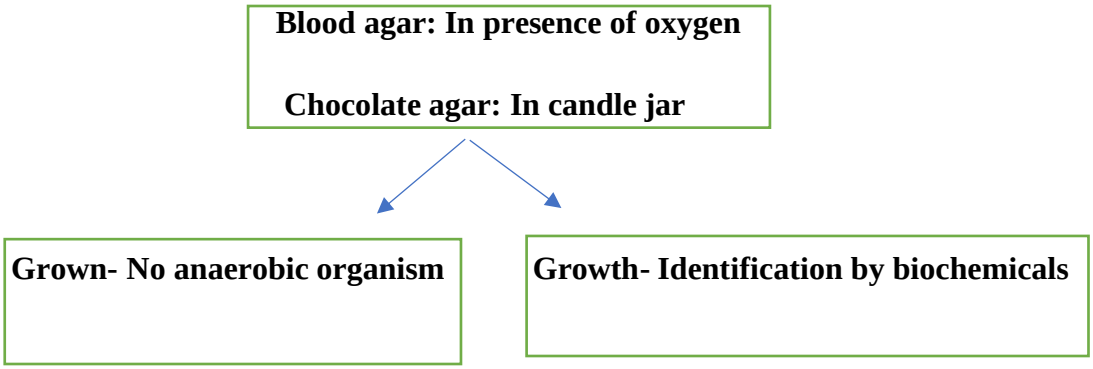
Selective Media- For all samples except Stool

- Neomycin Blood Agar
- Brain Heart Infusion Agar medium

After inoculation the cultures were put on Mc-Intosh jar with anaerobic gas pack and indicator Resazurin disc for 48hours along with biological control of obligate aerobe i.e., *Pseudomonas aeruginosa* cultured in MacConkey agar as shown in Fig 19.

Then after the incubation the colony morphology are observed and put for aerotolerance test.

Aerotolerance test: The isolated colonies are tested in the following manner.



1) Isolation & identification of Bacteroides species:

- Bacteroides Bile Esculin Agar: Tests the ability of the organism to grow in presence of 20% bile. Incubated for 24-72 hours after culturing onto BBE agar. It the interpreted as negative if there is no growth on the agar
- Indole test: Colony is inoculated into Tryptophan containing medium and observed for degradation by adding Kovacs reagent after overnight incubation. If there is development of pink of fuchsia ring it is interpreted as positive

- Glucose fermentation
- Trehalose fermentation
- Sucrose fermentation
- Arabinose fermentation

The carbohydrate fermentation test is used to differentiate bacteria according to the utilisation of the sugars. It tests for gas or acid that is the product of fermentation. The pH indicator used is Andrade's solution and Durham tube is used for detecting gas

2) Isolation and identification of *Peptostreptococcus* species:[8]

Antibiotic discs of Sodium Polyanethol Sulphonate (SPS) prepared in house was used for preliminary of *Peptostreptococcus anaerobius*. Suspected colony was streaked on Brucella Blood agar and SPS disk was put and incubated for 48 hrs in anaerobic environment. The sensitive GPCs were further identified based on the following biochemicals as shown in Table 14.

Biochemical reaction	Result
Catalase	-
Indole	-
Urease	-
Glucose fermentation	+
Lactose Fermentation	-
Mannose fermentation	-

Table 14: Biochemical reactions of *Peptostreptococcus* species

3) Isolation and identification of Clostridium species:[8]

- Indole test: Colony is inoculated into Tryptophan containing medium and observed for degradation by adding Kovacs reagent after overnight incubation. If there is development of pink of fuschia ring it is interpreted as positive
- Nitrate test: Organism is inoculated into indole nitrate medium and kept for overnight incubation. The test is interpreted as positive if there is development of red colour after adding the two reagents.
- Glucose, Lactose, Mannitol, Sucrose, Maltose fermentation test,
- Gelatin liquefaction: An actively growing gelatin tube (>2+ turbidity) was refrigerated for 1 hour along with an uninoculated tube (negative control). The tubes were later observed every 5 minutes for liquefaction. If it drops to the top of the inverted tube immediately then the test can be interpreted as positive.
- Litmus milk test: Anaerobic organisms produce four different reaction in milk medium namely acid, clotting, gas and digestion.The test begins by the inoculation of the litmus milk medium followed by incubating it at 35°C

d) Identification using special-potency disks

These disks were used as a method to determine the Gram reaction and separating different anerobic species. Generally, Gram positive organisms wee sensitive to Vancomycin and resistant to Colistin and Gram-negative organism were resistant to Vancomycin as shown in Table 15 and Fig 23

Organism	**Vancomycin (5µg)**	**Kanamycin (1000µg)**	**Colistin (10µg)**	**SPS**
Peptostreptococcus anaerobius	S	R	R	S
Bacteroides fragilis group	R	R	S	-
Clostridium species	S	V	R	-

Table 15: Presumptive identification using special-potency disks

e) Antimicrobial susceptibility testing of aerobic and Anaerobic isolates: [33]

It was done on Brucella Blood Agar by MIC detection with E-strip as in Fig 24, according to CLSI guidelines as shown in Table 16 [33].

Anaerobic

Test/ report group	**Antimicrobial agent**	**Interpretive categories and MIC breakpoints microg/mL**		
		S	**I**	**R**
Beta lactam combination				
A	Piperacillin/Tazobactam	≤16/4	32/4-64/4	≥128/4
A	Amoxicillin-Clavulonic acid	≤8/4	16/8.	≥32/8
Cephems				
C	Cefoperazone	≤16	32	≥64
Carbapenem				
A	Meropenem	≤4	8	≥16
A	Imipenem	≤4	8	≥16
Nitroimidazole				
A	Metronidazole	≤8	16	≥32

Phenicols				
C	Chloramphenicol	≤8	16	≥32
Lincosamides				
A	Clindamycin	≤2	4	≥8

Table 16: Antibiotics used for screening of resistance against anaerobes

AUTOMATED IDENTIFICATION OF AEROBIC AND ANAEROBIC ISOLATES [7,8]

a) VITEK 2 ID & AST system.

There are different panels available for the identification of different organism as follows:

1. GN- Gram- negative fermenting and non-fermenting bacilli

2. GP- Gram-positive cocci and non-spore-forming bacilli

3. ANC- Anaerobic organisms

4. N280- Lactose fermenter

5. N281- Non Lactose fermenter

Procedure:

1. A loop is used to pick 4-5 isolated colonies and then emulsified in 3.0 ml of normal saline (pH 5.0-7.2).

2. All the test tubes (polystyrene) should be arranged in Cassette, a special rack to hold the test tubes.

3. The turbidity is adjusted accordingly (Table 17) and can be measured with the help of a turbidity meter known as the DensiChek.

Card type	MacFarland turbidity
GN	0.5-0.63
GP	0.5-0.63
ANC	2.7-3.30

Table 17: Macfarland turbidity for VITEK 2

4) Followed this, identification cards are inoculated with the microorganism broth.

5) Then loaded the cards and suspension tubes into the Automated Transport system.

6. Taken the reading following the day. A VITEK 2 Compact takes about 8-12 hours to identify the organism and the susceptibility test

b) MALDI TOF MS

Anaerobic isolates were confirmed by MALDI TOF MS as Fig 25.

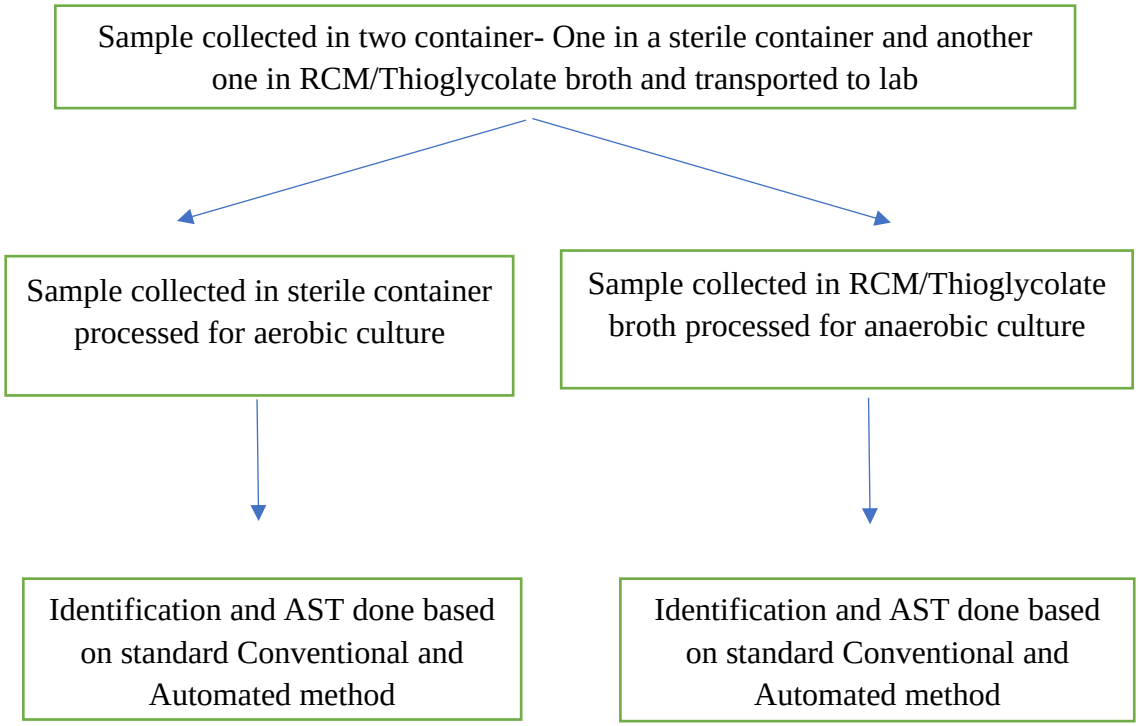


Fig 26: Flow chart of the method followed in this study

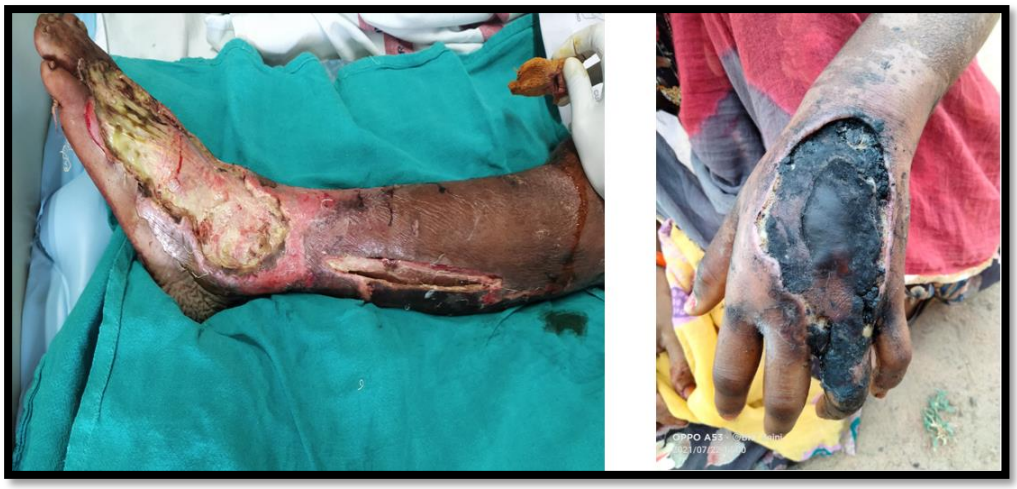


Fig 6: Patients having deep-seated infection

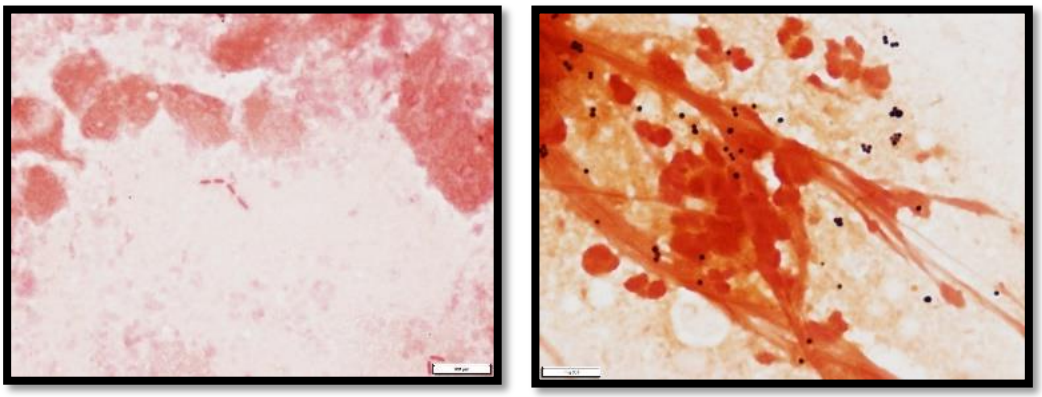


Fig 7: Gram-staining showing GNB and GPC

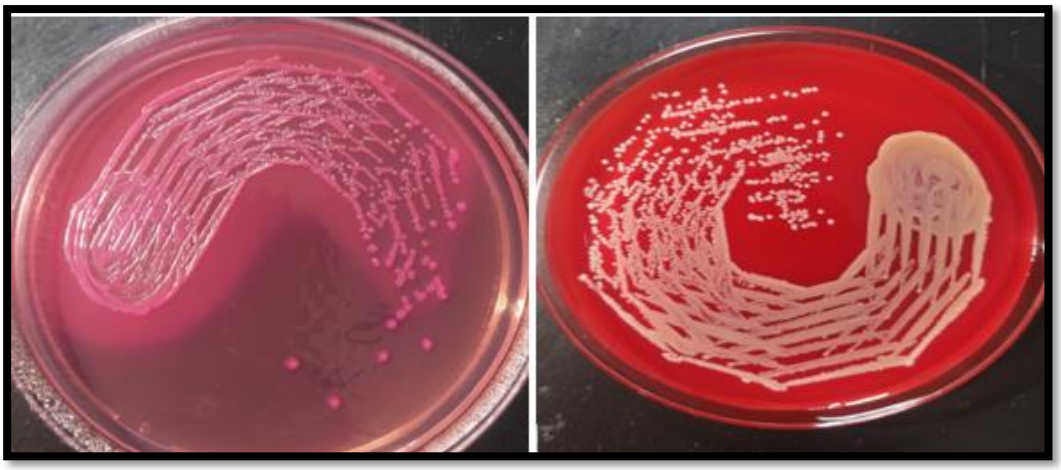


Fig 8: Culture showing growth of aerobic organisms

Fig 9: Biochemical reactions of various organisms

Fig 10: Vancomycin E strip method

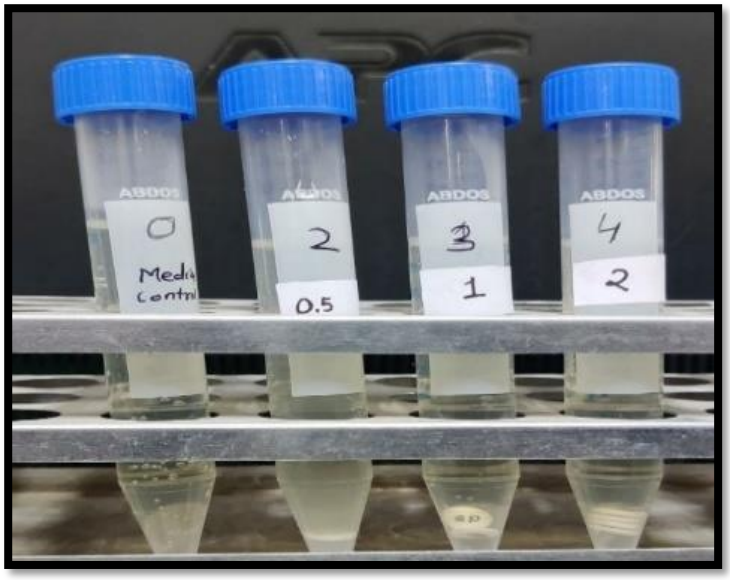


Fig 11: Colistin broth disc elution

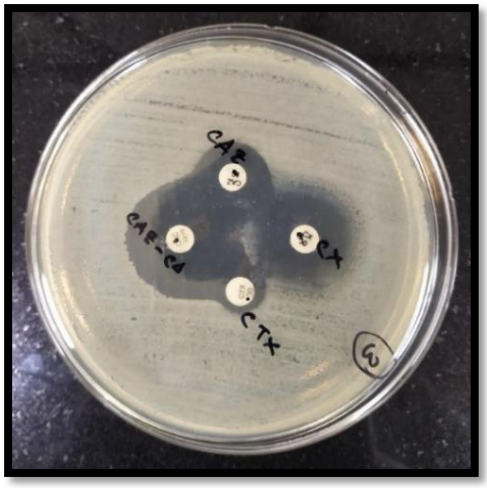


Fig 12: ESBL detection by combined disc diffusion test

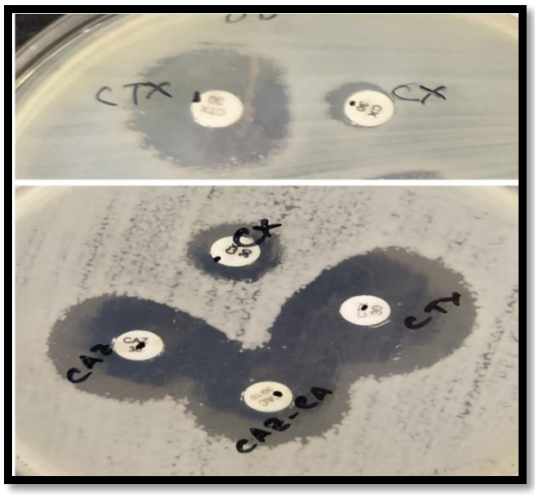


Fig 13: AmpC detection by Disc Antagonism test

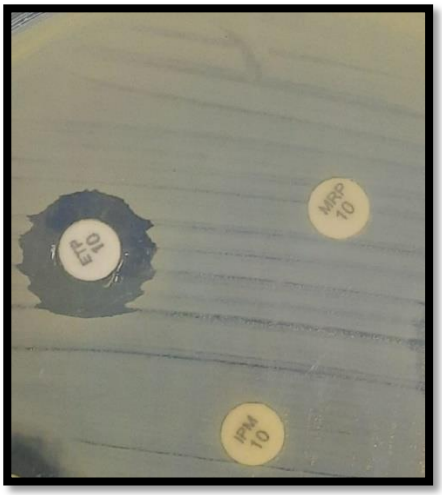


Fig 14: Screening of Carbapenemases Production

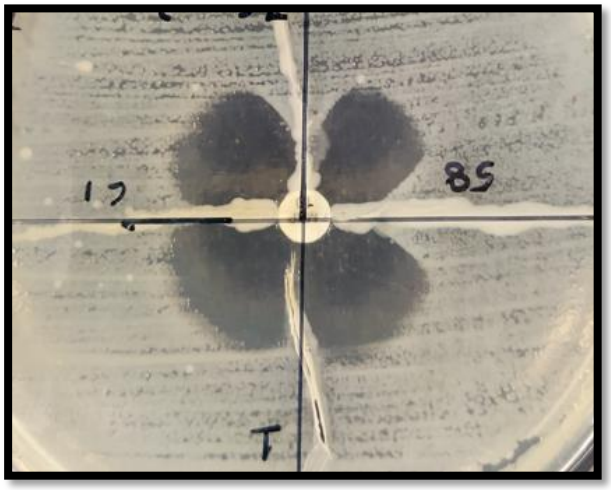


Fig 15: Detection of Carbapenemases production by Modified Hodge Test

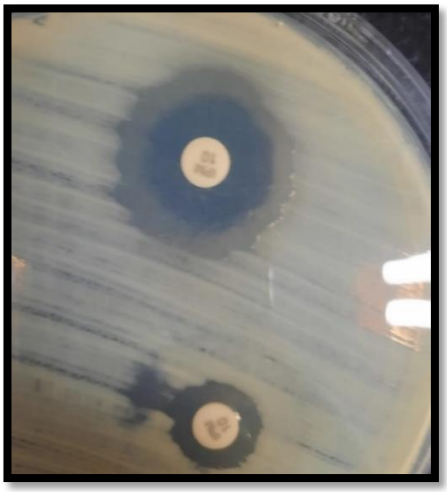


Fig 16: MBL detection by Disc Potentiation Test

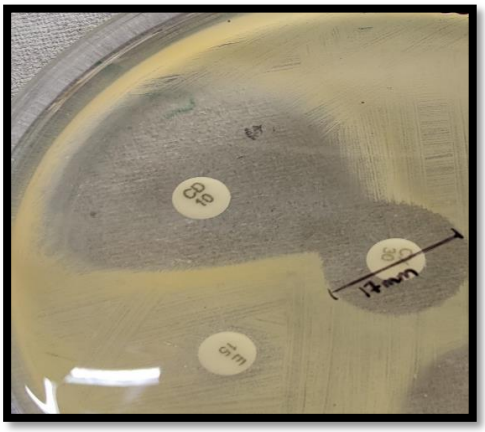


Fig 17: Methicillin resistance & D-Test positive (Inducible Clindamycin Resistance)

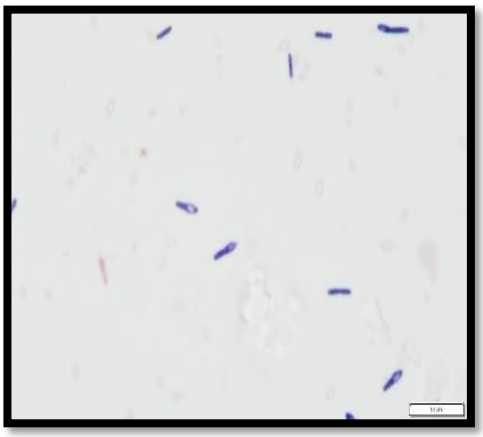


Fig 18: Gram-positive bacilli with spore

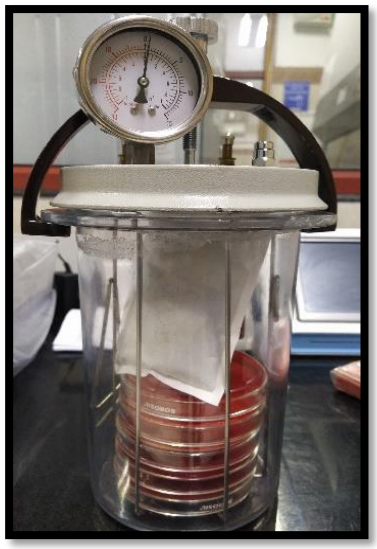


Fig 19: Anaerobic culture technique

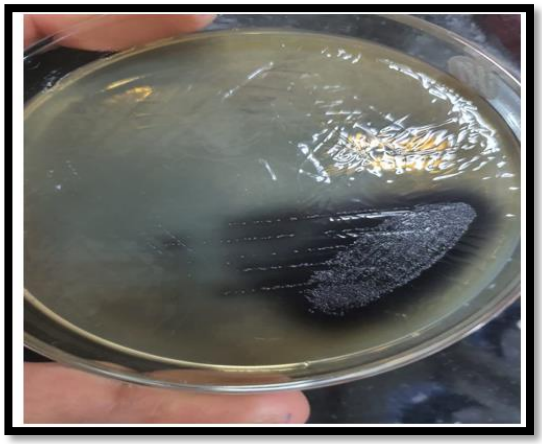


Fig 20: BBE agar showing isolation of *Bacteroides fragilis*

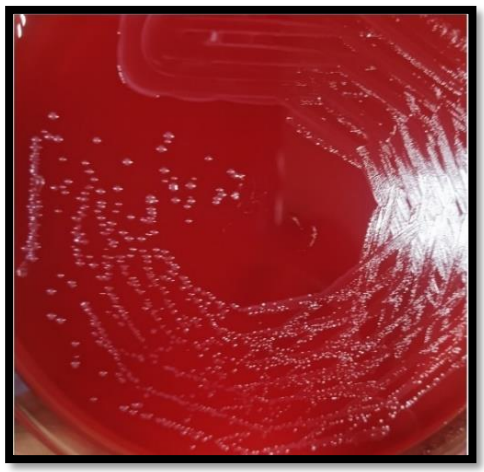


Fig 21: BBA agar showing growth of *Peptostreptococcus*

Fig 22: EYA showing growth of *Clostridium perfringes*

Fig 23: Presumptive identification using special-potency disks

Fig 24: MIC of anaerobe using E strip

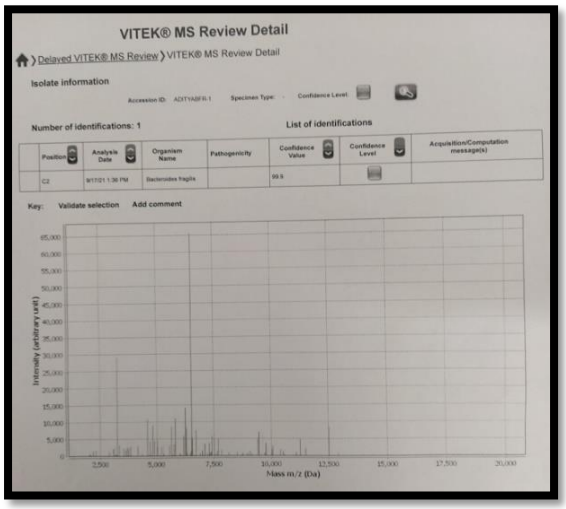


Fig 25: Identification of *Bacteroides fragilis* by MALDI TOF MS

RESULT

A total 107 patients with deep seated pyogenic infections were included in the study which is conducted from Jan 2020- July 2021 in the Department of Microbiology. All the 107 samples were processed by aerobic and anaerobic culture techniques.

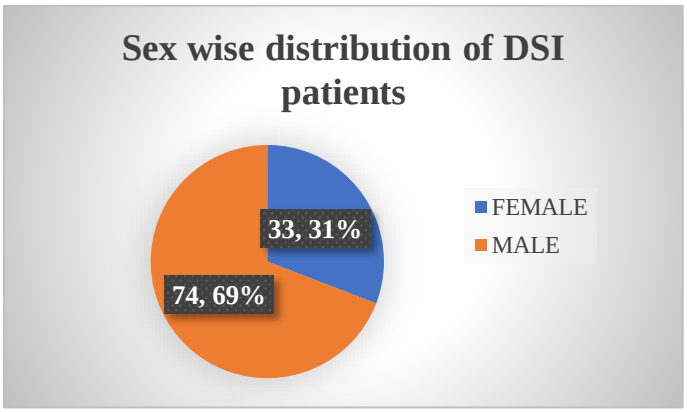


Fig 27: Sex wise distribution of the deep-seated infections

Out of total 107 deep seated infections processed during the study period, male patients 69% (n=74) were predominant compared to female patient 31% (n=33) as shown in Fig- 27

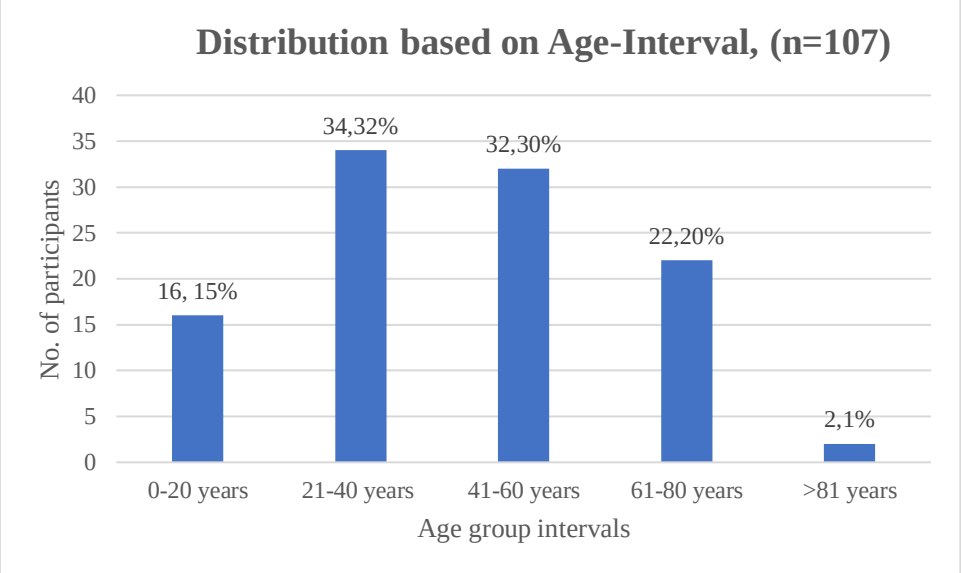


Fig 28: Age interval wise distribution of the participants

As shown in the Fig 28, majority of the participants 32% (n=34) were from adult age group ranging from 21-40 years. 15% (n=16) participants were in the age group 0-20 years. 30% (n=32) and 20% (n=22) were seen in the age interval of 41-60 years and

61-80 years of age group respective. >81 years age group there was only 1% (n=2) participants.

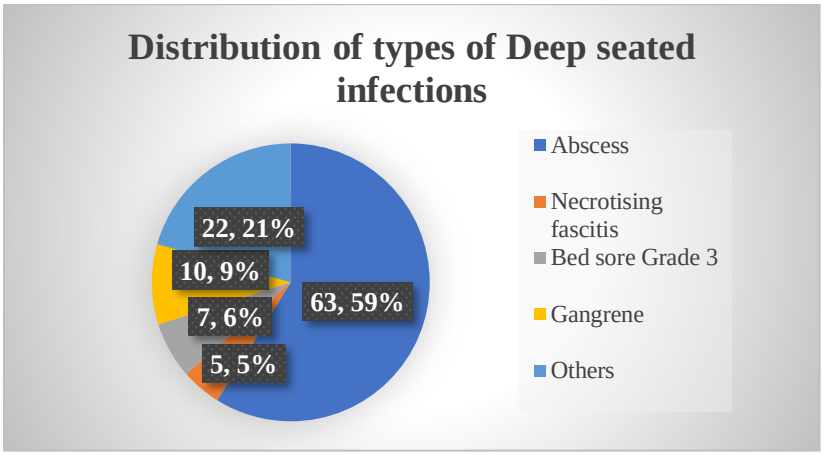


Fig 29: Distribution of types of deep-seated infections

In this study, deep abscesses 59% (n=63) were the most common deep seated pyogenic infections followed by gangrene were present in 9% (n=10) cases. Necrotizing fasciitis were observed in 5%(n=5) cases and Grade 3 Bed sore were present in 6% (n=7) cases. 22% (n=21) DSI cases were of diabetic foot, deep ulcers, gingivitis etc. cases as shown in Fig-29.

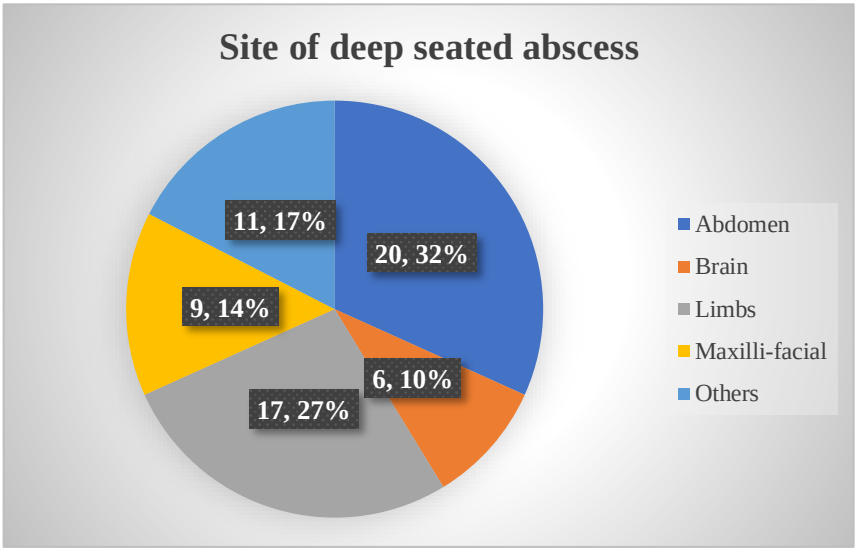


Fig-30: Site of deep-seated abscesses

As shown in Fig 30, most common abscess was the intraabdominal 32% (n=20) followed by abscesses on the limb which were about 27% (n=17). Brain abscess were there in 10% (n=16) cases. Maxillo-facial deep abscess was present in 14% (n=9) cases.

17% (n=11) were having abscesses on different site of the body other than the above-mentioned sites like back, gluteal region etc.

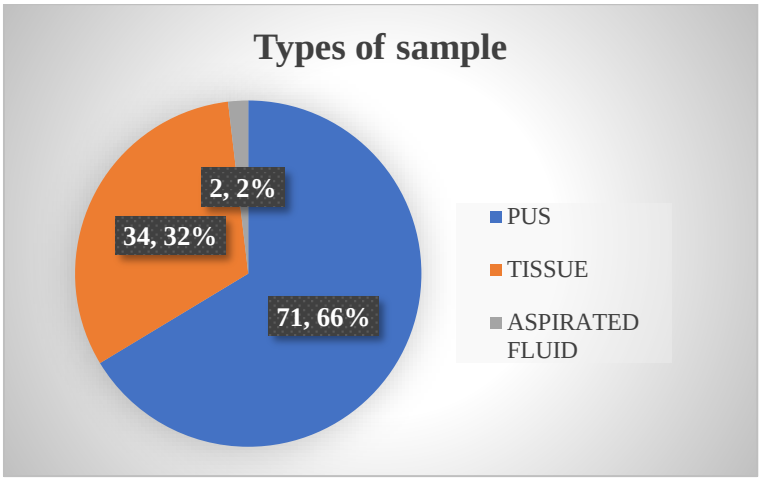


Fig 31: Types of samples

Various types of samples were collected in this study. As shown in Fig 31, the most common type of sample was pus 66% (n=71). 32% (n=34) were tissue biopsy sample and 2% were aspirated fluid.

Culture findings	Type of growth		Total (n=107)
Growth	Polymicrobial	15% (n=12)	74.8% (n=80)
	Monomicrobial	85% (n=68)	
No growth			25.2% (n=27)

Table 18: Distribution of the culture reports.

Out of the 107 samples processed, 25.2% (n=27) samples were sterile and growth were present on 74.8% (n=80) samples. Out of the 80 samples polymicrobial infections were in 15% (n=12) cases and monomicrobial infections were seen in 85% (n=68) cases as shown in Table 18.

Isolates	Polymicrobial growth (n=12)	No. of pathogen isolated (N= 24)
Escherichia coli + Klebsiella pneumoniae	4	8
Escherichia coli + Acinetobacter baumannii	2	4
Klebsiella pneumoniae + Pseudomonas aeruginosa	2	4
Escherichia coli + Proteus vulgaris	1	2
Klebsiella pneumoniae + Enterobacter species	1	2
Klebsiella pneumoniae + Bacteroides fragilis	1	2
Enterobacter aerogenes + Bacteroides fragilis	1	2

Table 19: Distribution of polymicrobial flora based on isolates isolated.

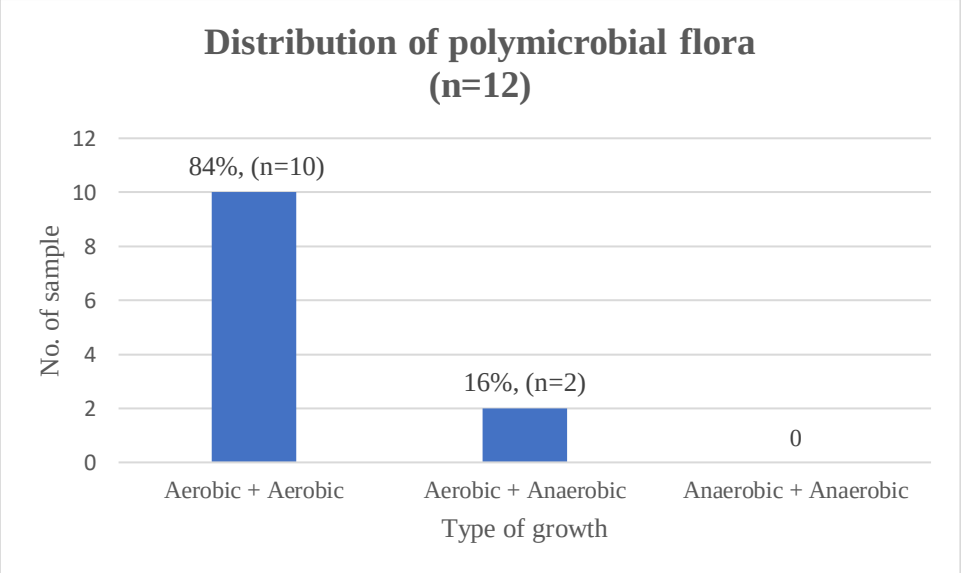


Fig 32: Distribution of pattern of polymicrobial growth.

In the present study, among 12 polymicrobial flora total 24 isolates were isolated as shown in Table 19. 84% (n=10) were having aerobic isolates and 16% (n=2) were having aerobic and anaerobic growth as shown in Fig 32.

No. of isolates	Aerobic	Anaerobic
92	92.3% (n=85)	7.7% (n=7)

Table 20- Distribution of isolates

As shown in Table 20, Out of 80 culture positive samples total 92 isolates were isolated from out of which aerobic bacteria were 92.3% (n=85) and anaerobic bacteria were 7.7% (n=7).

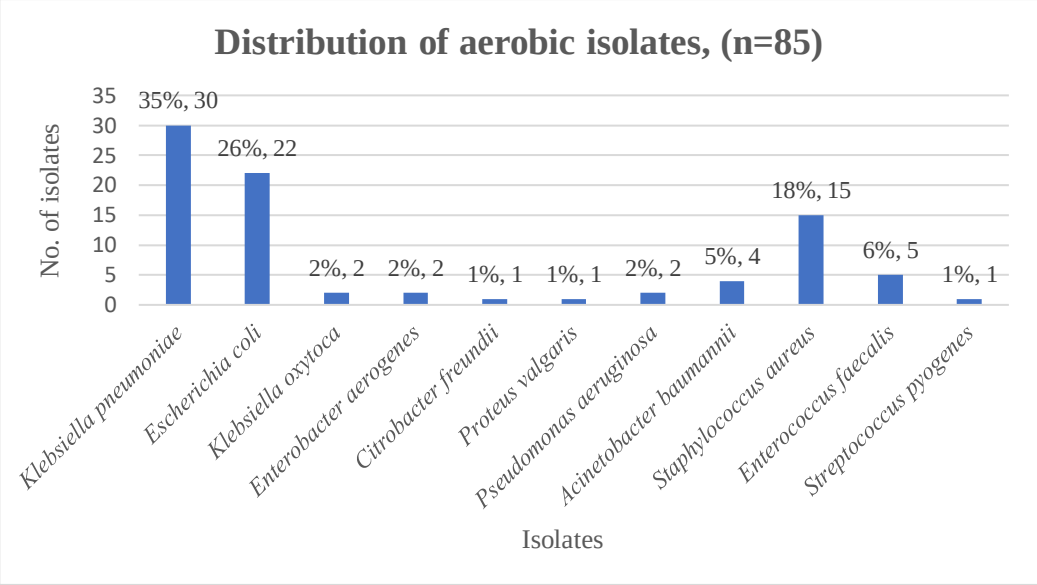


Fig 33: Distribution of aerobic isolates

As shown in the Fig 33, most common GNB isolated was *Klebsiella pneumoniae* 35% (n=30) followed by *Escherichia coli* which was isolated in 26% (n=22) cases. Other Gram-negative organisms like *Enterobacter aerogenes* 2% (n=2), *Citrobacter freundii* 1% (n=1), *Klebsiella oxytoca* 2% (n=2), *Proteus vulgaris* 1% (n=1), *Acinetobacter baumannii* 5% (n=4), *Pseudomonas aeruginosa* 2% (n=2) were also isolated. Among the GPCs *Staphylococcus aureus* was isolated in 18% (n=15) cases. *Enterococcus faecalis* was seen in 6% (n=5) cases. *Streptococcus pyogenes* was isolated in 1% (n=1) cases.

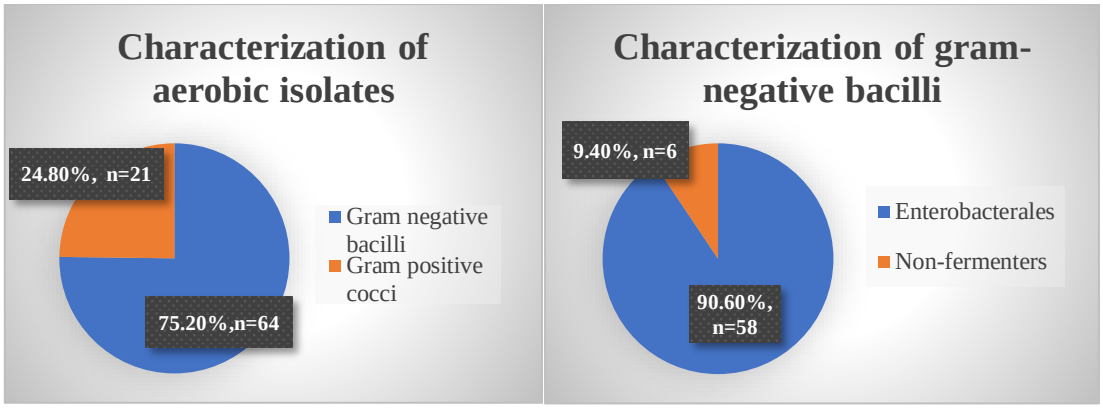


Fig 34: Characterization of aerobic isolates

Among the aerobic isolates most common was Gram negative bacteria (GNB) 75.2% (n=64) followed by Gram positive cocci (GPC) 24.8% (n=21) as per Fig 34. Enterobacterales were 90.6% (n=58) and non-fermenters were 9.4% (n=6)

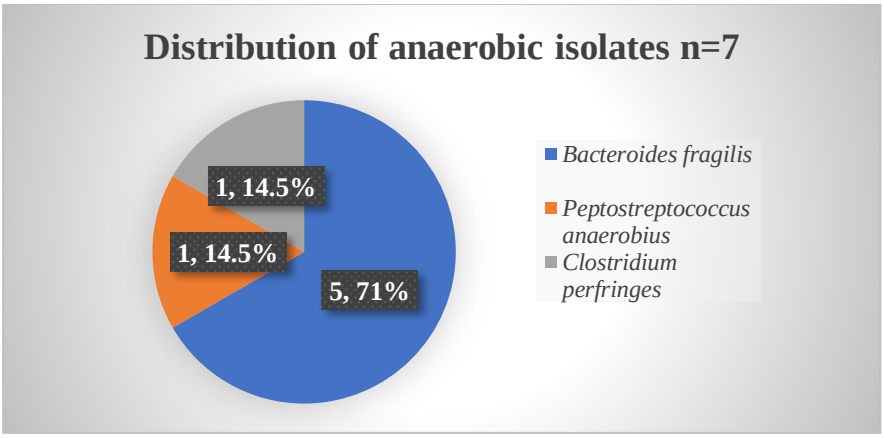


Fig 35: Distribution of anaerobic isolates.

Among the 7 anaerobes *Bacteroides fragilis* 67% (n=5) was the most commonly isolated followed by *Peptostreptococcus anaerobius* and *Clostridium perfringes* were isolated in 14.5% (n=1) cases each as shown in Fig 35.

Antibiotic resistance pattern of the aerobic bacteria in deep seated infections.

Antibiotics	*Klebsiella pneumoniae (n =30)*	*Escherichia coli (n = 22)*	*Enterobacter aerogenes*	*Klebsiella oxytoca (n=2)*	*Citrobacter freundii (n=1)*	*Proteus vulgaris (n=1)*	*Acinetobacter baumannii (n=4)* 2)	*Pseudomonas aeruginosa (n=2)*	*Total (n=64)*
Ceftriaxone	73.3% (n=22)	72.7% (n=16)	0	0	0	0	50% (n=2)	-	62.5% (n=40)
Cefepime	70% (n=21)	72.7% (n=16)	0	0	0	0	25% (n=1)	0	59.3% (n=38)
Piperacillin-Tazobactam	56.6% (n=17)	50% (n=11)	0	0	0	0	50% (n=2)	0	46.8% (n=30)
Cotrimoxazole	66.6% (n=20)	68.1% (n=15)	0	0	0	0	50% (n=2)	-	57.8% (n=37)
Amikacin	56.6% (n=17)	54.5% (n=12)	0	0	0	0	25% (n=1)	0	46.8% (n=30)
Gentamicin	60% (n=18)	59% (n=13)	0	0	0	0	50% (n=2)	0	51.5% (n=33)
Ciprofloxacin	66.6% (n=20)	72.7% (n=16)	0	0	0	0	50% (n=2)	0	59.3% (n=38)
Meropenem	63.3% (n=19)	45.4% (n=10)	0	0	0	0	25% (n=1)	0	46.8% (n=30)
Imipenem	63.3% (n=19)	45.4% (n=10)	0	0	0	0	25% (n=1)	0	46.8% (n=30)
Ertapenem	63.3% (n=19)	45.4% (n=10)	0	0	0	0	-	-	46.8% (n=30)
Aztreonam	60% (n=18)	54.5% (n=12)	0	0	00	00	-	0	46.8% (n=30)
Minocycline	40% (n=12)	40.9% (n=9)	0	0	0	0	0	-	32.8% (21)
Colistin	0	0	0	0	0	0	0	0	0

Table 21: Antibiotic resistance pattern in the Gram-negative bacilli

As described in Table 21, GNBs were highly resistant to 3rd and 4th generation cephalosporins like ceftriaxone 62.5% & cefepime 59.3%. piperacillin-tazobactam was resistant in 46.8% cases of GNBs. Aminoglycosides like amikacin, gentamicin were resistant in 46.8% and 51.5% respectively. Fluoroquinolones like ciprofloxacin was resistant in 56.3% cases of GNBs. Carbapenems were resistant in 46.8% cases of GNBs. Minocycline was sensitive in 32.8% cases while no resistance was detected in colistin except the intrinsic resistance ones. Most of the multidrug resistance organisms were *Klebsiella pneumoniae* and *Escherichia coli*.

Antibiotics	*Staphylococcus aureus (n = 15)*	*Enterococcus faecalis (n = 5)*	*Streptococcus pyogenes (n = 1)*
Cefoxitin	60% (n=6)	-	-
Erythromycin	33.3% (n=5)	0	0
Clindamycin	26.6% (n=4)	0	0
Vancomycin	0	0	0
Linezolid	0	0	0
Teicoplanin	0	0	0
Ciprofloxacin	13.3% (n=2)	0	0
Gentamicin	13.3% (n=2)	-	0

Table 22: Antibiotic resistance pattern of the Gram-positive isolates

As per Table 22, MRSA was detected in 60% cases. Erythromycin and clindamycin were resistant in 33.3% and 26.6% isolates of *Staphylococcus aureus*. Ciprofloxacin and gentamicin were resistant in 13.3% cases of *Staphylococcus aureus*. No resistance was detected to vancomycin, linezolid and teicoplanin. No resistance was detected in case of *Enterococcus feacalis* and *Streptococcus pneumoniae*.

Multidrug Resistance detection in aerobic isolates:

Organisms	ESBL	AmpC	MBL
Klebsiella pneumoniae (n=30)	16.6% (n=5)	3% (n=1)	60% (n=18)
Escherichia coli (n=22)	22.72% (n=5)	9% (n=2)	45.4% (n=10)
Enterobacter aerogenes	0	0	0
Citrobacter freundii	0	0	0
Klebsiella oxytoca	0	0	0
Proteus vulgaris	0	0	0
Total GNB (n=64)	15.7% (n=10)	4.6% (n=3)	43.7% (n=28)

Table 23: Distribution of β-lactamases in the Enterobacterales.

ESBL and MBL were detected in 15.7% and 43.7% of total GNBs. AmpC production was seen in 4.6% of GNBs. ESBL production was detected in 16.6% and 22.72% of *Klebsiella pneumoniae* and *Escherichia coli* respectively. AmpC was seen in 3% and 9% of *Klebsiella pneumoniae* and *Escherichia coli* respectively. 60% *Klebsiella pneumoniae* and 45.4% *Escherichia coli* produced Metallo beta lactamases as shown in Table 23.

	Methicillin Resistance	Inducible Clindamycin (D-Test)
Staphylococcus aureus (n=15)	60% (n=6)	26.6% (n=4)

Table 24: Resistance in *Staphylococcus aureus*.

60% isolates of *Staphylococcus aureus* were Methicillin Resistant *Staphylococcus aureus.* Inducible clindamycin resistance was detected in 26.6% cases of *Staphylococcus aureus* as per Table 24.

Antibiotic susceptibility testing of anaerobes

Antibiotics	*Bacteroides fragilis* (n=5)	*Peptostreptococcus anaerobius* (n=1)	*Clostridium perfringes* (n=1)
Piperacillin-Tazobactum	0 (n=0)	-	-
Amoxicillin-Clavulanic acid	0 (n=0)	0 (n=0)	0 (n=0)
Cefoperazone	0 (n=0)	0 (n=0)	-
Meropenem	0 (n=0)	-	-
Imipenem	0 (n=0)	-	-
Metronidazole	0 (n=0)	-	0 (n=0)
Chloramphenicol	0 (n=0)	-	-
Clindamycin	0 (n=0)	0 (n=0)	0 (n=0)

Table 25: Antibiotic resistance pattern of the anaerobic bacteria.

All the anaerobes were 100% sensitive to the antimicrobials used against them as shown in the Table 25.

DISCUSSION

Deep seated infections can occur in various parts of the body and are usually associated with high morbidity and mortality. Often are polymicrobial in nature and a vast group of both anaerobic and aerobic bacteria act as an etiological agent [1]. Proper identification of causative agent and its antimicrobial susceptibility pattern is required for better management of patient. Pyogenic infections including deep seated infections have a significant load in view of cost to the health services across the world. Due to irrational and overuse of antimicrobials, infections caused by MDR bacteria are increasing [1,7,42]. It has become biggest threat in world, as availability of newer antibiotics are less. Even evolution of resistance in the bacterial strains is natural, overuse and irrational use of antimicrobials have accelerated the speed of emergence of resistance.

In the present study out of 107 participants, male was 69% and female was 31% i.e., M: F is 2.2:1. The study conducted by Rijal BP *et al* [78] had 66.3% male subjects. The study conducted by JM Kalita *et al.* [24] had M: F of 1.6:1. In the study conducted by Rana *et al.* [79] male predominance was seen by 1.5:1 ratio. Mudasar *et al* [80]. also found comparable data of having 60% male participants. This can be explained that in our set up male are vulnerable to various types of wound and deep pyogenic infections due to their more involvement in outdoor professions as compared to females.

In the present study the most of the population were from the age group 21-40 years (32%) followed by 30% in the older age group 41-60 years. Study conducted by JM Kalita *et al* [24]. also had more participants 34.32% in the age group of 20-40 years. In the study done in Nepal by Rijal BP *et al* [78]. had participants of about 31.34% in the age group 20-40 years of age group. The study conducted by Manmeet kaur Gill *et al* [22]. have also found more participants in the age group of 20-40 years.

In this study of deep-seated abscesses were found to be the most common type of deep-seated pyogenic infections 59%. Saini *et al* [21]. on their studies had also documented abscesses 43% to be the most common form of infections.

In the present study, deep-seated abscesses were predominantly present in intra-abdominal sites (32% cases). Most of the studies have shown intra-abdominal abscess to be the common deep abscess probably due to having high blood supply in this region

and also might be having the highest number of commensal flora which can breach and cause infections [1,2,8]. It is comparable with the study conducted by Basireddy *et al.* [5], where intraabdominal abscesses were 28%. Pramodhini S *et al.* [14] also had similar finding of about 31% intraabdominal abscesses. Brook *et al*. [17] in his study of deep abscesses found 75% (585 out of 778) i.e., two-third of total cases having deep abscesses in the abdominal region. Out of the 585 intra-abdominal abscesses most were in the retroperitoneal (27%) region.

In our study out of the 107 samples processed growth was seen in 74.8% cases and remaining 25.2% were sterile. Study conducted by Basireddy *et al* [5]. had growth in 70% cases. Biradar *et al* [81] had shown growth in 66% of samples. In another study conducted by Rana *et al* [79] 72.9% of the samples were having growth. Comparable finding was also found in the study conducted by R Sharma *et al* [28]. as shown in Table 26. This may be due to variation in the prevalence of infection in different areas and culture method can also vary from place to place.

Studies	Basireddy *et al* [5]	Biradar *et al* [81]	Rana *et al* [79]	Kedar Mohan *et al* [25]	R Sharma *et al* [28]	Present study
Growth	70%	66%	72.9%	78%	85.02%	74.8%
No growth	30%	34%	27.1%	22%	14.98%	25.2%

Table 26: Comparison of the culture reports of various studies

In the present study 80 positive cultures grew 92 bacterial pathogen i.e., the rate of growth per sample is 1.16 which is comparable to the studies conducted by Basireddy *et al* [5]. where they found the rate to be 1.2 and the average rate of 1.45 isolates per sample were found in the studies conducted by Pramodhini S *et al* [14] and Saini *et al* [21].

As shown in Table 27, out of the 80 samples having growth, polymicrobial flora was present in 15% cases and monomicrobial isolates were there in 85% cases. Study conducted by Basireddy *et al* [5] have 79.1% monomicrobial flora and 20.9%

have polymicrobial flora. Other studies also have more or less comparable findings. Polymicrobial flora are usually associated with oral abscesses, diabetic foot ulcers, deep venous ulcers.

Studies	Basireddy *et al* [5]	Pramodhini S *et al* [14]	Saini *et al* [21]	Sukanya sudhaharan *et al* [83]	Beena *et al* [82]	Present study
Monomicrobial	79.1%	88%	85%	93.2%	84.33%	85%
Polymicrobial	20.9%	11%	15%	6.8%	15.66%	15%

Table 27: Comparison of types of growth in various studies

Among the polymicrobial flora isolated, isolates having both aerobic and anaerobic were 16% which is comparable with the study conducted by Kedar Mohan *et al* [25]. where the mixed infection with aerobic and anaerobic organisms was 20.52%. However, in the study conducted by Pramodhini S *et al* [14]. the rate of mixed infection having aerobic and anaerobic growth was around 43%.

In the present study out of the 92 isolates processed, aerobic organisms were 92.3% and anaerobic organisms are 7.7% isolates. In the other study conducted by Saini *et al* [21] has found 5% anaerobes in the study group. The study conducted by Nema S *et al.* [84] had shown 14.5% of anaerobes causing pyogenic infections. The study conducted by PA Shenoy *et al* [6]. had found the rate of anaerobes causing infections to be 12.48%. Whereas Pramodhini S *et al* and Basireddy *et al*. aerobic isolates were around 75% and anaerobic were around 25%. Variation in the rate of isolation of anaerobes is due to the fact anaerobes are fastidious organisms and are difficult to grow in culture. The prevalence of anaerobic infections could also be less in this region as there is no previous studies depicting the anaerobic infection in this region.

In this present study on deep seated pyogenic infections among the aerobes, Gram negative bacilli 75.2% were the predominant as compared to Gram-positive bacteria were 24.8%. The study conducted by R Sharma *et al* [28]. had also shown the predominance of Gram-negative bacilli 76.44% in their study. Study done by JM Kalita *et al* [24] in a tertiary care hospital in Rajasthan found Gram negative bacilli 70.59% to be the most predominant group among the aerobic isolates. Although in some studies

like Basireddy *et al.* [5] and Pramodhini S *et al.* [14] there was predominance of gram-positive organisms in causing pyogenic infections. The reason might be the prevalence of GNB causing pyogenic infection is much higher in this region as shown by J M Kalita *et al* [24] on their previous study.

As per Table 28, in the present study *Klebsiella pneumoniae* 35% was the most common aerobic isolate followed by *Escherichia coli* which was isolated in 26% cases. Study conducted by Poonam Verma *et al.* [23] also found *Klebsiella pneumoniae* 33% to be the most common Gram-negative isolate. Other studies like Manmeet kaur Gill *et al* [22], JM Kalita *et al.* [24] have shown *Escherichia coli* to be the most common Gram-negative bacteria to be isolated 29.2%, 24.7% respectively. The isolation rate of *Escherichia coli* in the present study is comparable with the above-mentioned studies. Non- Fermenters like *Pseudomonas aerginosa, Acinetobacter baumannii* which were isolated in 2%, and 5% respectively. Acinetobacter baumannii isolated is also compared with various studies like Manmeet Kaur Gill *et al.* [22] and JM Kalita *et al.* [24]. *Staphylococcus aureus* was the most common Gram-Positive bacteria 18% followed by *Enterococcus faecalis* 6% cases. This finding is comparable with the study done by Manmeet Kaur Gill *et al*. [22] where the prevalence of Staphylococcus aureus was 20%. Pramodhini *et al.* [14], Saini *et al*. [21], Itzhak Brook *et al.* [17] *also* found the isolation rate of *Staphylococcus aureus* to be 38%, 30%and 26% respectively. *Enterococcus feacalis* isolated in 6% cases is quite similar to the study done by Basireddy *et al.* [5] where they found the rate of isolation to be 6%.

	Itzhak Brook *et al* [17]	Pramodhini S *et al* [14]	Saini *et al* [21]	Manmeet kaur Gill *et al* [22].	Basireddy *et. al* [5]	Poonam Verma *et al* [23]	JM Kalita *et al* [24]	Present study
Klebsiella pneumoniae	2%	9.7%	9%	12.3%	12%	33%	14.4%	35%
Escherichia coli	15%	17.9%	13%	29.2%	24%	16%	24.7%	26%
Enterobacter aerogenes	-	-	-	4.9%	-	-	1.7%	2%
Klebsiella oxytoca	-	-	-	-	-	-	-	2%
Citrobacter freundii	-	-	-	1.3%	4%	-	1.4%	1%
Proteus vulgaris	9%	4.4%	4%	3.1%	4%	7%	1.5%	1%
Pseudomonas aeruginosa	3%	-	11%	11.1%	9%	18%	16.6%	2%
Acinetobacter baumannii	-	-	-	8%	-	-	8.3%	5%
Staphylococcus aureus	26%	38%	30%	20%	29%	40%	30%	18%
Enterococcus faecalis	7%	-	-	1%	6%	-	4.6%	6%
Streptococcus pyogenes	-	16%	6%	0.2%	6%	-	0.5%	1%

Table 28: Comparison of aerobic isolates in various studies.

As shown in Table 29, in the present study among the anaerobic isolates most common isolate was *Bacteroides fragilis* 71% which is comparable with the finding in the studies by Pramodhini *et al.* [14] and Saini *et al.* [21] where they also found the most common anaerobe to be isolated as *Bacteroides fragilis* 59% and 66% respectively. Other anaerobes like *Peptostreptococcus anaerobius* (14.5%) and

Clostridium perfringes (14.5%) were also isolated in the present study. *Bacteroides* being a predominant commensal flora of the oral cavity and abdomen, the chance of it to breach the mucosal membrane when there are appropriate host factors and causing deep seated pyogenic infections is high.

	Itzhak Brook *et al.* [17]	Pramodhini S *et al.* [14].	Saini *et al.* [21].	Basireddy *et. al* [5].	Kedar Mohan *et al.* [25].	Present study
Bacteroides fragilis	16%	59%	60%	23%	-	71%
Peptostreptococcus anaerobius	35%	41%	-	41%	38%	14.5%
Clostridium perfringes	10%	-	-	9%	50%	14.5%

Table 29: Comparison of anaerobic isolates in different studies

Staphylococcus aureus and HAI causing Gram negative bacteria produce a very large amount of potent virulence factors, which are responsible for causing and maintaining the infection and delay in the process of healing [85]. The major concern of the study is the high prevalence of multi drug resistance pathogenic bacteria causing pyogenic infections which focuses us to rethink about judicious use of antimicrobial agent. Among the BRICS countries (Brazil, Russia, India, China and South America) India has an increase in 23% of retail sale of antibiotics. With 10.7 units of antibiotic being consumed per person India was the highest antibiotic consumer on 2010 [86]. Thus, easily availability of the over-the-counter antibiotics and irrational use of antimicrobials has led to increase in the emergence of Multi drug resistant superbugs in the community and it is becoming a global threat now a days [87].

In the present study many Gram-negative bacteria were seen to be resistant to most of the commonly used antibiotics. Third generation cephalosporins like ceftriaxone was resistant in 73.3%, 72.7%, 50% cases of *Klebsiella pneumoniae, Escherichia coli* and *Acinetobacter baumannii* respectively. Cefepime was resistant in 70% *Klebsiella pneumoniae,* 72.7% *Escherichia coli* and 25% cases of *Acinetobacter*

baumannii. 56.6% *Klebsiella pneumoniae,* 50% *Escherichia coli* and 50% *Acinetobacter baumannii* were resistant to Beta- lactam inhibitors like piperacillin-tazobactam. Aminoglycosides like amikacin, gentamicin was resistant to *Klebsiella pneumoniae* in 56.6% and 60% respectively. Gentamicin and amikacin were resistant in 50% and 25% of *Acinetobacter baumannii* respectively. In case of *Escherichia coli,* the resistance to amikacin and gentamicin were 54.5% and 59% respectively. Fluoroquinolones like ciprofloxacin were resistant in 66.6%, 72.7%, 50% cases of *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii* respectively. Carbapenems like meropenem, imipenem, ertapenem were resistant in 63.3% cases of *Klebsiella pneumoniae,* 45.4% case of *Escherichia coli* and 25% cases of *Acinetobacter baumannii*. Aztreonam were resistant in 60% and 54.5% (n=12) in case of *Klebsiella pneumoniae* and *Escherichia coli* respectively. Minocycline was sensitive in around 60% cases of the GNBs. Colistin was 100% sensitive in all the Gram-negative bacteria except the intrinsic resistant ones like *Proteus vulgaris*. *Enterobacter aerogenes, Citrobacter freundii, klebsiella oxytoca, Pseudomonas aeruginosa, Proteus vulgaris* were 100% sensitive. All the Gram-negative isolates were 100% sensitive to polymyxin except the intrinsic resistance ones. The recent pan Indian study on the nation-wide antimicrobial resistance surveillance 2019 showed high resistance of antibiotics towards the Gram-negative bacteria which were commonly isolated. The study showed resistance towards cephalosporins to be >70% and >60% towards the fluoroquinolones [88]. The present study found ESBL producing 16.6% and 22.72% *Klebsiella pneumoniae* and *Escherichia coli* respectively. 60% *Klebsiella pneumoniae* and 45.4% *Escherichia coli* produced Metallo beta lactamases. The findings of the antimicrobial resistance pattern are also comparable to the finding of the Basireddy *et al.* [5], JM Kalita *et al.* [24]., R Sharma *et al.* [28].

Present study findings also corelates with other studies conducted by different authors like Pramodhini *et al.* [14] where the occurrence of ESBL producing Enterobacterales was about 32.6% with majority of the 47% of Klebsiella pneumoniae.

In this study MRSA was detected in 60% cases. The variability in the findings could be due to the reason that the above-mentioned studies were done on various types of samples. In the study conducted by Manpreet kaur Gill *et al*. [22] in Punjab, 75% isolates were MRSA. The present study findings were also in agreement with those studies done in Nepal, Italy, and Ethiopia which shows high prevalence of MRSA

[89,90,91]. Inducible Clindamycin resistance was detected in 26.6% in the present study. Comparable to the finding with 23.6% seen on a multicentric study done by Walia K *et al* [88]. Vancomycin and Linezolid were 100% sensitive in this study which is in correlation with the studies done by R Sharma *et al* [28] and Rijal BP *et al.* [78].

All the anaerobe isolated were 100% sensitive to the antimicrobials used against them like beta- lactam inhibitors, imidazole, macrolides, carbapenems etc which corresponds to the finding by Pramodhini *et al* [14] and Saini *et al* [21]. In the study conducted by Ritu Garg et al [92] most of the anaerobic isolates were 100 % sentitive to the antimicrobials, however metronidazole was resistant in 16% cases.

Hence, there is a high multidrug resistance seen among aerobic isolates causing deep seated pyogenic infections. Environment of the abscess also have impact on many antimicrobials. The capsule of the abscess, low pH level, presence of the binding proteins and inactivating enzymes like β-lactamase impairs the activity of antimicrobials. Management of the mixed anaerobic and aerobic infections requires drainage of the pus along with the administration of antimicrobial agents which are effective against both aerobic and anaerobic isolates. Without adequate treatment infection will still persist. As it is a tertiary level health care system, so most of the patients come on referral basis i.e., referred from primary health care settings where there is pre-exposure of antibiotics. Other causes like irrational over use of antibiotics, long hospital stays can also lead to emergence of MDR strains.

CONCLUSION

Deep seated pyogenic infections are generally characterized by inflammation of soft tissue, skin and internal organs which are usually caused due to multiplication and invasion of the pathogenic microorganism. The pathogens release toxic metabolites and various leucocidins which kills the neutrophils forming various abscesses and pus. This DSPIs are usually associated with both mortality and morbidity worldwide by prolonged hospital stay and causing disability in the patients.

DSPIs are caused by various etiological agents consisting both aerobic and anaerobic organisms. Aerobic organisms like *Staphylococcus aureus, Escherichia coli, Klebsiella spp. And anaerobic organisms like Bacteroides* species, *Peptostreptococcus* species are the common etiological agents. Over and irrational use of antibiotics is one of the important causes which transform these organisms as a superbug.

In the present study most common aerobic isolate causing DSPI was found to be *Klebsiella pneumoniae* followed by *Escherichia coli*. Among the gram-positive organism most common was *Staphylococcus aureus. Bacteroides fragilis* was the most common anaerobic isolate. Most of the aerobic gram-negative isolates were multidrug resistant. ESBL and MBL was detected in 15.7% and 43.7% of total GNBs respectively Methicillin resistance was also detected in majority of *Staphylococcus aureus*. However, no resistance was detected in the anaerobic isolates.

This prospective study gives an idea about prevalence of the common etiology causing deep seated pyogenic infection and also the antibiotic resistance pattern of the isolates in the western part of Rajasthan. In this study most of the MDR superbugs are the Gram-negative bacteria. Anaerobic bacteria are also shown to cause deep seated pyogenic infections although isolating anaerobes are very difficult and cumbersome for which it is usually neglected. Proper Antimicrobial stewardship programme to be followed to combat the emergence of the MDR superbugs. Local antibiotic policy should be made for proper treatment of the pyogenic infections so that it can be managed early.

This study will help the institute in selecting the antibiogram against the common isolates isolated as a part of good antimicrobial stewardship programme to control the MDR superbugs causing deep seated pyogenic infections.

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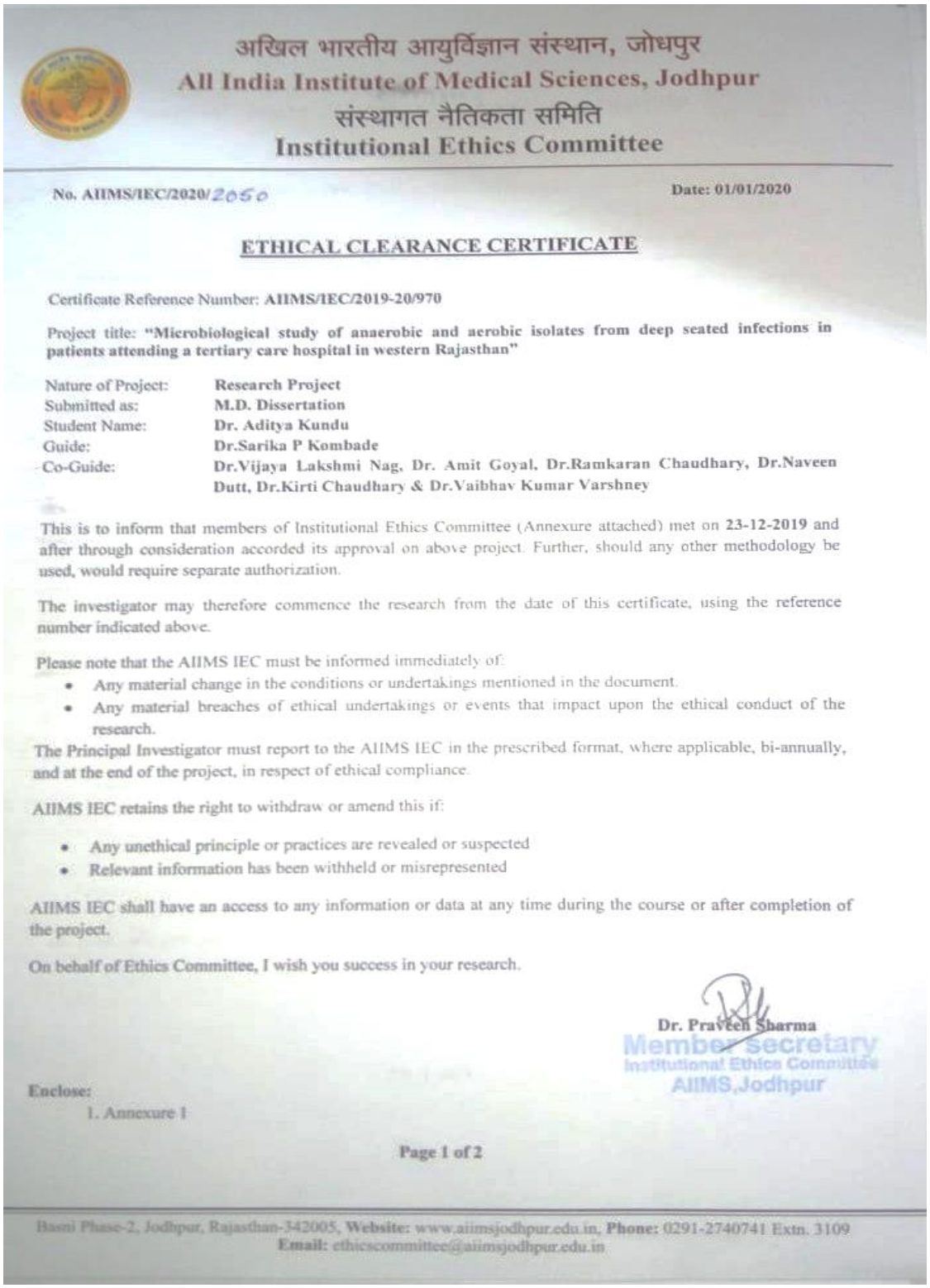
ANNEXURE - 1

Institutional Ethical Committee certificate



अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2020/2050 Date: 01/01/2020

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2019-20/970**

Project title: **"Microbiological study of anaerobic and aerobic isolates from deep seated infections in patients attending a tertiary care hospital in western Rajasthan"**

Nature of Project:	**Research Project**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Aditya Kundu**
Guide:	**Dr.Sarika P Kombade**
Co-Guide:	**Dr.Vijaya Lakshmi Nag, Dr. Amit Goyal, Dr.Ramkaran Chaudhary, Dr.Naveen Dutt, Dr.Kirti Chaudhary & Dr.Vaibhav Kumar Varshney**

This is to inform that members of Institutional Ethics Committee (Annexure attached) met on **23-12-2019** and after through consideration accorded its approval on above project. Further, should any other methodology be used, would require separate authorization.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member Secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Enclose:
1. Annexure 1

Page 1 of 2

Basni Phase-2, Jodhpur, Rajasthan-342005, **Website:** www.aiimsjodhpur.edu.in, **Phone:** 0291-2740741 Extn. 3109
Email: ethicscommittee@aiimsjodhpur.edu.in

ANNEXURE - 2

All India Institute of Medical Sciences, Jodhpur

Informed Consent Form

Title of the project: MICROBIOLOGICAL STUDY OF THE ANAEROBIC AND AEROBIC ISOLATES FROM DEEP SEATED INFECTIONS IN PATIENTS ATTENDING A TERTIARY CARE HOSPITAL, WESTERN RAJASTHAN.

Name of the Principal Investigator: Dr Aditya Kundu Tel no.8974371954

Patient ID NO : _____

I,_____S/o or D/o_____ R/o _____give my full, free, voluntary consent to be a part of the Microbial study of the anaerobe and aerobic isolates from deep seated infections and their antimicrobial susceptibility pattern in a tertiary care center, the procedure and nature of which has been explained to me in my own language to my full satisfaction.

I confirm that I have had the opportunity to ask questions. I understand that my participation is voluntary and I am aware of my right to opt out of the study at any time without giving any reason.

I understand that the information collected about me and any of my medical records may be looked at by responsible individual from AIIMS Jodhpur or from regulatory authorities. I give permission for these individuals to have access to my records.

Date: _____ _____

Place: _____ Signature/Left thumb impression of patient/caregiver

This to certify that the above consent has been obtained in my presence.

Date: _____ _____

Place_____ Signature of Principal Investigator

1. Witness 1 2. Witness 2

Signature Signature

ANNEXURES- 3

अखिल भारतीय चिकित्सा विज्ञान संस्थान

सूचित सहमति प्रपत्र

अन्वेषक का नाम : डॉ आदित्य कुंडू.
मोबाइल न 8974371954

रोगी आईडी नं.

मैं. ---------- एस / ओ या डी / ओ.

अपनी पूरी स्वैच्छिक सहमति देता हूं, पश्चिमी राजस्थान में तृतीयक देखभाल अस्पताल में जाने वाले रोगियों में गहरे बैठे संक्रमण से एनारोबिक और एरोबिक का सूक्ष्मजीवविज्ञानी अध्ययन का हिस्सा बनने के लिए ।अपनी भाषा में अपनी पूर्ण संतुष्टि के लिए मुझे समझाया गई है। मैं पुष्टि करता हूं कि मुझे सवाल पूछने का अवसर मिला है।

मैं समझता हूं कि मेरी भागीदारी स्वैच्छिक है और मुझे बिना कोई कारण बताए किसी भी समय अध्ययन से बाहर निकलने के मेरे अधिकार के बारे में पता है।

मैं समझता हूं कि मेरे और मेरे किसी भी मेडिकल रिकॉर्ड के बारे में एकत्रित जानकारी को एम्स, जोधपुर के जिम्मेदार व्यक्ति या नियामक अधिकारियों से देखा जा सकता है।

दिनांक.
स्थान.
प्रिंसिपल जांचकर्ता के हस्ताक्षर

1. साक्षी.

2.साक्षी

ANNEXURE 4

All India Institute of Medical Sciences, Jodhpur

PATIENT INFORMATION SHEET

Deep seated infections are one of the important causes if not treated properly can cause morbidity and mortality to the patient. It can be caused by anaerobic or aerobic or can be of mixed origin.

PURPOSE OF STUDY: To study the anaerobic and aerobic isolates of deep seated infections and antimicrobial susceptibility pattern of the isolates in patients attending a tertiary care hospital, Western India.

METHODS INVOLVED: Samples like pus aspirates, tissue biopsy, body fluids, drain fluid will be collected in a sterile container and transported to the laboratory as early as possible within 30 minutes and will be processed for gram staining, culture will be done to isolate the organisms and identification of the species will be done by VITEK, and antimicrobial susceptibility pattern will be seen.

DURATION OF SUBJECT PARTICIPATION: A total of presenting to of AIIMS, Jodhpur presenting with deep pyogenic infections.

BENEFIT OF STUDY TO THE PATIENT: The isolation of the anaerobic or aerobic bacteria from deep seated infections and their antimicrobial susceptibility pattern testing will be helpful in the proper diagnosis and treatment to the patient and will reduce the misuse of antibiotics. As misuse of antibiotics is one of the leading causes of antimicrobial resistance.

RISK INVOLED TO THE PATIENT: There is no risk of any kind to the patient in this study. No drug or vaccines are being tested in the study.

CONFIDENTIALITY OF RECORDS: The patient's records/reports/ shall be kept confidential.

FREEDOM TO PARTICIPATE AND WITHDRAW FROM THE STUDY: The patient is free to participate and to withdraw from the research at any time. He/She shall not experience any kind of loss in the time of enrolment into the study.

ANNEXURES- 5

अखिल भारतीय चिकित्सा विज्ञान संस्थान

रोगी सूचना पत्र

, पश्चिमी राजस्थान में तृतीयक देखभाल अस्पताल में जाने वाले रोगियों में गहरे बैठे संक्रमण से एनारोबिक और एरोबिक का सूक्ष्मजीवविज्ञानी अध्ययन

अध्ययन का उद्देश्य : गहरे बैठे संक्रमण से अवायवीय और एरोबिक बैक्टीरिया को अलग करने के लिए और आइसोलेट्स के रोगाणुरोधी संवेदनशीलता की स्थिति का अध्ययन करने के लिए।

अध्ययन में शामिल तरीके : नमूने दो सेटों में एकत्र किए जाएंगे और ग्राम स्टेनिन के लिए संसाधित किए जाएंगे। जीवाणुओं के अलगाव के लिए एरोबिक और एनारोबिक संस्कृति मीडिया दोनों में नमूने सुसंस्कृत किए जाएंगे और रोगाणुरोधी संवेदनशीलता परीक्षण किया जाएगा।

रोगी को अध्ययन का लाभ : यह हमें संक्रमण में अवायवीय और एरोबिक बैक्टीरिया के प्रसार का विचार देगा और उनके साथ जुड़े रोगाणुरोधी संवेदनशीलता पैटर्न के बारे में भी। यह त्रासदी को आसान बना देगा।

रोगी के लिए जोखिम : रोगी को किसी प्रकार का कोई खतरा नहीं है। रोगी में कोई भी दवा या वैक्सीन का परीक्षण नहीं किया जाता है।

परिणामों की गोपनीयता : मरीजों के रिकॉर्ड / रिपोर्ट को गोपनीय रखा जाएगा।

अध्ययन से भाग लेने और वापस लेने की स्वतंत्रता : रोगी किसी भी समय अध्ययन से भाग लेने और वापस लेने के लिए स्वतंत्र है।

ANNEXURES- 6

Case record form:

Patient Details: Name:

Sex: OPD/WARD No: Registration No: Age:

Clinical History:

Patient Complaints:

Type of sample: Pus/ Tissue /Exudate /Aspirated fluid / any other

History of Injury: Yes/No Traumatic/ Surgical/ Intravascular injection/ Any other

Date and Time of Injury:

Any other underlying disease:

Local Examination:

1 Swelling/ blebs

2 Discharge — Purulent/ Blackish brown/ Foul smelling/ Sulphur granules/ Any other

3 Crepitus/ Crackling sound

4 Any other (if specify)

Other Laboratory investigation:

Microscopic *exams* nation:

Aerobic culture:
Anaerobic culture
Treatment given:

1. Antibiotics given: Yes/No
2. Name of Antibiotics given:
3. Debridement- Done/ Not done

Recovery:

Final diagnosis:

ANNEXURES- 7

Abstract presented in E-microcon 2020

Microbiological study of anaerobic and aerobic isolates in deep seated infections in patients attending a tertiary care hospital in Western Rajasthan.

Dr Aditya Kundu[1], Dr Sarika P Kombade[1], Dr V L Nag[1]

1: All india institute of medical sciences, jodhpur

INTRODUCTION Deep seated infections (DSI) are one of the common cause of hospitalization which results in high mortality & morbidity due to MDR superbugs. DSI-mostly pyogenic in nature –superficial / deep seated. DSI are generally polymicrobial in nature which are caused by anerobic and aerobic bacteria. As Anaerobic culture is neglected and DSI are mostly polymicrobial in nature cause of DSI, so this study is formulated to determine both aerobic & anaerobic bacteria from deep seated infections in patients attending in this Institution.

AIM & objectives: To study the anaerobic and aerobic bacterial isolates from deep seated infections in patients attending a tertiary care hospital and to study the antimicrobial susceptibility pattern of the isolates.

Material and methods: This prospective, cross sectional observational study was conducted in the laboratory of the department of microbiology of a tertiary care hospital of Jodhpur. All age groups patient with deep seated infections from OPD, IPD, OT were included in this study. The sample from deep seated infections were collected and processed in both anaerobic and aerobic condition and isolates were identified by both conventional and automated method and their antibiotic susceptibility was done according to CLSI 2019.

Result: Out of the 25 samples collected during the period 24 samples have shown growth of either aerobic, anaerobic or mixed growth. 3 samples have growth of obligate anaerobic organisms and 22 samples have aerobic/facultative anaerobic growth. 1 sample have mixed growth of both aerobic and anaerobic growth. *Klebsiella pneumoniae* is the most common isolated among the aerobes and *Bacteroides* among the anaerobic isolates.

Conclusion: Gram negative organisms were commonly isolated from deep seated infections. Anaerobic organisms were also isolated. Majority of aerobic isolates were multi drug resistance including resistance to carbapenems by gram negative bacilli and vancomycin by gram positive organisms.

ANNEXURES- 8

Abstract presented in Micro-d-con 2020

Title: To study the profile of aerobic and anaerobic isolates in deep seated infections in patients attending a tertiary care hospital in Western Rajasthan.

Aditya Kundu, Sarika Kombade, Vijayalakshmi Nag

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

AIM & OBJECTIVES: To study the microbiological profile and drug susceptibility pattern of culture isolates from deep seated infections in patients attending a tertiary care hospital

MATERIAL AND METHODS: This prospective, cross-sectional study was conducted in the Microbiology Department of a tertiary care hospital in Jodhpur. Patients from all age groups with DSI from OPD, IPD, OT were included in this study. The sample from deep seated infections like aspirated pus, tissue, biopsy material, etc. were collected and processed in both anaerobic and aerobic condition and isolates were identified by both conventional and automated method and their antibiotic susceptibility was done according to CLSI 2020.

RESULT: Around 120 samples collected during the period Jul 2020- Dec 2020, out of which 32 shown monomicrobial and 7 polymicrobial growth pattern. Out of the 47 isolates isolated 42 samples have aerobic/facultative anaerobic growth, 5 samples have growth of anaerobic organisms Among aerobes *Klebsiella pneumoniae* (36%) was the most common isolated organism while *Bacteroides fragilis* (6%) was commonest among the anaerobic isolates. Most of the organisms were resistant to the usual drugs.

CONCLUSION: MDR *Klebsiella Pneumoniae* were most commonly isolated organism from DSI while anaerobic organisms were isolated in around 11%. This study will help to formulate local antibiotic policy and help to implement anaerobic profile of isolates in DSPI this region.