Original Article

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Characterisation of Anaerobes Isolated from Various Clinical Samples: A Cross-sectional Study

Microbiology Section

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ABSTRACT

Introduction: Anaerobes are an important cause of infections but are often neglected. These infections can range from simple abscesses to life-threatening infections. The isolation of anaerobes is crucial for administering appropriate antibiotic therapy.

Aim: To investigate the profile of anaerobes in various clinical samples, including deep-seated skin and tissue infections, aspirated body fluids, and tissue biopsies.

Materials and Methods: A cross-sectional study was conducted on a total of 100 samples at the Department of Microbiology, ABVIMS and Dr. RML Hospital, New Delhi, from November 2019 to March 2021. Aspirations from deep-seated abscesses, body fluids, intraoperative samples, and tissue biopsies meeting the criteria for anaerobic culture were included. Simultaneous processing for the detection of aerobes was also performed. Anoxomat III anaerobic culture system was used to create an anaerobic environment. Robertson Cooked Meat (RCM) broth was used, and subculture was conducted on 10% Blood Agar (BA). Presumptive identification was performed using gram stain, catalase test (15% hydrogen peroxide), metronidazole disc (5 µg), special potency disc (vancomycin 5 µg, kanamycin 1000 µg, colistin 10 µg), and aerotolerance test. The Vitek 2 compact ID system was used for the final identification of anaerobes. Statistical Package for Social Sciences (SPSS) software, IBM manufacturer, Chicago, USA, ver 21.0, was utilised.

Results: The isolation rate of anaerobes was 17 (17%), with *Bacteroides fragilis* being the predominant organism (6; 35.29%), followed by *Actinomyces* (2; 11.76%), *Clostridium* (2; 11.76%), *Peptostreptococcus* (2; 11.76%), and *Prevotella* species (2; 11.76%). Isolation was observed from diverse anatomic sites, with pus aspirates constituting the majority of the isolates (9; 52.94%), followed by brain abscesses (3; 17.65%), liver abscesses, peritoneal fluid (2; 11.76%), and tonsillar abscess (1; 5.89%). Five (29.41%) infections were polymicrobial, while 12 (70.59%) were monomicrobial in nature.

Conclusion: Anaerobes are emerging as an important causative agents in a variety of diverse and heterogeneous pyogenic infections. This study demonstrates their isolation from various infection sites. Therefore, routine anaerobic cultures should be conducted alongside aerobic cultures, and the importance of anaerobes in clinical infections should not be underestimated.

Keywords: Abscess, Anatomical sites, Metronidazole, Species

INTRODUCTION

Anaerobes are organisms that fail to grow in the presence of oxygen. They constitute a significant proportion of the normal microbiota and convincingly outnumber aerobes [1]. Anaerobes have been reported to cause infections in practically every organ and anatomical location of the body. The spectrum of infection ranges from local abscesses to life-threatening emergency situations. Historically, anaerobic infections from exogenous sources have been well-described, but recent data demonstrate a gross predominance of endogenous infections, possibly due to the expanding population of patients receiving immunosuppressive drugs or improved recovery of these pathogens in labs [1].

Anaerobic infections are primarily diagnosed based on the suspicion of their presence, since most anaerobic infections are endogenous and result from the breakdown of mucocutaneous barriers or the release of powerful toxins [2,3]. Reduced blood supply and tissue necrosis play a notable role in the aetiopathogenesis of anaerobic infections. The presence of foul smell, gas, discoloration of exudates, etc., strongly suggests an anaerobic infectious aetiology [1,4]. Establishing a diagnosis may be difficult because anaerobes frequently contaminate collected samples, leading to misleading results.

Limited data is available regarding the extent of the problem of anaerobic infections, and it needs to be shared with the medical community for future therapeutic strategies. Commonly encountered anaerobes in clinical samples include Gram-negative anaerobes like *Bacteroides fragilis*, *Porphyromonas*, and *Prevotella*. Gram-positive anaerobes such as *Actinomyces*, *Propionibacterium*, and *Bifidobacterium* are commonly associated with oral/dental infections, dental caries, bacteraemia, and abdominal infections [5]. Diagnosing anaerobic infections is challenging because they are difficult to culture, their identification is demanding, expensive, time-consuming, and are mostly overlooked in microbiology labs unless the lab's resources and capacity align with the requirements of anaerobic bacteriology. Anaerobes are among the most commonly missed or overlooked organisms in clinical samples. Failure to suspect anaerobes in clinical materials and initiate appropriate antimicrobial therapy may result in therapeutic failures and reinforce the essentiality of identifying anaerobes. Anaerobes are hypothesised to be significant pathogens in causing infections in deep-seated tissues and sterile body fluids. This study was undertaken to characterise the profile of anaerobes from various clinical samples of deep-seated skin and tissue infections, aspirated body fluids, and tissue biopsies.

MATERIALS AND METHODS

A cross-sectional study was conducted in the Department of Microbiology at ABVIMS and Dr. RML Hospital, New Delhi, from November 2019 to March 2021. The samples were simultaneously processed for the detection of aerobes. The study was approved by the Institutional Ethics Committee (IEC) with reference IEC no. 32/2019.

Inclusion criteria: Samples were taken from deep-seated infections without surface exposure, abscesses from various body sites, aspirated body fluids, intraoperative samples, and tissue biopsies.

Exclusion criteria: Samples collected on swabs, urine samples, and respiratory samples like sputum, small volume, and small-sized biopsy samples were excluded from the study.

Sample size: According to a previous study, it was found that anaerobes had an isolation rate of 41.1% [6]. Taking this as a reference, the minimum required sample size with a 10% margin of error and a 5% level of significance was 93. Therefore, a sample size of 100 samples was taken to meet the study objectives.

Study Procedure

Aspirations from abscesses, body fluids, and intraoperative samples were collected in sterile containers and immediately transported to the laboratory for anaerobic processing. They were also inoculated into RCM broth for enrichment and subculture after 48 hours and five days of incubation. Some samples like tissue biopsies were collected in RCM broth for further processing. Gram stain was performed on all the samples to obtain a presumptive idea about the possible infecting organisms. The samples were then plated on 10% sheep BA with a metronidazole disc (5 µg) for anaerobic culture. Anoxomat III, an automated anaerobic culture system (Advanced Instruments Inc., Norwood, MA) [7], was used to create an anaerobic environment. The anaerobic jars were incubated for 48 hours, after which the plates were inspected for any growth. All suspected anaerobic colonies were subjected to Gram stain and Catalase test using 15% hydrogen peroxide (H_2O_2) [8]. The inoculated RCM broths were checked daily for growth until five days, and subculture was done on 10% BA in case of turbidity. Simultaneous culture on 10% BA and MacConkey agar was also performed following aerobic microbiological protocol. Basic lab procedures like Gram stain, metronidazole disc (5 µg), special potency discs like vancomycin (5 µg), kanamycin (1000 µg), colistin (10 µg) aided in the preliminary identification of anaerobes [1]. The final identification of the anaerobic isolates was done using the VITEK 2 Compact ID system (ANC card) (bioMerieux) [9], which is a long and tedious process for identifying anaerobes.

STATISTICAL ANALYSIS

The presentation of categorical variables was done in the form of numbers and percentages (%). On the other hand, quantitative data were presented as means±SD and medians with 25th and 75th percentiles (interquartile range). The data entry was done in Microsoft Excel spreadsheet, and the final analysis was performed using SPSS software, manufactured by IBM in Chicago, USA, version 21.0.

RESULTS

A total of 100 samples were collected from deep-seated infections, body fluids, and tissue biopsies from patients attending OPDs, wards, and ICUs over a period of 17 months. Seventy-seven (77%) samples were received from indoor patients, 16 (16%) from OPDs, and 7 (7%) were from intensive care facilities. Among the 100 samples tested, 45 (45%) of the samples were pus aspirates, which constituted the majority, followed by liver abscess (23, 23%), peritoneal fluid (13, 13%), and tissue samples (6, 6%), as mentioned in [Table/Fig-1].

Sample	n (%)
Pus aspirates	45 (45)
Liver abscess	23 (23)
Peritoneal fluid	13 (13)
Tissue	6 (6)
Pleural fluid	3 (3)
Brain abscess	5 (5)
Tonsillar abscess	1 (1)
Bile	3 (3)
CSF	1 (1)

[Table/Fig-1]: Source and site of samples (n=100).

The growth positivity for primary culture plates was 5 (5%), while 95 (95%) did not show any growth. Among the 5 (5%) that showed growth on primary culture plates, 3 (3%) of the organisms were identified as anaerobes and 2 (2%) as facultative anaerobes.

For inoculated samples in RCM broth, turbidity was seen in 51 (51%) samples. Upon subculturing to culture plates, 41 out of 51 broths showed growth, while no growth was observed in the remaining ten broths [Table/Fig-2]. The remaining 49 (49%) broths were clear after re-incubation, and hence, no further processing was done.

RCM broth		Subculture plate
Turbid	Growth	41 (41%)
	No growth	10 (10%)
No turbidity		49 (49%)
Total		100 (100%)

[Table/Fig-2]: Growth on subculture plate from RCM broth.

Among the 100 clinical samples processed in this study, 17 (17%) anaerobes were isolated. Anaerobes were predominantly isolated from pus aspirates, with 9 (52.94%) isolates out of the total 17 isolates, as highlighted in [Table/Fig-3].

The predominant anaerobe isolated was *Bacteroides fragilis* (6, 35.29%), followed by *Actinomyces* (2, 11.76%), *Clostridium* (2, 11.76%), *Peptostreptococcus* (2, 11.76%), and *Prevotella* (2, 11.76%) species [Table/Fig-4].

Out of the 17 isolates, 14 (82.35%) anaerobes were isolated after inoculation in RCM broth, and the remaining 3 (17.65%) were isolated from primary culture plates.

Polymicrobial infections were seen in 5 (29.41%) cases, while 12 (70.59%) were monomicrobial in nature [Table/Fig-5].

A metronidazole disk (5 µg) was placed in each of the culture plates and checked for the inhibition zone around the disk. Three (17.65%) anaerobes were resistant to the disk with no inhibition, whereas 14 (82.35%) of the isolated anaerobes were sensitive to the disk. Two isolates of *Actinomyces* and one *Bifidobacterium* were resistant to the metronidazole disk [Table/Fig-6].

Anaerobes isolates in different sample	Pus aspirates	Brain abscess	Liver abscess	Peritoneal fluid	Tonsillar abscess	Total
Actinomyces	1 (50%)	0	0	0	1 (50%)	2 (100%)
Anaerococcus prevotti	0	0	1 (100%)	0	0	1 (100%)
Bacteroides fragilis	2 (33.33%)	2 (33.33%)	0	2 (33.33%)	0	6 (100%)
Bifidobacterium	1 (100%)	0	0	0	0	1 (100%)
Clostridium spp.	1 (50%)	0	1 (50%)	0	0	2 (100%)
Peptococcus	0	1 (100%)	0	0	0	1 (100%)
Peptostreptococcus	2 (100%)	0	0	0	0	2 (100%)
Prevotella oris	2 (100%)	0	0	0	0	2 (100%)

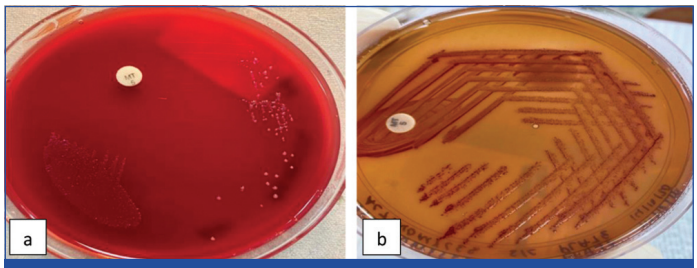
[Table/Fig-3]: Distribution of anerobic isolates in different samples.

Organisms	Frequency	Percentage (out of total 17 anaerobes isolated)
Actinomyces	2	11.76%
Anaerococcus prevotti	1	5.89%
Bacteroides fragilis	6	35.29%
Bifidobacterium	1	5.89%
Clostridium spp.	2	11.76%
Peptococcus	1	5.89%
Peptostreptococcus	2	11.76%
Prevotella	2	11.76%
Total	17	100%

[Table/Fig-4]: Profile of anaerobic isolates.

Samples	Anaerobes mixed with aerobes	Pure anaerobes	Total
Pus aspirates	3	6	9 (52.94%)
Brain abscess	1	2	3 (17.65%)
Liver abscess	0	2	2 (11.76%)
Peritoneal fluid	1	1	2 (11.76%)
Tonsillar abscess	0	1	1 (5.89%)
Total	5 (29.41%)	12 (70.59%)	17 (100%)

[Table/Fig-5]: Distribution of polymicrobial infections in anaerobes.



[Table/Fig-6]: a) *Bacteroides fragilis* showing zone of inhibition around metronidazole disc (5 µg); b) *Actinomyces* showing no inhibition around metronidazole disc (5 µg). Cultures done on Blood Agar (BA).

The isolated gram-positive anaerobes constituted 9 (53%) out of 17 isolated anaerobes and were found to be sensitive to vancomycin (5 µg) and resistant to colistin (10 µg). The isolated gram-negative anaerobes (8, 47%) were resistant to vancomycin (5 µg), and variable findings were observed with colistin (10 µg) and kanamycin (1000 µg) discs.

Among the aerobes, a total of 29 isolates were obtained. *Escherichia coli* and *Staphylococcus aureus* were the predominant organisms with 8 (27.59%) isolates each, followed by 5 (17.24%) isolates of *Klebsiella* species, 2 (6.89%) isolates each of *Acinetobacter* and CoNS. Organisms with the least frequency (1, 3.45%) were *Micrococcus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Sphingomonas* species [Table/Fig-7].

Organisms	n (%)
Acinetobacter	2 (6.89)
CoNS	2 (6.89)
Escherichia coli	8 (27.59)
Klebsiella species	5 (17.24)
Micrococcus	1 (3.45)
Proteus mirabilis	1 (3.45)
Pseudomonas aeruginosa	1 (3.45)
Sphingomonas spp.	1 (3.45)
Staphylococcus aureus	8 (27.59)

[Table/Fig-7]: Profile of aerobic isolates (n=29).

The majority of the aerobic isolates were from pus aspirates (15, 51.72%), 4 (13.79%) isolates each from liver abscess and tissue samples. The remaining isolates were from peritoneal fluid, bile, and brain abscess with 3 (10.34%), 2 (6.89%), and 1 (3.45%), respectively.

DISCUSSION

Anaerobes are an important part of the normal flora that inhabit mucosal surfaces and play a key role in preventing colonisation by pathogenic microbial populations. The isolation rate of anaerobes in the present study was 17% (17 cases). These findings appear to be consistent with other studies conducted in India, where anaerobic isolation rates were reported as 12.48% [10] and 19% [11], respectively. However, there are various studies that have shown higher rates of anaerobic culture positivity, such as 61.05% [12] and 74.6% [13]. The variation in isolation rates could be attributed to the lack of uniform protocols for sample collection and processing of anaerobic samples.

In this study, the isolation of anaerobes in primary culture plates was extremely low, with only three isolations out of a total of seventeen. However, the majority of isolations (14 cases) were obtained through subculture from RCM broth. RCM broth appears to be a good enrichment medium for resuscitating organisms present in original specimens, which may explain its widespread use in various labs [10,14].

In this study, anaerobes were isolated from diverse anatomic sites with varying recovery rates. The majority of anaerobes were isolated from aspirated pus samples, accounting for 9 cases (52.94%), where the exposure to oxygen is minimal or absent. Other isolates were obtained from deep-seated abscesses, such as brain abscess (3 cases, 17.65%) and liver abscess (2 cases, 11.76%). A study conducted by Shenoy PA et al., reported 73.4% anaerobic isolations from tissue samples and 23.4% from aspirated pus samples [15]. The isolation rate from brain abscess was reported as 8.6% [16] and 41.1% [6] in Indian studies. This study falls between the two aforementioned studies, with a brain abscess isolation rate of three (17.65%). Samples collected through swabs and drains were not included for anaerobic isolation and were rejected for further processing. However, Shenoy PA et al., included these samples in two of their studies, as wound swabs were the only feasible samples [10,15]. In those instances, the authors collected the samples bedside and then directly inoculated them into RCM broth.

Anaerobic infections typically present as either monomicrobial or polymicrobial infections, often in the form of abscesses. These infections can originate either endogenously, through autoinfections caused by the microbiota of the affected site, or exogenously. In the present study, out of the 17 anaerobes isolated from different clinical sites, 12 (70.59%) exhibited monomicrobial growth, while 5 (29.41%) grew in mixed culture. This finding, with a predominance of monomicrobial infections, was consistent with a study conducted in India by Beena A et al., where monomicrobial and polymicrobial growth accounted for 70 (84.33%) and 13 (15.66%) cases, respectively [12].

A significant number of anaerobes have been implicated as the causative agents of deep-seated abscesses, skin and soft tissue infections, and life-threatening emergencies associated with toxin-producing anaerobes. Virulence factors that may facilitate anaerobes in establishing infections include adhesion factors like fimbriae, antiphagocytic capsular polysaccharides, and invasion-aiding enzymes such as collagenase and fibrinolysin, as well as toxins like tetanus and botulinum toxin [1,4,5].

The main anaerobic organisms isolated in these infections were *Bacteroides fragilis*, *Prevotella*, *Clostridium* species, *Peptostreptococcus*, and *Actinomyces*. In this study, the isolation rate of gram-positive anaerobes was higher at 9 (53%) compared to gram-negative anaerobes at 8 (47%). However, the most commonly isolated anaerobe was gram-negative *Bacteroides fragilis*, accounting for 6 (35.29%) cases. The high prevalence of *Bacteroides fragilis* could be attributed to its invasive virulence factors. Two international studies also reported similar findings, where *Bacteroides fragilis* exhibited prevalence rates of 33.8% [17]

and 31% [18], respectively. *Bacteroides fragilis* is a normal resident of the gastrointestinal tract and is commonly isolated from intra-abdominal infections, as well as infections originating from the gut flora. Other infections associated with *Bacteroides fragilis* may include skin and soft tissue infections, and wound infections [19].

Although hundreds of species of anaerobes have been recognised, only a relatively small number are actually involved in causing infections. Identifying anaerobes is a challenging task that requires the use and application of a battery of media and tests, including molecular methods and mass spectrometry (MALDI). However, it is not practical for most laboratories to employ all these systems, so they often rely on basic techniques such as staining, colony morphology, sensitivity patterns to vancomycin, colistin, and kanamycin, as well as results from automated identification systems.

In this study, a metronidazole disk (5 µg) was used for susceptibility testing to differentiate strictly anaerobic organisms from aerotolerant, microaerophilic, or capnophilic organisms [1]. This approach aided in the preliminary identification of anaerobes, where 14 (82.35%) of the isolated anaerobes were sensitive to the disk, exhibiting a zone of inhibition around it.

Emphasising the isolation and identification of anaerobes is essential due to the significant morbidity and mortality associated with some of these infections. Treating anaerobic infections requires an individualised approach, considering the site, organ, and severity of the infection. Medical treatments usually need to be complemented with surgical debridement or aspiration of abscesses, and in some cases, major procedures such as limb amputation. Initiating empiric therapy is a common practice since anaerobes may take several days to grow, but treatment cannot wait. As antimicrobial sensitivity patterns are no longer predictable, microbiology laboratories would need to upgrade their systems to meet the challenge of antimicrobial sensitivity testing for anaerobes.

Limitation(s)

The incidence of anaerobic isolations may be significantly higher than reported in this study because the study period overlapped with COVID-19 pandemic-associated restrictions, and most invasive procedures were better avoided. It was also observed that the aetiological diagnosis of anaerobic infections is rarely sought by treating clinicians due to the unacceptably long turnaround time for anaerobic cultures.

CONCLUSION(S)

Anaerobes are emerging as important causative agents of a variety of diverse and heterogeneous pyogenic infections. Identifying anaerobes with conventional systems is a difficult proposition, and laboratories need to upgrade their capacity to include molecular and spectrometric modalities for aiding in the accurate and faster identification of anaerobes. Although anaerobic infections tend to be polymicrobial, the majority of infections in the present study were monomicrobial. Since antimicrobial resistance among anaerobic bacteria is increasingly being reported, knowledge about their identification and site distribution would help guide clinicians in selecting appropriate empirical therapy for better management of pyogenic infections. This also underscores the urgent need to sensitise clinicians to the increasing role of anaerobes in pyogenic infections to ensure due diligence during the collection of clinical samples.

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