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PHENOTYPIC CHARACTERIZATION OF ANAEROBIC MICROBIAL COMMUNITY ISOLATED FROM HUMAN INFECTIONS IN THE SUBURBAN POPULATION OF COASTAL KARNATAKA – A THREE YEAR STUDY

Medical Microbiology

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ABSTRACT

Anaerobes exist as normal flora in various body sites and also cause a variety of invasive infections. Though the pathogenic potential of anaerobes is proved beyond doubt, they have not received much attention among the Microbiologists in India, as conventional anaerobic culture techniques are time consuming, relatively expensive and needs a lot of expertise. Of the 1334 specimen included in this study of 3 years duration, 1086 culture positive specimen were obtained which yielded 772 anaerobes, predominated by *Bacteroides fragilis* group. Maldi Tof, a rapid automated technique is very helpful in speciating all bacteria including slow growing and unusual anaerobes. The findings of this study and the similar ones from India emphasize the magnitude of the anaerobes and the necessity to include anaerobic culture techniques routinely to identify these potential pathogens.

KEYWORDS

Anaerobes, Phenotypic Characterization, Maldi Tof

INTRODUCTION:

Anaerobic bacteria are the most diverse microbial community of the human body inhabiting mainly in female genital tract, oral cavity and gastrointestinal tract as normal flora.[1] As resident microbiota, anaerobes lead a harmonious life with aerobes, however during impairment of host defense mechanisms they express their true colour and exhibit the pathogenic potential. Anaerobic infections are usually polymicrobial in etiology and endogenous in origin and they act synergistically with co-existing bacteria to cause damage.[2,3] Due to the low oxygen concentration in many of the areas especially periodontal pockets, urogenital tract and gastrointestinal tract, anaerobes flourish and also outnumber the aerobes by 10-1000 times in these body sites.[4,5] A well-documented role of these notorious pathogens in various clinical infections such as intra-abdominal infections,[6] brain abscess,[7] female genital tract infections,[8] orodental infections,[9,10] bone and soft tissue infections [11] bacteremia[12] have been reported by various investigators. Many virulence factors contribute to the pathogenesis of anaerobes, of which biofilm formation plays an important role in initiation of colonization.[13,14]

These organisms are overlooked or under estimated in clinical set up as well as in diagnostic laboratories due to many reasons. Conventional Anaerobic culture techniques are laborious, time consuming and relatively expensive. Many technical factors like improper collection of specimen, delay in transport, exposure to oxygen are likely to reduce the frequency of isolation rate of anaerobes. The lack of expertise in the field of anaerobic bacteriology is another factor which hinder the set-up of anaerobic laboratories. The common belief of the clinicians that antibiotic coverage with metronidazole will take care of all anaerobes, is also adding agony to this problem. Taxonomy of Anaerobes is changing frequently with the addition of new genera and species from various infections, using 16SrRNAgene sequencing.[15] Introduction of a rapid automated method, Matrix Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry Analysis (Maldi Tof) has simplified the time consuming classical identification techniques. The development of more recent non culture techniques in the field of Molecular biology made the microbiologists to deviate from the difficult field of anaerobes.

Emergence of resistance to Metronidazole in many anaerobes are reported from various countries including India.[16-18] A new hurdle is also arising regarding the role of Anaerobes in COVID as well as new outbreaks which will be overlooked by the clinicians. *Clostridioides difficile* causing antibiotic-associated diarrhoea and life-threatening colitis due to broad-spectrum antibiotics as well as new emerging anaerobes are arising as diagnostic challenges.

The present study investigates the phenotypic characterization of Anaerobic bacteria isolated from the human Infections in the suburban population of coastal Karnataka for a duration of 3 years.

MATERIAL & METHODS:

This study was conducted in a tertiary care hospital in Mangalore for a duration of 3 years starting from January 2020 to December 2022 supported by the K- FIST L2 grant (GRD 650) sanctioned by Vision Group of Science & Technology, Karnataka Government for the development of Infrastructure in Anaerobic laboratory. Institutional Ethical Clearance Certificate was obtained before starting the study. The samples were processed according to the standard protocol. The various specimen suggestive of anaerobic infections were inoculated directly into Reduced Transport Fluid (RTF) Medium[19] and Robertson's Cooked Meat Medium (RCM), an anaerobic medium to reduce exposure to oxygen.

RTF were vortex mixed and subcultured onto the anaerobic media such as Laked Blood Agar (LBA), Neomycin BA (NBA) as well as Cefoxitin, Cycloserine Fructose Agar (CCFA) for *C. difficile* and Bacteroides Bile Esculin agar for *B. fragilis* group depending on the specimen. To differentiate the pathogenic and commensal status, a semi quantitative culture technique was employed. The plates were divided into 4 quadrants and the specimen were streaked according to Wadsworth Manual.[20] Growth in 1st &2nd quadrants only were considered as commensals while growth in 3rd & 4th were indicating pathogenic status. After an incubation of 24 hours, RCM media were inoculated onto the above mentioned media. A metronidazole disc (5µg) was placed in the plates for the presumptive identification of anaerobes in all media and incubated in **DonWhitley-DG 250 Anaerobic Workstation or BD Gaspak Jar.** For the cultivation of capnoic organisms, the specimen were inoculated on Blood agar and Chocolate agar and incubated in **CO_2 incubator** at 37^{0}C for 48 hours. To find out the association of aerobes and fungi, the samples were simultaneously inoculated onto Blood agar, MacConkey agar and Sabourauds Dextrose agar and processed aerobically.

After an incubation of 48-72 hours, the plates that showed a definite zone of inhibition to metronidazole were presumptively identified as anaerobes and the isolates were further characterized based on aerotolerance (No growth in the aerobic incubation or under CO_2), Gram stain, colony morphology and biochemical reactions according to the standard procedures.[20,21] As soon as the plates were taken out of anaerobic jar or chamber, the isolated colonies suggestive of Anaerobes were subjected to **Maldi Tof** (Matrix Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry Analysis (Bruker Daltonics India.Pvt Ltd.) by **Extended Direct Transfer Technique**. Briefly, an isolated colony was smeared as a thin film

directly onto the MALDI target plate and overlaid the material with 1.0µL of 70% formic acid. After drying at room temperature, the smear was overlaid with 1.0µL of matrix solution, α-Cyano-4-hydroxycinnamic acid (HCCA) and dried at room temperature. The sample plate was placed inside the MALDI-TOF apparatus after entering the patient information. Based on the spectra acquired by summing the laser shots, the data was processed automatically by the instrument software and the spectra were compared with reference libraries for bacterial identification to obtain the best matching.

For biochemical characterization, Viande-Levure Broth was used as the basal medium and the tests include carbohydrate fermentation, Indole, Esculin hydrolysis, bile tolerance and H_2S production[21]

RESULTS:

Of the 1334 specimen included in the study, predominant samples were from pus specimen from deep abscesses, 492(36.88 %), followed by high vaginal swabs 279 (20.91%) and Blood and Body fluids 260 (19.49%). **Table 1** describes the clinical entities included in the study. Among these 1334 specimens, no growth was obtained in 248 while 1086 showed the growth of Aerobes or Anaerobes. 301 specimen showed mono microbial growth (235 aerobes alone and 66 anaerobes alone). Polymicrobial growth (yielding 2 or more organisms of aerobe or anaerobe) was seen in 785 with 543 showing mixed growth of aerobe and anaerobe, 217 with 2 aerobes each and 25 with 3 organisms.

Table 1 - Details of Specimen Included In the Study

Sl. No.	Specimen	No	%
1.	Pus from deep abscess	492	36.88
2	High vaginal Swabs	279	20.91
3.	Blood & Body fluids	260	19.49
4.	Faeces	171	12.82
5	Orodental Infections	85	6.37
6.	Respiratory Secretions	47	3.52
	Total	1334	99.99

Table -2 denotes the Anaerobic Bacteria obtained in the study. 772 anaerobes were isolated during this duration. *Bacteroides fragilis group*,191(24.74%) was the most common anaerobic isolate followed by *Peptostreptococcus* sps. 117(15.16%). From 171 faecal specimen 61 isolates of *Bifidobacterium* and 13 *Clostridioides difficile* were isolated. Whenever species identification was not possible, isolates were subjected to MALDI TOF Analysis.

Table 2- Details of Anaerobic Bacteria Isolated In the Study (772)

Sl. No	Anaerobes	No.	%
1.	*B. fragilis* group – (*B. fragilis, B .thetaiotamicron, B. vulgatus, B. ovatus, B. uniformis*)	191	24.74
2	*Peptostreptococcus* – (*P. anaerobius, P. stomatitis*)	117	15.16
3	*Peptoniphilus* (*P. harei, P. anaerobius, P. gorbachi*)	12	1.55
4	*Anaerococcus* (*A. hydrogenalis, A. vaginalis, A. octavius, A. murdochii*)	8	1.04
5	*Porphyromonas* (*P. gingivalis, P. asacharolytica*)	43	5.57
6	*Prevotella* spp (*P. buccae, P. melaninogenica, P. denticola, P. bivia, P. oralis*)	84	10.88
7	*Fusobacterium* spp (*F .varium, F. nucleatum, F. canifelinum*)	44	5.70
8	*Finegoldia magna*	48	6.22
9	*Lactobacilli* spp (*L.acidophilus, L.fermentum, L.rhamnosus, L.vaginalis, L.iners, L.gasseri, L.paracasei*)	32	4.15
10	*Cutibacterium* (*Propionibacterium) C.acnes, C.lymphophilum, C.avidum, C.granulosum, C.propionicum*)	40	5.18
11	*Actinomyces* spp *(A.odontolyticus, A.naeslundii, A.urogenitalis*)	14	1.81
12	*Bifidobacterium* spp (*B.bifidum, B.longum, B.dentium*)	61	7.90
13	*Clostridium* spp (*C.butyricum, C.perfringens, C.tertium, C.sporogenes, C.sordelli*)	14	1.81
14	*Clostridioides difficile*	13	1.68
15	*Mobiluncus* spp	9	1.17
16	*Veillonella* (*V. parvula, V. atypica, V. dispar*)	42	5.44
	Total	**772**	**100**

Association of Aerobic, Microaerophilic bacteria and Fungi in these specimen was shown in Table **3.** Among the 911 isolates which included 666 aerobes, 162 microaerophilic bacteria and 83 fungi were obtained. α-hemolytic *Streptococci* was the predominant isolate (110), followed by *Staphylococcus aureus* (102) and *E. coli* (93). Of 54 fungi, 41 isolates of *Candida* and 11 *Rhizopus* species were isolated. Unusual isolates of aerobes include strains of *Corynebacterium striatum* from Pus samples, *Burkholderia pseudomallei* and *Brucella* species from blood *Granulicatella adiacens* from orodental infections.

Table 3- Association of Other Bacteria& Fungi In the Study

Sl. No	Aerobes	No	%
1.	*Staphylococcus aureus*	102	11.20
2.	Coagulase Negative *Staphylococci (S. haemolyticus, S.epidermidis)*	46	5.05
3.	Group A *Streptococcus*	36	3.95
4.	Group B *Streptococcus*	53	5.82
5.	*Enterococci*	47	5.16
6.	*Corynebacterium* spp (*C.striatum, C.jeikeium, C.amylocalatum, C.urealyticum*)	43	4.72
7.	*E.coli*	93	10.21
8.	*Klebsiella* spp *(K.pneumoniae, K.aerogenes, K.oxytoca)*	63	6.92
9.	*Citrobacter* spp (*C.freundii, C.koseri)*	17	1.87
10.	*Proteus* spp(*P.mirabilis, P.vulgaris*)	18	1.98
11.	Non Typhoidal *Salmonellae*	53	5.82
12.	*Pseudomonas aeruginosa*	40	4.39
13.	*Burkholderia pseudomallei*	4	0.44
14.	*Acinetobacter* spp (*A.baumannii, A.pitti, A.nosocomialis*	51	5.59
	Microaerophilic bacteria		
15.	α- Haemolytic *Streptococci* (*S. mitis, S. salivarius, S.anginosus, S.constellatus*)	110	12.07
16.	*Brucella* spp	3	0.33
17.	*Gardnerella vaginalis*	25	2.74
18.	*Aggregatibacter actinomyctemcomitans*	20	2.19
19.	*Granulicatella adiacens*	4	0.44
	Fungi		
20.	*Candida* spp (*C.albicans, C.tropicalis, C.auris, C.glabrata, C.parapsilosis)*	66	7.24
21.	*Rhizopus*	11	1.21
22.	*Aspergillus* spp	6	0.66\
	Total	911	100

During this duration a total of 47 respiratory secretions and 10 faeces from COVID patients were tested as a Pilot study. Gram **Positive Cocci** *(Peptostreptococci* sps & *Finegoldia)* predominated the growth followed by Gram **Negative Bacilli** (*Prevotella* spp, *Porphyromonas spp*, *Fusobacterium nucleatum*). Three strains of *C. difficile* were also isolated from COVID patients with diarrhoea.

DISCUSSION:

Anaerobes are known to cause a variety of invasive infections with high morbidity and mortality in the head and neck region such as chronic sinusitis, chronic otitis media and Ludwig's angina, orodental infections including periodontal abscesses, infections of central nervous system like brain abscesses and subdural empyema, pleuropulmonary diseases such as aspiration pneumonia, necrotizing pneumonia, lung abscess and empyema, various intra-abdominal infections like peritonitis, intra-abdominal and liver abscesses, female genital tract infections such as salpingitis, pelvic inflammatory diseases, tubo-ovarian abscess, vulvovaginal abscess, septic abortion and endometritis[5] Anaerobes are also associated with bacteremia and infections of the skin, soft tissues and bones.[7]

Though the pathogenic potential of anaerobes is proved beyond doubt, they have not received much attention among the Microbiologists in India due to various reasons and they still remain unexplored. These 'sour grapes' were always kept aside underneath the 'protective umbrella' of metronidazole. Time consuming and relatively expensive conventional anaerobic culture techniques coupled with lack of expertise, not many microbiologists in India were keen on working with anaerobes. It is a herculean task to provide exacting growth requirements to create anaerobic environment and to maintain it, which demands a lot of dedication, patience and expertise. Despite the scientific recognition and increased enthusiasm in the field of

anaerobic bacteriology had been extended to India during 1970s under the able guidance of few pioneers, it lost its charm in the next few years. In the recent past many Indian workers are showing increased interest in the field of anaerobes, resulting in quite a few research articles related to various aspects such as prevalence of Anaerobes,[22-24] Antimicrobial Susceptibility pattern,[25,26] *Clostridioides difficile* etc.[27,28] Still, the present trend is adopting culture-independent methods based on molecular techniques.

In this study of 3 years duration, 1334 specimen were processed for the isolation of anaerobes with predominant samples belong to pus specimen from deep abscesses. 1086 showed the growth of Aerobes or Anaerobes with 301 specimen showed mono microbial growth and 785 Polymicrobial growth. It is a well-known fact that anaerobic infections are of polymicrobial etiology constituting anaerobes with aerobes or microaerophilic bacteria. Foul odour, black discoloration, presence of gas or sulphur granules in specimen, proximity to mucosal surface are some of the indications of anaerobes in the specimen. In addition, presence of organisms in the direct smear while 'No growth' obtained in the aerobic culture, may be having an anaerobe hiding.[20,21] Though the emergence of Metronidazole is reported from various countries including India, zone of inhibition to metronidazole disc (5µg) in the primary isolation culture plates gives a good correlation to the presence of anaerobes. However, it is also observed that metronidazole resistance detected by *nim* genes in *B. fragilis* may not be expressed fully by phenotypical tests.[18]

Repeated isolation, clinical suspicion, correlation of the direct smear with organisms having unique morphology, appropriate specimen and collection techniques to avoid normal flora are some of the factors to assess the clinical significance of anaerobes as they occur as resident flora in various sites.[20] Among the 1334 specimen included in the study, 772 anaerobes were isolated predominated by *Bacteroides fragilis* group (191isolates) followed by Peptostreptococcus spp. No doubt that these are the predominant anaerobes encountered in majority of the infections and reported by various investigators. The findings of this study suggest that numerous anaerobes can be isolated if anaerobic cultures are employed routinely, if not, we are missing these prominent culprits in many cases that are reported as 'No Growth'.

Species identification of anaerobes by conventional techniques is very confusing and time consuming as well it is not possible for many of the strains even if we take utmost care. In this context, Maldi Tof, an automated technique has revolutionized and sealed the gap by identifying proteomic signature of all the anaerobes, including slow growing and unusual ones.[29] As soon as the colonies appear on culture plates, it requires only less than half an hour to obtain the identification of the species, according to the recent taxonomical revision, whereas conventional biochemical characterization may take at least 4-5 days. In this study many new species and genera of anaerobes could be identified. However, the initial high cost of the instrument remains as a major problem. If facility is available Maldi Tof will reduce the 'Turn Around Time' and in turn will improve the patient care.

Anaerobic infections in Covid patients were reported by certain investigators. Poor oral hygiene, cough, increased inhalation under normal or abnormal conditions and mechanical ventilation were some of the risk factors which allow the anaerobic components of oral microbiome to enter the lower respiratory tract and to cause severe infections in those patients. [30]Hypoxia due to many factors might favor the formation of a reduced oxygen containing environment favoring the growth of anaerobes in the lungs**.** As reported by Spigaglia , during the Covid pandemic over usage of broad spectrum antibiotics and disinfectants might have led to a selection of resistant *Clostridioides difficile* strains both in hospitals and in the community.[31] In addition, the patients who survived COVID-19 might be a new group of susceptible patients, at a higher risk of hospital acquired C difficile colitis & diarrhea.[31] Hence in the emerging and reemerging cycles of COVID, anaerobes might pose a major public health threat.

In conclusion, the pathogenic potential of anaerobes is documented in many studies. As conventional techniques for characterization of anaerobes are time consuming and cumbersome, Maldi Tof, a rapid and simple technique will be very advantageous to overcome this difficulty. At present, many investigators are showing interest in anaerobic culture techniques, which is a promising trend. Findings of this article and other literature pertaining to anaerobes, emphasize the magnitude of the problem with anaerobes and the necessity to include anaerobic culture techniques routinely to identify these potential pathogens.

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