Original Article

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Microbiology Section

Anaerobic Bacteria in Clinical Specimens – Frequent, But a Neglected Lot: A Five Year Experience at a Tertiary Care Hospital

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ABSTRACT

Introduction: Anaerobic bacteria which constitute a significant proportion of the normal microbiota also cause variety of infections involving various anatomic sites. Considering the tedious culture techniques with longer turnaround time, anaerobic cultures are usually neglected by clinicians and microbiologists.

Aim: To study the frequency of isolation of different anaerobic bacteria from various clinical specimens.

Materials and Methods: A retrospective study to analyse the frequency of isolation of different anaerobic bacteria, was conducted over a period of five years from 2011 to 2015 including various clinical specimens submitted to anaerobic division of Microbiology laboratory. Anaerobic bacteria were isolated and identified following standard bacteriological techniques.

Results: Pathogenic anaerobes (n=336) were isolated from 278 (12.48%) of overall 2227 specimens processed with an average yield of 1.2 isolates. Anaerobes were isolated as polymicrobial flora with or without aerobic bacterial pathogens in 159 (57.2%) patients. Anaerobic Gram-negative bacilli (140, 41.7%) were the predominant isolates. *B. fragilis* group (67, 19.9%) were the most commonly isolated anaerobic pathogens. Anaerobes were predominantly isolated from deep seated abscess (23.9%).

Conclusion: Pathogenic anaerobes were isolated from various infection sites. Unless culture and susceptibility tests are performed as a routine, true magnitude of antimicrobial resistance among anaerobic pathogens will not be known. Knowledge of the distribution of these organisms may assist in the selection of appropriate empirical therapy for anaerobic infections.

Keywords: Abscess, Anaerobe, Gram-negative bacilli, Gram-positive cocci, Polymicrobial infections

INTRODUCTION

Anaerobic bacteria constitute a significant proportion of the normal microbiota colonizing skin and various mucosal surfaces of human body [1]. Anaerobes are more commonly found in polymicrobial aerobic and anaerobic infections of endogenous origin. Breach in mucosal barriers due to surgery, trauma, tumours, or ischemia lead to infections by these microbes following entry of endogenous flora into normally sterile sites [2,3]. Infections by *Clostridium* spp. are mainly of exogenous origin [2]. The most commonly encountered anaerobes in clinical specimens include *Bacteroides fragilis* group, pigmented *Prevotella* spp. and *Porphyromonas* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Clostridium* spp. and *Actinomyces* spp. [3]. The pathogenic anaerobes may cause variety of infections ranging from mild to severe life threatening ones, involving various anatomic sites [3,4]. Varying rates of anaerobic bacterial isolation have been reported across the globe from different clinical infection sites [5-8].

Whenever there is high suspicion of anaerobic aetiology, the management of such infections is often dependent only on empirical antibiotic therapy. The reason could be attributed to tedious anaerobic culture techniques, cost and more importantly longer turnaround time for intimation of anaerobic culture reports to treating clinician. However, resistance to metronidazole, the empiric drug of choice for anaerobic coverage is on the rise [9].

Anaerobes are the most overlooked microorganisms in many of the clinical specimens. Failure to identify them and provide antimicrobial coverage may result in therapeutic failure. Therefore, it is important to know the microbial pathogen responsible for the infectious process. A study was undertaken to determine the frequency of isolation of anaerobes from various clinical specimens in the Microbiology laboratory attached to a tertiary care teaching hospital in coastal Karnataka, India.

MATERIALS AND METHODS

A retrospective study was conducted over a period of five years from January 2011 to December 2015 in the department of Microbiology of Kasturba Medical College, Manipal, a tertiary care teaching hospital in Southern India. For microbiological analysis, specimens including tissue, pus aspirate, body fluids, corneal scrapings, wound swabs and stool for *C. difficile* were aseptically inoculated into a wide mouth sterile container and/or Robertson's Cooked Meat (RCM) medium soon after collection and transported immediately to the laboratory. In cases where wound swabs were the only mode of sampling, samples were collected and inoculated at bedside into RCM broth. The specimens were processed for Gram stain and the anaerobic cultures were done on 5% sheep blood agar, neomycin blood agar and phenyl ethyl alcohol agar with metronidazole (5 µg, Oxoid) disc. The specimens were inoculated into RCM if bedside inoculation was not performed. The inoculated culture plates were incubated in anaerobic Gaspak jars (BD Diagnostics, Sparks, MD, USA) (from January 2011 to July 2013) or anaerobic workstation (Whitley A35 Anaerobic workstation, Don Whitley Scientific, Shipley, UK) (from August 2013 to December 2015). The Gaspak jars were opened after 48-72 hours for inspection of plates, whereas plates were inspected daily for anaerobic growth when anaerobic chamber was used for incubation. The inoculated RCM broth was incubated till seven days and subcultures were done on to 5% sheep blood agar if any additional bacterial morphotypes were noted on Gram stain from the broth. The specimens were also cultured aerobically on 5% sheep blood agar and MacConkey agar and isolates were identified following standard methods [10].

Preliminary identification of the anaerobic isolates was done by colony morphology and Gram stain, aerotolerance test on chocolate agar, fluorescence under long-wave (365 nm) ultraviolet light (UVP, LLC), antibiotic identification discs (vancomycin 5 µg, kanamycin 1000 µg and colistin 10 µg), biochemical tests and susceptibility to Sodium Polyanethol Sulfonate (SPS) [11]. Automated microbial identification systems, VITEK 2 (ANC card, bioMerieux) (from January 2011 to May 2015) or Matrix Assisted Laser Desorption/ Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (VITEK MS, bioMerieux) (from June 2015 to December 2015) were used for species level identification.

RESULTS

A total of 2227 samples were received in anaerobic division of Microbiology laboratory over a period of five years involving diverse infections in our tertiary care hospital. Pathogenic anaerobes (n=336) were isolated from 278 (12.48%) patients with an average yield of 1.2 isolates per specimen showing anaerobic growth [Table/Fig-1].

Anaerobic Gram-negative bacilli (140, 41.7%) were the predominant isolates. *Bacteroides fragilis* group (67, 19.9%) were the most commonly isolated anaerobic pathogens. Amongst Gram-positive Anaerobic Cocci (GPAC), *Finegoldia magna* (43, 12.8%) was the most frequently isolated pathogen followed by *Peptostreptococcus anaerobius* (20, 6%) [Table/Fig-2]. The most commonly isolated anaerobe in monomicrobial flora were *Clostridium* spp. (39, 32.8%) [Table/Fig-3]. In 159 (57.2%) patients, anaerobes were isolated as polymicrobial flora with or without aerobic bacterial pathogens [Table/Fig-1].

Anaerobes were predominantly isolated from deep seated abscess (23.9%), followed by diabetic foot infection (20%), necrotizing fasciitis (15.6%), chronic osteomyelitis (7.8%), infected non healing ulcer (7.3%), antibiotic associated diarrhea (6.8%), corneal ulcer (4.4%), fournier's gangrene (3.4%), gas gangrene (2.9%), empyema (2.4%), cellulitis (2.4%), pyometra (1.9%) and endophthalmitis (0.9%).

Characteristic	No. of patients	Percentage (%)
Age (yrs)		
0-20	16	5.75
21 to 40	91	32.7
41 to 60	116	41.7
61 to 80	52	18.7
> 80	3	1.0
Gender		
Male	199	71.6
Female	79	28.4
Specimen		
Tissue	130	46.8
Pus aspirate	88	31.7
Body fluids	22	7.9
Wound swabs	15	5.4
Stool for *C. difficile* culture	14	5.0
Corneal scrapings	9	3.2
Nature of growth with anaerobic infections (n=278)		
• **Pure anaerobic growth (n=147, 52.9%)**		
Monomicrobial anaerobic growth	119	42.8
Polymicrobial anaerobic growth	28	10.1
• **Mixed anaerobic and aerobic growth (n=131, 47.1%)**		
1 Aerobic + 1 Anaerobic growth	82	29.5
1 Aerobic + 2 Anaerobic growth	20	7.2
2 Aerobic + 1 Anaerobic growth	19	6.8
2 Aerobic + 2 Anaerobic growth	10	3.6

[Table/Fig-1]: Demographic and microbiological profile of study subjects (n=278).

Isolates (n=336)	Number	Percentage
Anaerobic Gram-Positive Cocci (n=85, 25.3%)		
Finegoldia magna	43	12.8
Peptostreptococcus anaerobius	20	6.0
Peptoniphilus asaccharolyticus	18	5.4
Parvimonas micra	3	0.9
Anaerococcus prevotii	1	0.3
Anaerobic Gram-Negative Cocci (n=24, 7.1%)		
Veillonella parvula	24	7.1
Anaerobic Gram-Negative Bacilli (n=140, 41.7%)		
Bacteroides fragilis subsp. fragilis	56	16.7
Bacteroides fragilis subsp. thetaiotaomicron	7	2.1
Bacteroides fragilis subsp. ovatus	3	0.9
Bacteroides fragilis subsp. vulgatus	1	0.3
Parabacteroides distasonis	1	0.3
Prevotella spp.	25	7.4
Prevotella bivia	8	2.4
Prevotella buccae	5	1.5
Prevotella disiens	5	1.5
Prevotella melaninogenica	2	0.6
Fusobacterium nucleatum	13	3.9
Fusobacterium necrophorum	5	1.5
Fusobacterium varium	3	0.9
Fusobacterium mortiferum	2	0.6
Porphyromonas asaccharolytica	4	1.2
Anaerobic Gram-Positive Bacilli (n=87, 25.9%)		
Clostridium spp.	17	5.1
Clostridium difficile	14	4.2
Clostridium bifermentans	9	2.7
Clostridium sporogenes	9	2.7
Clostridium perfringens	6	1.8
Clostridium clostridioforme	5	1.5
Clostridium ramosum	3	0.9
Clostridium baratii	3	0.9
Clostridium septicum	2	0.6
Clostridium cadaveris	1	0.3
Clostridium subterminale	1	0.3
Clostridium sordelli	1	0.3
Clostridium innocuum	1	0.3
Clostridium histolyticum	1	0.3
Propionibacterium acnes	11	3.3
Bifidobacterium spp.	1	0.3
Eggerthella lenta	1	0.3
Lactobacillus gasseri	1	0.3
TOTAL	**336**	**100**

[Table/Fig-2]: Distribution of anaerobic bacterial pathogens isolated during the study.

Anaerobic Gram-negative bacilli (n=7) were the predominant isolates found in polymicrobial anaerobic infections (n=28). Among infections with mixed aerobic and anaerobic bacterial flora (131, 47.1%), anaerobes were mainly isolated in association with *E. coli* in 43 (32.8%) patients followed by *K. pneumoniae* in 31 (23.7%) patients. *B. fragilis* group (47, 35.9%) was the most common anaerobic bacteria found in association with aerobic bacterial pathogens.

DISCUSSION

Anaerobic bacteria constitute a large majority of commensal flora

Anaerobes	Number (%)
Clostridium spp. including *C. difficile*	39 (32.8%)
Bacteroides fragilis group.	21 (17.6%)
Finegoldia magna	16 (13.4%)
Prevotella spp.	13 (10.9%)
Peptostreptococcus anaerobius	9 (7.6%)
Propionibacterium acnes	7 (5.9%)
Veillonella parvula	7 (5.9%)
Fusobacterium spp.	4 (3.4%)
Porphyromonas asaccharolytica	2 (1.7%)
Peptoniphilus asaccharolyticus	1 (0.8%)

[Table/Fig-3]: Anaerobic bacteria isolated as monomicrobial flora: (n=119).

Study Investigator	Year	Clinical profile	Total No. of specimens	Isolation rate
Brook I et al., [14]	1998	Retroperitoneal abscesses	161	78.9%
De A et al., [6]	2001	Diverse clinical infections	2591	8%
De A et al., [15]	2002	Pleuropulmonary infections	100	72%
De A et al., [16]	2003	Gas gangrene	580	26.8%
Saini S et al., [17]	2004	Surgical infections	117	50.4%
Tanaka K et al., [18]	2005	Bartholin's gland abscess	224	53.1%
Boyanova L et al., [19]	2006	Deep-space head and neck infections	118	74.6%
Gadepalli R et al., [20]	2006	Diabetic foot ulcer	80	35%
Huang TT et al., [21]	2006	Deep neck infections	128	59.3%
Singhal R et al., [22]	2006	Anaerobic bacteremia	1743	1.14%
Citron DM et al., [5]	2007	Diabetic foot infections	454	45.2%
Ng LS et al., [8]	2008	Diabetic foot infections	38	78.9%
López VN et al., [23]	2009	Iliopsoas abscess	124	15.1%
Mathew A et al., [24]	2010	Necrotising fasciitis	50	18.5%
Al-Benwan K et al., [25]	2011	Breast abscess	114	28%
Ingle M et al., [26]	2011	*Clostridium difficile* infection	99	17%
Vishwanath S et al., [27]	2012	Chronic suppurative otitis media	94	19.14%
Urban E et al., [28]	2012	Anaerobic bacteremia	43992	0.69%
Vishwanath S et al., [29]	2013	*Clostridium difficile* infection	25	16%
Kamble S et al., [30]	2014	Cutaneous and subcutaneous wound infections	50	18%
Garg R et al., [7]	2014	Diverse clinical infections	100	19%
Antony B et al., [31]	2016	Surgical infections	393	39.9%
Sudhaharan S et al., [32]	2016	Brain abscess	430	41.1%
Shenoy PA et al., [33]	2016	Surgical infections	261	24.5%

[Table/Fig-4]: Isolation rates of anaerobic bacterial pathogens from different infection sites.

which inhabit various body sites, including mucosal surfaces of oral cavity, pharynx, gastrointestinal tract, genitourinary tract orifices and skin. This microbiome serves as source for majority of infections involving anaerobes [12]. Anaerobes as pathogens are isolated from various anatomic sites with variable recovery rates. Anaerobic bacterial pathogens are isolated in high frequency (50-100%) from gas gangrene, diabetic foot infections, infections after colorectal surgery and appendectomy, perianal abscess, non-clostridial crepitant cellulitis, lung abscess, aspiration pneumonia, brain abscess, intraperitoneal/pelvic abscess, soft tissue/subcutaneous abscess, dental/oral infections, chronic sinusitis and mammary abscess [13]. Our data shows isolation of various anaerobic bacteria from diverse infections. We isolated anaerobes mainly from abscesses (23.9%) and diabetic foot infection (20%). Some of the reported isolation rates of anaerobic bacteria from different infection sites are summarized in [Table/Fig-4] [5-8,14-33].

Successful isolation of anaerobes depends on specimen collection and transportation procedures, anaerobic incubation system and the quality and selection of the primary isolation media. For optimal recovery, it is necessary that specimens are transported within 30 minutes after collection and if anaerobic transport media are used, within 2-3 hours to the laboratory [34]. The commonly used culture media for isolating anaerobes from clinical specimens include, 5% sheep blood agar with hemin (5 µg/mL) and vitamin K1 (1 µg/mL), kanamycin-vancomycin laked blood agar, phenyl ethyl alcohol sheep blood agar, Columbia nalidixic acid agar, *Bacteroides* bile esculin agar, cycloserine-cefoxitin fructose agar, egg yolk agar, supplemented thioglycollate broth with hemin, vitamin K1 and sodium bicarbonate and RCM broth [11].

In the recent years, major taxonomic changes of anaerobic bacteria have occurred, more so among Gram-negative bacilli and Gram-positive cocci. It is essential for both microbiologists and the clinician to be updated with the changes in bacterial names for better description and recognition of the bacterium-disease associations [35]. Anaerobic Gram-negative bacilli were the predominant pathogens in our study as also reported in various studies [6,15,36-39]. However, GPAC are isolated as most frequent pathogens in other reports [5,7,40,41].

B. fragilis which forms about only 0.5% of normal commensal flora in the colon is the most commonly isolated anaerobic bacterial pathogen as reported in literature by virtue of its virulence factors. These factors include, tissue adherence by fimbriae and agglutinins; polysaccharide capsule, lipopolysaccharide and a variety of enzymes which help in evading oxygen toxicity and phagocytosis; and histolytic enzymes which cause tissue destruction [42].

B. fragilis group (19.9%) were the predominant isolates, mirroring the finding of other studies [6,37,39]. The capsule production by *B. fragilis* helps in abscess formation [42]. Majority of our *B. fragilis* strains (n=25) were isolated from deep seated abscess.

Infections caused by *Veillonella parvula* are seldom reported. They are commonly found in head and neck infections, skin and soft tissue infections, infections in the respiratory tract, peritoneal fluid, blood and abdominal infections [11]. In our study, majority of *V. parvula* were isolated from necrotising fasciitis (n=7).

The Gram-Positive Anaerobic Bacilli (GPAB) which are seen in the laboratory include the spore forming *Clostridium* spp. and the nonsporing, *Actinomyces, Bifidobacterium, Eggerthella, Eubacterium, Lactobacillus and Propionibacterium* spp. Identification of the non-sporing GPAB in the clinical microbiology laboratories is difficult. Gas liquid chromatography is helpful in accurately identifying these bacilli when the basic information of Gram stain reaction, spore status, oxygen susceptibility, catalase and indole reactions are available. These GPAB can also be missed due to their complex transport and growth requirements and being often seen along with non-fastidious aerobic bacteria as part of polymicrobial flora. Their epidemiology, clinical significance and pathogenic potential needs further understanding [43,44]. Among the spore forming GPAB, *Clostridium* spp. was commonly found in association with diabetic foot infection (n=20) and *C. perfringens* (1.9%) was isolated from six cases of gas gangrene. *C. difficile* which is being increasingly recognized and isolated from patients with antibiotic associated

colitis mainly in a nosocomial setting was isolated in 4.2% (n=14) cases. Gorbach SL et al., in their analysis found intra-abdominal sepsis associated with trauma or prior intestinal surgery as a major source for Clostridial infections [45]. *Clostridium* spp. have also been reported to be predominantly isolated from wound infections, abscesses, abdominal infections, and blood [30,36].

GPAC are frequently isolated from clinical specimens and account for 24-31% of anaerobic isolates [1]. In our study, 25.3% (n=85) isolates were found to be GPAC. *Peptostreptococcus, Finegoldia, Parvimonas, Anaerococcus and Peptoniphilus* are the more commonly reported GPAC [1]. Zone of inhibition of ≥15 mm around a 5 µg metronidazole disc differentiates GPAC from microaerophilic Gram-positive cocci. Infections involving GPAC are usually polymicrobial and are isolated mainly from abscesses, infections of oral cavity, skin and soft tissues, bone and joints, upper respiratory and female genital tract [46]. However, *F. magna* is reported to be isolated as monomicrobial flora from various infection sites [1,46]. *F. magna* is found in high frequency in chronic wounds like diabetic ulcers and pressure ulcers [1]. Majority of *F. magna* which were the most frequent GPAC in our study, were obtained from diabetic foot infection (n=13) and necrotising fasciitis (n=8).

Anaerobic blood stream infections are relatively uncommon and contribute to 0.5%-12% of all positive blood cultures which corresponds to an occurrence of 0.5 – 1.0 cases per 1,000 hospital admissions [22,47]. There are conflicting data on the incidence and trends of anaerobic bacteremia over time, and the clinical significance of isolating anaerobic bacteria from blood cultures [22,48]. However, it is recommended that anaerobic blood cultures are performed as a routine in all patients with suspected blood stream infections [48]. Commercial automated blood culture bottles can also be used for inoculation of non-blood specimens like sterile body fluids for isolation of anaerobic bacteria.

Anaerobes were predominantly isolated as polymicrobial flora involving aerobic and anaerobic pathogens from clinical specimens (159, 57.2%). *B. fragilis* (n, 47) was the common anaerobe found in association with facultative aerobes such as *E. coli* and *Klebsiella* spp. Microbial synergy leads to enhanced pathogenicity and severity of infection in polymicrobial infections with aerobic and anaerobic bacterial pathogens. *B. fragilis* which is known to be the most frequent anaerobic pathogen in polymicrobial infections is associated with a mortality rate of more than 19% [42].

It is essential that the clinicians recognize the importance of anaerobic bacteria as pathogens and utilize the expertise of the laboratories having facilities for anaerobic culture and susceptibility testing for infections with suspected anaerobic aetiology. Performance of anaerobic cultures, along with aerobic cultures will provide complete bacterial work-up of specimens from infectious sites. With increasing instances of antimicrobial resistance amongst anaerobic bacteria to commonly used antimicrobials and the inherent drug resistance amongst some of these bacteria, knowledge of the distribution of these organisms may assist in the selection of appropriate empirical therapy for anaerobic infections.

LIMITATION

As we do not perform routine anaerobic blood cultures, incidence of anaerobic bacteremia could not be obtained. We could not obtain the follow-up clinical data on antibiotic prescription practices based on anaerobic culture reports and therapeutic response of the patients.

CONCLUSION

Anaerobes as pathogens are isolated from diverse infection sites. Unless they are cultured and susceptibility tests are performed as a routine, true magnitude of antimicrobial resistance among anaerobic pathogens will not be known.

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