

Isolation of obligate anaerobes from clinical samples received for routine bacterial culture and sensitivity: a cross sectional study

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ABSTRACT

Background and Objectives: Obligate anaerobic bacteria are known to cause various infections in human beings. We aimed to determine the prevalence and spectrum of obligate anaerobes encountered in pus aspirate, sterile fluids and tissue samples received for routine bacterial culture and sensitivity.

Materials and Methods: A total of 160 samples including tissue, sterile body fluids and pus aspirate were collected, analysed for prevalence and spectrum of obligate anaerobes. Identification of obligate and facultative anaerobes was done by automated MALDI-TOF and Vitek 2 method.

Results: Among 160 samples, 75 samples (46.8%) yielded obligate anaerobes out of which 41 samples (26%) yielded obligate anaerobes along with facultative anaerobes which was significant (p value=0.031) and 34 samples (21%) yielded only obligate anaerobes. 90 obligate anaerobes were isolated from 75 samples among which only 34 (37.7%) samples yielded only obligate anaerobes and 56 (62.2%) yielded both obligate and facultative anaerobes. Gram stain with polymicrobial appearance (p value 0.02) was found to be significantly associated with growth of obligate anaerobes. Clinical conditions where obligate anaerobes were commonly associated were appendicular abscess, empyema, fourmier's gangrene, diabetic foot, ludwigs angina and deep abscess. Out of 75 positive samples 30 (40%) patients had predisposing conditions like diabetes mellitus, hypertension etc. Total of 90 obligate anaerobes and 49 facultative anaerobes were isolated. The common obligate anaerobes were *Bacteroides fragilis* 18 (20%), *Prevotella* spp. 20 (22.2%), and *Clostridium* spp. 8 (8.88%). Facultative anaerobes like *Escherichia coli* 25 (34.7%), *Klebsiella* species 15 (20.8%), *Enterococcus faecalis* 19 (26.3%) were isolated. Antibiotic sensitivity was performed for facultative anaerobes by Kirby bauer disc diffusion method. Out of 15 *Escherichia coli* isolates resistance was commonly seen for ampicillin 13 (86.6%), cephalosporins 11 (73.3%), ciprofloxacin 10 (66.6%) and *Piperacillin tazobactam* 8 (53.3%). In *Klebsiella* species resistance were commonly seen to Ampicillin 6 (100%), cephalosporins 2 (33.3%) and ciprofloxacin 2 (33.3%).

Conclusion: There was significant isolation of obligate anaerobes along with facultative anaerobes in clinical samples received for aerobic culture and sensitivity. There is a need for isolation of these bacteria routinely and a scope for doing antibiotic susceptibility testing, which will help in evidence-based medicine and a better clinical outcome by giving appropriate therapy.

Keywords: Obligate anaerobes; Facultative anaerobes; Polymicrobial appearance

INTRODUCTION

Anaerobes are important pathogens in various human clinical infections (1). The infection caused

by anaerobic organisms are often endogenous and are caused by tissue invasion of the bacteria, mostly resident near to mucosal surfaces, upper respiratory tract, gastrointestinal tract, Genitourinary tract

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and skin etc (2). Most common organisms responsible for anaerobic infections are *Bacteroides* species, *Prevotella* species, *Clostridium* species and *Cutibacterium* spp. Though the anaerobes can live in complete harmony with the host and lead a mutual beneficial relationship with aerobic commensal, they can still become pathogenic when hosts defence mechanism gets impaired (1).

A study done by Antony et al. who aimed isolation of bacteria that are encountered in the surgical infections with an emphasis on anaerobes and to create an awareness among the clinicians regarding the potential role of these silent pathogens. Among total 393 specimens (surgical infections), 193 anaerobes and 311 aerobes were obtained. Majority of the cases (226) exhibited polymicrobial etiology. Hence, concluding the need to employ anaerobic culture techniques routinely in microbiology laboratories and to create an awareness among the clinicians regarding the potential role of anaerobes (1).

There is high likely tendency to see the anaerobic infection in samples showing polymicrobial appearance in Gram stain from clinical samples (3). Polymicrobial infection can be more virulent than those involving single organism (1). Synergism between aerobic and anaerobic organism has been recognized in various clinical infections (4).

From culture of contaminated samples obligate anaerobic organisms are recovered infrequently because of short comings in collection and transport of specimen (4). In the era of cost containment, most clinical laboratories neither identify obligate anaerobes to the species level nor perform antimicrobial susceptibility studies on these organisms. The reason being difficulty in isolating these organisms as it is time consuming and involves various technical skills and hence it is overlooked (5).

Routine processing of the samples by anaerobic methods should be practiced by the laboratories to look for the presence of obligate anaerobes as it can be fatal when untreated (4, 6). The diagnostic importance has to be given for obligate anaerobic organisms in various clinical samples as they are often overlooked (5). So, there is a need to look for these organisms in the routine clinical sample as they play a predominant role in causing infection. Indication for presence of obligate anaerobes help the clinician to provide appropriate treatment (4). A study by Viswanath B.G. et al. conducted on emergence of antimicrobial resistance among anaerobic bacteria. In

their study, totally 33 samples showed the presence of obligate anaerobes with a rate of isolation of 66%. The obligate anaerobes isolated were *Bacteroides* spp. *Prevotella* spp. *Fusobacterium* spp. *Porphyromonas* spp. *Peptococcus* spp. *Peptostreptococcus* spp. and *Bifidobacterium* spp. *Bacteroides* spp. showed resistance to penicillin G (76.9%), ciprofloxacin (61.5%), erythromycin (61.5%), metronidazole (46.1%), amoxicillin clavulanic acid (46.1%) and clindamycin (38.4%). *Prevotella* spp. showed resistance to penicillin G (69.2%), erythromycin (30.7%), metronidazole (15.3%) and clindamycin (7.6%). *Porphyromonas* spp. *Peptostreptococcus* spp. and *Bifidobacterium* spp. showed susceptibility to all the drugs tested. *Fusobacterium* spp. showed resistance to penicillin (63.6%), metronidazole (54.5%), ciprofloxacin (36.3%) and erythromycin (27.2%). Their sensitivity pattern has to be studied as there are several reports of the emergence of resistance to various antibiotics. This antibiogram pattern helps the clinician to treat these infections with appropriate and effective therapy resulting in excellent clinical outcomes (7).

We aimed to determine the spectrum of obligate anaerobes encountered in clinical samples like pus aspirate and tissue sample received for routine bacterial culture and sensitivity. Also to look for the prevalence and speciation of all the obligate and facultative anaerobes isolated from pus aspirate and tissue sample received for routine bacterial culture and sensitivity along with its clinical correlations.

MATERIALS AND METHODS

A prospective descriptive cross-sectional study was conducted from November 2019- December 2020. All the pus aspirates and tissue samples received for routine aerobic culture and sensitivity received in Microbiology laboratory, St Johns Medical college and hospital, Bengaluru were included. Pus aspirate, sterile body fluids or tissue samples indicating any of the selection criteria were taken and processed for anaerobic culture. Exclusion criteria were any swabs like eye swab, ear swab, sputum and urine samples were not included as they were not suitable samples for routine anaerobic culture and sensitivity. Sample selection criteria were foul odor of specimen, necrotic and gangrenous changes, unique morphology on Gram stain, failure of organisms seen on Gram stain of original exudate to grow aerobically, presence of

“sulfur granules” in discharges (actinomycosis) and location of infection in proximity to a mucosal surface. IEC approval with reference number IEC Study No 397/2019 was obtained from Institutional Ethics Committee.

A pilot study was done in our department in the year 2011 wherein we found the isolation of obligate anaerobes in anaerobic culture was 26%. Using this prevalence of 26% with 7% precision and 95% confidence interval the required sample size was 150. A total of 160 samples including tissue, sterile body fluids and pus aspirate were collected. All samples were analyzed for macroscopic appearance details like foul odour, foul smelling, mucosal involvement, gas gangrene and black discoloration. The samples were subjected to Gram stain and looked for polymicrobial appearance. Anaerobic culture was performed using anaerobic blood agar, neomycin blood agar (NBA) and Brucella blood agar (BBA) using Gaspak jar method for anaerobiosis. Aerotolerance was put up for each anaerobically isolated colony for confirmation (4). Aerotolerance is the reliable method to know whether the sample has obligate anaerobes in it or not. Each colony type from the anaerobic isolation plate was subculture to an aerobic (5-10% CO₂, or candle jar) and anaerobic blood agar plate for overnight incubation. No growth in the 5-10% CO₂ aerobic incubation indicated presence of obligate anaerobes and growth indicates facultative anaerobic bacteria or microaerophilic bacteria. For facultative anaerobes routine antibiotic susceptibility test was performed by Kirby Bauer disc diffusion method or Automated Vitek method. Identification of obligate anaerobes was done by automated method using Matrix Assisted Laser Desorption Ionization -Time of Flight (MALDI-TOF) and Vitek 2 ANC card. Isolated colonies from Brucella Agar with Hemin and vitamin K were picked up and subjected to mass spectrometry (MALDI-TOF) or Vitek ANC card identification. Presently MALDI-TOF method is currently being employed to greatly reduce the time to identification. Fig. 2 represents the algorithm of methodology of sample processing and identification of bacteria.

Matrix Assisted Laser Desorption Ionization -Time of Flight (MALDI-TOF) (biomeuriex Vitek Ms). A portion of the test colony is taken from anaerobic blood agar plate and applied onto a spot of the target slide. A matrix solution is then added onto the smear. Smear is allowed to dry and then loaded into

the system. The sample spectra are compared to a database of spectra developed from a number of microbial species fed in the system. The sample spectra are then interpreted to provide organism identification results associated with a confidence level.

Vitek 2 ANC Card (bioMérieux Vitek 2). Each isolate was inoculated onto an anaerobic blood agar plate and incubated in 5-10% CO₂ atmosphere for aerotolerance testing. Inoculum suspensions were prepared from isolated colonies of Brucella blood agar with 0.45% aqueous NaCl until a turbidity of between 2.70 and 3.30 McFarland standards was reached by using a calibrated Vitek 2 Densichek instrument. Final results were available in approximately 6 hours.

Preliminary report was released in 72 hrs with an indication of obligate anaerobes and final report in 5-7 days with complete identification of bacteria. Statistical analysis was done using SPSS version 25 for Descriptive statistical analysis and p value calculated by using Chi square test for categorical variables.

RESULTS

A total of 160 samples were collected from November 2019 - December 2020, satisfying at least one of the indications for anaerobic infection like black discoloration, foul smell, mucosal involvement, presence of “sulfur granules” in discharges (actinomycosis) or unique morphology on Gram stain. The maximum samples collected were between 30-60 yrs ie 115 samples and 54 (51.4%) samples yielded maximum obligate anaerobes. Among total samples collected M:F ratio was 1.9:1 whereas in positive samples the ratio was 1.3:1. The different samples from which the samples received were common from surgery 33 (44.0%), medicine 22 (29.3%), ENT 7 (9.3%), other wards 11 (14.6%) and out-patients were 2 (2.6%). Out of 160 samples, 50 (51%) out of 98, 11 (55%) out of 20 Body fluid samples and 14 (33.3%) out of 42 tissue samples yielded obligate anaerobes.

Table 1 represents different clinical conditions involved with an isolation of obligate anaerobes. Out of 160 samples 75 (46.8%) samples yielded obligate anaerobes commonly isolated from gastrointestinal system (20.0%) with clinical conditions like appendicular abscess and peritonitis, followed by respiratory system (14.6%) with clinical conditions like empyema, non resolving pneumonia and pulmonary ac-

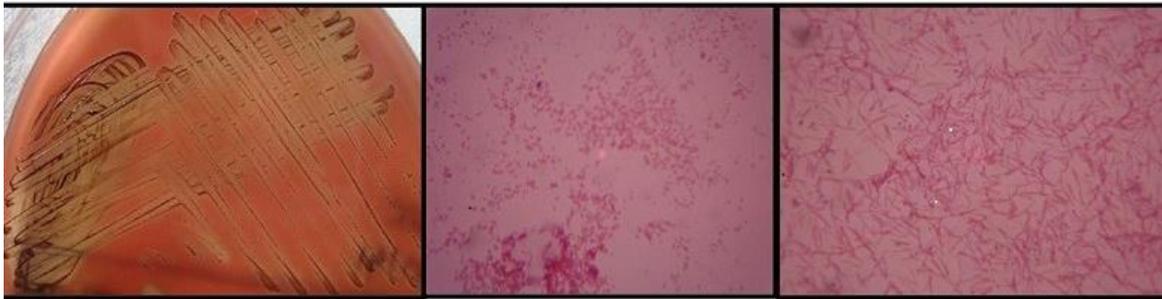


Fig. 1. (a) Showing black pigmented colonies of *Prevotella melaninogenica* on *Brucella* Blood Agar. (b) Gram stained colony smear of *Prevotella melaninogenica* showing Gram negative coccobacilli (GNCB) under 1000×. (c) Gram stained colony smear of *Fusobacterium nucleatum* showing slender gram negative bacilli (GNB) with tapering ends under 1000×

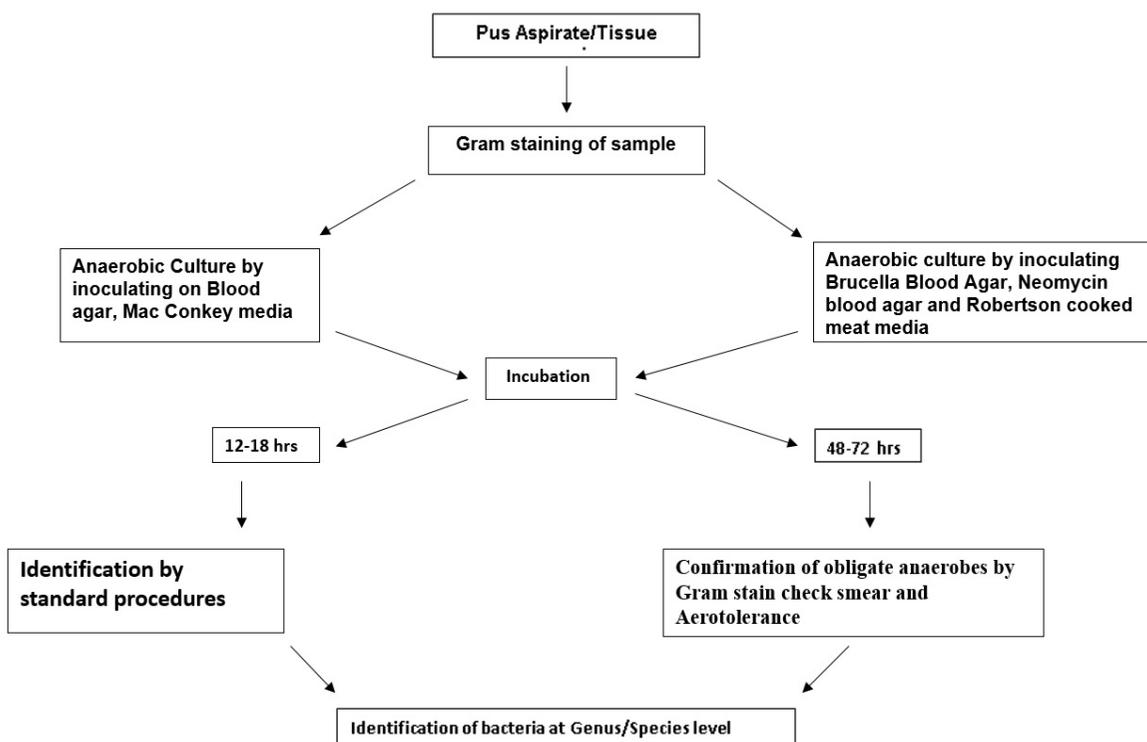


Fig. 2. Flow chart showing the algorithm of sample processing and identification of bacteria.

tinomycosis. The other commonly involved systems were musculoskeletal system (26.6%) like necrotic fasciitis and gas gangrene. Genitourinary system (18.6%) like ovarian cyst, bartholin abscess and teratoma etc, orofacial maxillary system (16.0%) commonly involving deep abscesses, Ludwig angina and preauricular sinus. Central nervous system (1.8%) like brain abscess. A total of 30 (40%) samples were associated with predisposing factors like diabetes mellitus 16 (39%), hypertension 12 (29.2%), hypothyroidism 3 (0.07%), anaemia 3 (0.07%), liver disease 2 (0.04%) and immunosuppressive conditions like cancer, HIV

5 (12.1%).

All 160 samples, received were analysed for macroscopic features indicative of an anaerobic infection like foul smell, gas presence, gangrene changes, mucosal involvement and granules formation. Involvement of mucosa, gangrenous changes and foul smelling samples were strongly associated with anaerobic infections with an odds ratio >1 (Table 2).

The Gram stain feature like polymicrobial appearance in direct smear from sample was analysed. Among 74 samples which showed polymicrobial appearance in the Gram stained direct smear, 42 (56.7%) samples

Table 1. Isolation of obligate anaerobes from various clinical conditions and systemic involvement

SL.NO.	Systems involved	Conditions	No.	
1	Gastrointestinal tract (20%)	Appendicular abscess	10	
		Perianal abscess	4	
		Peritonitis	1	
2	Respiratory tract (14.6%)	Empyema	4	
		Non resolving pneumonia	2	
		Pulmonary actinomycosis	1	
		Pneumonia	4	
3	Subcutaneous and soft tissue (26.6%)	Fourniers gangrene	3	
		Necrotising fasciitis	1	
		Psoas abscess	2	
		Pilonidal abscess	2	
		Other deep abscess	10	
		Gluteal abscess	2	
		Diabetic foot & ulcer	5	
		Brain abscess	1	
4	Central nervous system (1.3%)	Brain abscess	1	
		Kidney failure	4	
5	Genito urinary tract (18.6%)	Ovarian cyst	2	
		Perineal abscess	1	
		Teratoma	1	
		Bartholins abscess	1	
		Abscess in genito urinarytract	5	
		Nasal obstruction	1	
6	Orofacial mandibular region (12%)	Sebacious cyst	1	
		Cheek abscess	2	
		Pre-auricular sinus	1	
		Ludwigs angina	2	
		Submandibular abscess	2	
		Total (n=75)		75

Table 2. Table showing analysis of the macroscopic/gross features indicative of anaerobic infection.

SL NO.	Indications	Total Samples	Positive	Negative	Odds ratio	P value	Interpretation of p value
1	Mucosal involvement	103	54	49	1.89	0.059	not significant
2	Black discoloration	10	7	3	2.81	0.131	not significant
3	Gas gangrene	8	8	0	-	-	-
4	Foul smell	14	11	3	4.7	0.013	Significant

yielded obligate anaerobes. The growth of obligate anaerobes in culture and its association with polymicrobial appearance was statistically significant with P value of <0.02 (CI,1.12-2.31) by Chi square test as shown in Fig. 3. Among them, 34 (45.9%) samples had growth of facultative along with obligate anaerobes and 8 (10.8%) samples had an exclusive growth

of obligate anaerobes in cultures. Among 86 samples which didn't indicate polymicrobial appearance in the Gram stained direct smear, totally 33 (38.3%) samples yielded obligate anaerobes in which 26 (30.2%) samples had exclusive growth of obligate anaerobes and 7 samples (8.1%) yielded both obligate and facultative anaerobes.

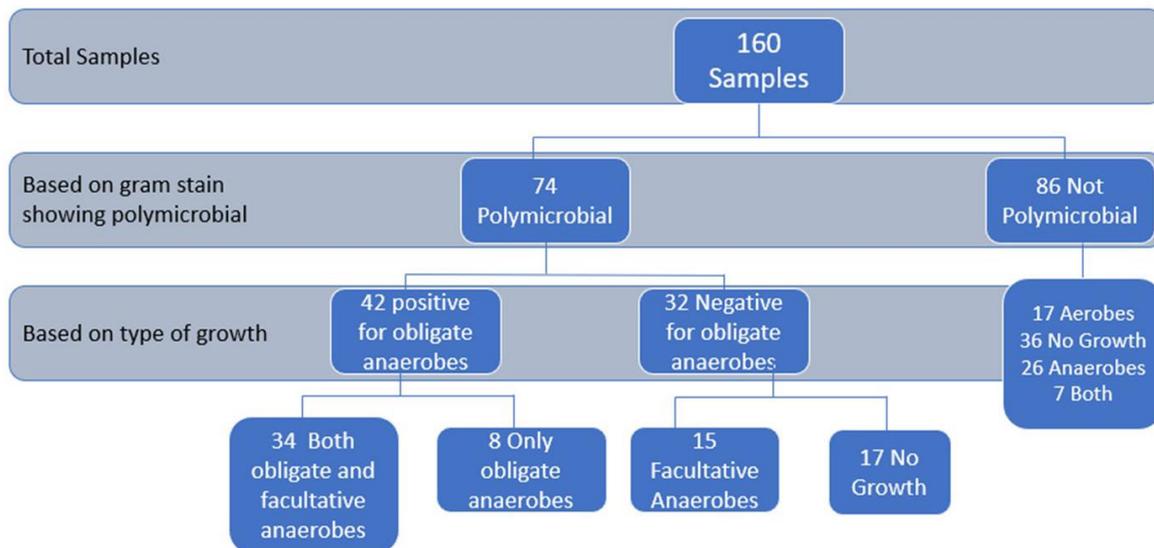


Fig. 3. Growth of different types of organisms in culture and their direct Gram stain showing polymicrobial appearance.

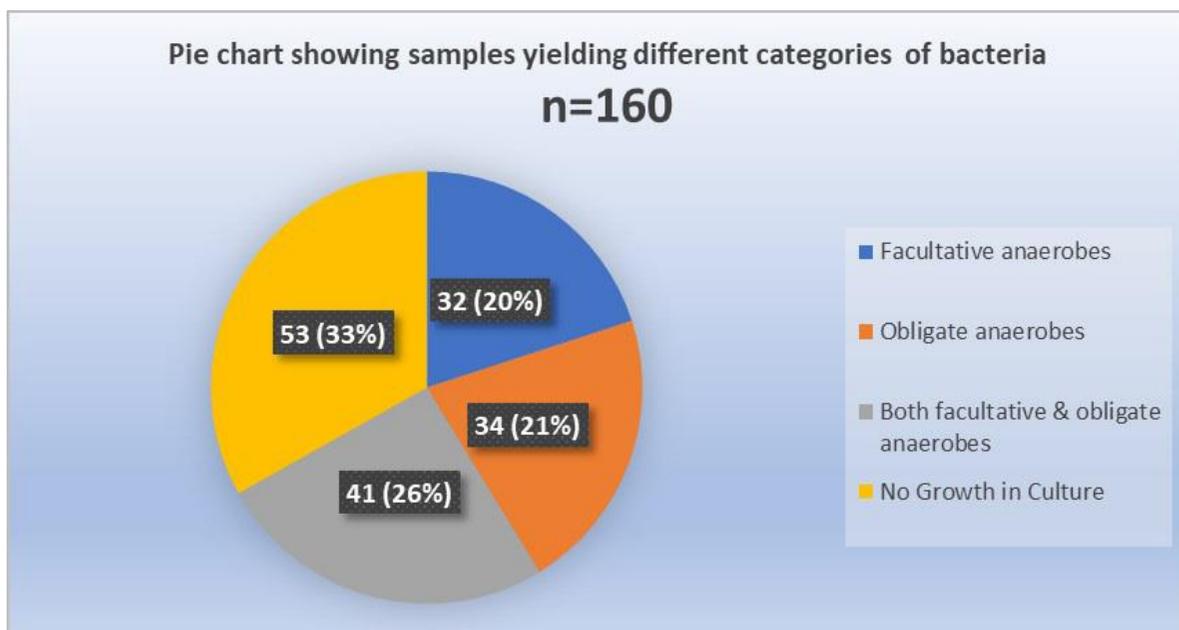


Fig. 4. Pie chart showing clinical samples yielding different categories of bacteria in culture.

The pie chart (Fig. 4) depict different types of bacterial growth in culture. Among 160 samples a total of 41 (26%) samples yielded both facultative and obligate anaerobes where in synergism was found to be statistically significant with P value of (<0.031) CI (1.06,3.75) by chi square test. Exclusive obligate anaerobes were seen in 34 (21%) samples contributing for total of 75 (46.8%) samples yielding obligate anaerobes. 34 samples yielded only obligate anaerobes which were *Bacteroides fragilis* (5), *Clostridium spe-*

cies (6) *Prevotella* species (5), *Actinomyces* species (4) *Anaerococcus prevotii* (3), *Viellonella* species (1), *Cutibacterium acne* (3), *Finegoldia magna* (1), *Lactobacillus fermentum* (3), *Peptostreptococcus anaerobium* (1), *Peptinophilus assacharolyticus* (2).

A total of 90 obligate anaerobes were isolated. Most commonly Gram negative bacilli (GNB) 40 (44.4%), followed by Gram positive cocci (GPC) 13 (14.4%), Spore bearing Gram positive bacilli (GPB) 8 (8.8%) and Gram negative cocci (GNC) 1 (1.1%) were isolat-

Table 3. Spectrum of obligate anaerobes yielded from the clinical samples in anaerobic culture.

Microscopic appearance	Genus	Species	Number of isolates	
Gram positive cocci GPC (27.7%)	Atopobium	nimae	1	
	Anaerococcus	prevotii	6	
	Finegoldia	magna	2	
	Petostreptococcus	anaerobium	3	
	Peptococcus	saccarolyticus	1	
	Peptinophilus	assacharolyticus	4	
	Unidentified GPCs		4	
	Gram Negative Bacilli GNB (44.4%)	Bacteroides	uniformis	1
thetaitaomicron			2	
Prevotella		fragilis	12	
		ovatus	2	
		vulgatus	1	
		bivia	3	
		nigrescens	3	
		intermedia	7	
		loescheii	1	
		bergensis	1	
		oralis	1	
		melaninogenica	3	
		disiens	1	
		Fusobacterium	nucleatum	2
		Gram Positive Bacilli. GPB (14.4%)	Cutibacterium	acne
not identified	1			
Lactobacillus	fermentum		3	
Actinomyces	israelii		2	
	naeslundii		1	
	odontolyticus		1	
Unidentified GPB			1	
Gram Positive Bacilli with spore GPB with spore	Clostridium		perferingens	3
		septicum	1	
		innocuum	1	
		clostridioforme	1	
		butyricum	1	
		paeniclostridium sordellii	1	
		Gram Negative cocci.GNC (1.1%)	Vielonella	Not identified
Gram negative coccobacilli (1.1)	Not identified		1	
Obligate anaerobes grown (2.2%)	Not identified		2	
Total (n= 90) OBLIGATE ANAEROBES			90	

ed. Most commonly species were *Bacteroides fragilis* group, *Prevotella* spp. and *Clostridium* spp. as shown in the Table 3. Some of the isolates were not completely identified and they were specified at Gram stain level, genus level or mentioned as obligate anaerobes.

A total of 49 facultative anaerobes had grown in synergism with obligate anaerobes among which 29

(59.%) were Gram negative bacilli and 20 (40.8%) were Gram positive cocci. *Escherichia coli* (15), *Enterobacter* spp. (1), *Klebsiella* spp. (6), *Providencia* species (1), non fermented Gram negative bacteria (NFGNB) (3), *Proteus mirabilis* (3), *Enterococcus faecalis* (12), Methicillin resistant *Staphylococcus aureus* (MRSA) (2), *Streptococcus pneumoniae* (1) and

other beta haemolytic streptococci (5).

DISCUSSION

Anaerobic bacteria are the common commensals seen in skin and mucosal surfaces of the human body. Normally, commensals do not enter the protective barriers, but in case of breach due to wound or in case of immunocompromised patients these commensals become pathogens. It is necessary to identify these pathogens but often due to high cost, technical skills, requirement of prolonged incubation and time-delay in identification, these obligate anaerobes are often overlooked.

Few clinical conditions are highly suggestive of anaerobic infection with obligate anaerobes. This study aimed to look for obligate anaerobes in samples based on selection criteria. Samples received for routine aerobic culture and sensitivity were taken for anaerobic culture and looked for isolation of obligate anaerobes. In our study out of 160, total males were 104 (65.0%), females were 56 (35.0%) with M:F ratio of 1.9:1 in total samples and 1.3:1 in positive samples. Most of the samples were from surgery ward (n,22) followed by medicine wards.

Most obligate anaerobes were seen from subcutaneous and soft tissue infection, which were found in skin and soft tissue infection mostly being surgical emergencies contributing for 26.6%. Hannah Zhao-Fleming et al. reviewed 16 articles on soft tissue and subcutaneous infection and demonstrated an average mortality rate of 20.7% (8). There was one Fournier's gangrene case seen in our study as shown in Table 1 and patient was type 2 diabetic on long term treatment. In a study done by Kuzaka et al. (2018), diabetes mellitus was present in 4 out of 13 (30.8%) patients of skin and soft tissue infections (10).

Viswanathan et al. performed a study to determine the prevalence of pathogens in diabetic foot infections, in relation to parameters like Wagner's grading, duration of diabetes and healing time. Among 654 diabetic patients, 728 pathogens were isolated. Aerobic pathogens were isolated in 437 (66.8%) patients and anaerobic pathogens were isolated in 217 (33.2%). Healing time was longer when strict aerobic pathogen *Pseudomonas* spp. and strict anaerobic pathogens were present. Diabetic foot accounts for 20% of hospital admissions (11). In our study 5 pa-

tients had diabetic foot infections among which all 5 samples had foul smelling and 3 had gangrenous changes. Cultures yielded combination of facultative anaerobic and obligate anaerobic bacteria in 4 patient samples. Major anaerobes were *Prevotella* species and *Bacteroides fragilis*, *Clostridium* species and *Anaerococcus* isolated from diabetic foot ulcers.

In a similar study by Louie TJ et al. 1976, certain observations suggest that anaerobic bacteria play a significant role: the exudate from the foot lesion may be putrid, soft-tissue gas is sometimes noted, and aerobic cultures may fail to show a likely pathogen (12). Their study defined the microbiology of diabetic foot ulcers using optimal culture techniques. Among 20 samples of diabetic foot infection 18 cases reported both aerobic and anaerobic bacterial growth. Out of 20, 16 samples yielded *Peptococcus* species other major isolates were *Bacteroides* species and *Clostridium* species. 20 of the patients were diabetic among which 9 were insulin dependent, fifteen of the specimens had a foul odor and soft tissue gas was seen in 2 patients (12). This defines importance to look for indications like foul odour, black discoloration, gangrenous changes with presence of gas in the sample for isolation of obligate anaerobes. In our study, total samples like pus aspirate, fluid and tissue received for routine bacterial culture were taken and looked for indications like mucosal involvement, foul smelling and gangrenous changes. Statistical significance was found with foul smelling samples with p value of 0.013, macroscopic features indicating anaerobic infection like mucosal involvement, blackish discoloration and foul smell were strongly associated with isolation of obligate anaerobes with an odds ratio more than one (Table 2). So, this signifies that these are the good indicators for all the purulent samples to be taken for anaerobic culture when indicated to look for complete identification of obligate anaerobes.

Zhao-Fleming et al. studied based on necrotizing soft tissue infections (NSTIs) are associated with high morbidity and mortality. The goal of their study was to address the gap in knowledge by characterizing NSTI microbial communities through molecular diagnostics. Based on their data (>70% bacterial population yielded obligate anaerobes) they concluded that obligate anaerobes are abundant in NSTIs and a high relative abundance of anaerobes is associated with a worse outcome and recommend antibiotics against anaerobe during the treatment of NSTIs even when anaerobes are not found by traditional

clinical microbiology methods especially when there is a clinical suspicion for anaerobic involvement (2), Gastrointestinal tract was source in almost half of anaerobic infections in study done by Hannah Zhao-Fleming et al. and similar findings were seen in our study wherein gastrointestinal tract infection was found in 20.0% of the cases (Table 1) (8).

In a study done by Bussutil et al. Post appendectomy infections were seen in the majority of patients, caused by a polymicrobial flora consisting of facultative aerobes and obligate anaerobes. *E. coli* was the most frequently cultured aerobe. Of the anaerobes, *B. fragilis* was the most common pathogen. A positive peritoneal culture in Bussutil et al. study yielded *E. coli* in 93% of the patients and *B. fragilis* in 62%. In addition, there was a direct correlation with the degree of appendiceal inflammation and the percentage of positive peritoneal and subcutaneous tissue cultures (9). Similar findings were seen in our study, Appendicular abscess was seen in 10 patients, out of which 6 cases yielded *Escherichia coli* in common along with *Bacteroides* spp. and Gram positive cocci like *Peptostreptococcus* spp.

Diabetes mellitus, hypertension, organ transplant, immunocompromised conditions, post surgical infections were the common predisposing factors for anaerobic infection. A total of 33 (40%) patients were having predisposed conditions in our study. In the similar study done by Choi, Y 27% patients were having comorbidities mostly seen in some diabetic foot infection, human chronic wounds, and underrepresentation of anaerobes in chronic wounds presented with autoimmune disease such as vasculitis, ankylosing spondylitis, Sjogrens syndrome, scleroderma, pyoderma gangrenosum, and livedoid vasculopathy (13).

Among 160 samples, based on Gram stain 74 samples were showing polymicrobial indication in which 42 (56.0%) samples yielded growth of obligate anaerobes. In Fig. 3. the association of polymicrobial appearance in the direct smear with isolation of obligate anaerobes were found to be statistically significant with the p value of 0.02 (CI 1.12,3.9) indicating good isolation of obligate anaerobes when the direct Gram stained smear shows polymicrobial appearance. However, 32 (44.0%) samples did not yield obligate anaerobes which showed polymicrobes in direct Gram stain (Fig. 3). This could be because of delay in the sample collection and receipt of the samples in the laboratory, hence delay in processing of samples

with exposure of oxygen affecting the yield of obligate anaerobes. However in our study, utmost care was taken to process the sample at the earliest and inoculate Robertson's cooked meat broth and thioglycolate broth as a backup media. So, this is an important finding in our study which indicates about the presence of obligate anaerobic infections among samples showing polymicrobial appearance in direct smear indicating for anaerobic culture at the earliest and without any delay. In a study done by Park YS et al. in 2009, clinical significance of a broad range of pathogenic obligate anaerobic organisms was examined, they found polymicrobial anaerobic infections represented 80.3% of all clinically significant cases which included clinical conditions such as sinusitis, otitis media, retropharyngeal abscess, lung and thorax lung abscess or empyema, intraabdominal infection, burn, DM foot, necrotizing fasciitis, septic arthritis, pressure sore (14). In the same study it was also found that the mean number of pathogens, including aerobes and anaerobes, was 3.7 ± 1.0 (minimum 1, maximum 5), and the mean number of anaerobic organisms was 1.0 ± 0.3 in each specimen (14).

In our study, out of 160 samples 75 (46.8%) samples yielded obligate anaerobes among which 34 samples yielded only obligate anaerobes and 41 samples yielded both facultative anaerobes and obligate anaerobes (Fig. 4). In a study by Holland et al. comparison of 14 years of cumulative data with data from current studies covering 1- to 2-year periods was done, out of which 826 specimens from 562 patients were cultured between 1960 and 1974. Among total 826 specimens, 689 (83%) yielded bacterial growth and 403 (58.5%) of these positive cultures contained anaerobic bacteria. Further breakdown of the data from the 689 positive specimens showed that anaerobes alone were found in 11.8% and anaerobes mixed with aerobes were found in 11.8% and anaerobes mixed with aerobes were found in 46.7% (15). In the present study, the total prevalence 46.8% which was more when compared to the previous study done in department of microbiology ie 26%. The raise in the prevalence can be explained due to the selection of the samples based on the selection criteria applied exclusively in this study. So routinely any indication of anaerobic infection like foul smell, gangrenous changes, proximity to mucosal layer and polymicrobial slender Gram stained organisms always indicates anaerobic infection and should be looked for obligate anaerobes. Anaerobes were isolated more frequently with facultative anaerobes. This can be explained on the

basis of intake of the oxygen by the facultative anaerobes and hence creating an anaerobic environment facilitating the growth of obligate anaerobes.

In the present study, the growth of obligate anaerobes with the facultative anaerobes was found to be strongly associated with an odds ratio of 1.997 and the p value was 0.031 which is considered to be significant. Among 75 positive samples, 90 obligate anaerobes were isolated (Table 3), in which GNB were 44.4% (Fig. 1), GPC 27.7%, GPB 14.4% (Table 3). In a similar study done by Park et al. the most common pathogens were *B. fragilis* (41.8%) followed by *Clostridium* spp. (14). Similar findings were seen in our study, the most common pathogens were *Bacteroides fragilis* (13.3%), *Prevotella intermedia* (7.7%) and *Anaerococcus prevotti* (6.6%). Antony et al. study was aimed at isolating bacteria encountered in the surgical infections with an emphasis on anaerobes. Out of 393 specimens, 311 aerobic bacteria and 193 anaerobes were obtained. Among these more frequently isolated organisms were nonsporulating Gram-negative bacilli such as *Bacteroides fragilis* group, *Prevotella-Porphyrromonas* group, and *Fusobacterium*, which constitutes 68.91% (i.e., 133 out of 193). Among these, *B. fragilis* group was the most common isolate [48 (24.87%)] (1).

Actinomycosis is a rare and slowly progressive disease. In our study pulmonary actinomycosis was commonly associated with *Actinomycosis*. We found 4 isolates of *Actinomyces* species, which is a rare organism and difficult to grow. In our study, 3 cases were of pulmonary actinomycosis and 1 case was of ovarian cyst from which *Actinomyces* spp was isolated. All the patients of pulmonary actinomycosis were more than 30 yrs with symptoms of cough with expectoration and hemoptysis. Though pulmonary actinomycosis is said to be in high percentage among patients with coexisting diabetes mellitus. A study done by Katsenos et al. 2015 data demonstrated a significant proportion of nearly 50% of actinomycosis patients without suffering from any comorbidity, thus suggesting that thoracic actinomycosis does not occur only in comorbid patients (16, 17).

A total of 49 facultative anaerobes were isolated along with obligate anaerobes. The common Gram negative bacilli (GNB) isolated were *Escherichia coli* 15 (30.6%), *Klebsiella* species 6 (12.2%) *Proteus mirabilis* 3 (6.1%), NFGNB 3 (6%) and each of *Providencia* and *Enterobacter* species. Gram positive cocci (GPCs) commonly isolated were *Enterococcus*

faecalis 12 (24.4%) and Beta haemolytic streptococci 5 (10.2%). In a similar study done by Park et al. isolation of *Escherichia coli* was found to be more in their study with 41.8% and *Klebsiella* species isolation with 21.0%, among GPCs isolation of *Staphylococcus aureus* was commonly seen with 7.5% (14).

Antibiotic sensitivity was performed for facultative anaerobes by Kirby Bauer disc diffusion method. Out of 15 *Escherichia coli* isolates resistance was commonly seen for ampicillin 13 (86.6%), cephalosporins 11 (73.3%), ciprofloxacin 10 (66.6%) and piperacillin tazobactam 8 (53.3%). In *Klebsiella* spp. resistance was commonly seen to ampicillin 6 (100%), cephalosporins 2 (33.3%) and ciprofloxacin 2 (33.3%). According to a study done by Jardim et al. the most active antimicrobial drugs were carbapenems (meropenem and imipenem), and resistance to these drugs was 1.6-2.3% seen among the isolates (18). In our study among Gram-positive cocci, most of the isolates of *Enterococcus* species were found to be sensitive except one which was vancomycin resistant *Enterococcus* (VRE). Among *Staphylococcus* isolates 2 were methicillin resistant *Staphylococcus aureus* (MRSA) and were sensitive to vancomycin. All the streptococcus species in our study were sensitive to beta lactams and other commonly used antibiotics. In a study done by Jardim et al. *Enterococcus* spp. were mostly resistant to amikacin, nalidixic acid and tetracycline whereas *Staphylococcus* spp. were resistance to amikacin and chloramphenicol (18).

However, antibiotic susceptibility testing could not be done for obligate anaerobes which can be a limitation in our study but our study provides further scope to perform antibiotic susceptibility testing as there has been a significant isolation of obligate anaerobe in clinical samples received for routine culture and sensitivity.

CONCLUSION

Our study signifies the importance of both macroscopic and microscopic examination of the samples and to look for indications of anaerobic infections. It is observed that obligate anaerobes and facultative anaerobes mostly grows together with an indication of polymicrobial appearance. Our study also signifies to look for the obligate anaerobes as pathogens causing clinical diseases like deep abscesses, diabetic ulcer and gas gangrene. Anaerobic culture should

be integrated with routine aerobic culture and to be looked for whenever there is a clinical indication. This holistic approach from both clinicians and clinical microbiologist helps in providing an appropriate report supporting evidence-based medicine. This study also gives scope to perform antibiotic susceptibility testing as there has been significant isolation of obligate anaerobes from the routine samples to look for emergence of resistance for commonly used antibiotics.

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