Advancements in methods to detect and culture medically important anaerobic bacteria

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Abstract

Anaerobic bacteria are one of the most important bacteria, involved in a number of diseases and infections. These are also involved in food borne illness. Due to their fastidious nature, culturing anaerobic bacteria is a bit difficult task. Moreover, anaerobic bacteria can take several days and weeks to grow in laboratories. Apart from this, most bacteria just cannot be cultured in laboratories using standard (anaerobic) cultivation techniques known so far. Difficulties in microbiological detection result in delayed diagnosis of the diseases. Many patients suffer due to these facts, as rapid identification is not only difficult, but in many cases, is almost impossible. Thus, there is a need to develop novel techniques for the cultivation and identification of clinically important anaerobes. Rapid detection of foodborne pathogens is necessary for the prevention of foodborne disease and for the safe supply of food. Present article reviews and discusses advance techniques, both culture-dependent and culture-independent, that allow rapid detection of such important anaerobic

bacteria. Advancements in culturing techniques has reduced the time to grow the anaerobic bacteria in laboratories. Whereas advancements in molecular techniques have enabled the rapid detection of medically important anaerobes including *Clostridium*, *Bacteroides*, and many others.

Keywords

Anaerobes, *Clostridium*, Rapid diagnosis, Foodborne illness, Pathogens

Introduction

Anaerobes are the dominant members of normal human microbiota, predominantly residing on mucosal membranes of oral cavity, gastrointestinal tract, and female genital tract. Being a major component of oral microbiota, their concentration ranges from 10^2 ml^{-1} in saliva to 10^{12} ml^{-1} in gingival scrapping. Gastric acidity is responsible for minimizing their number in stomach and upper intestine, however, in colon their concentration reaches up to 99% of the total bacterial burden which is 10^{12} organisms per gram of stool. *Clostridium, Pepetostreptococcus, Fusobacterium* and *Bacteroid* species are the culturable anaerobes however many of the anaerobes are found to be uncultivable by conventional laboratory techniques known so far. Breakdown of mucosal barrier can lead to the contamination of anaerobes in the sterile sites of the body leading to the severe infections Bennett et al. [1] and can cause for 1–17% positive blood cultures [2]. Moreover, they are also involved in food-born infection, lung infections and brain abscesses. According to Center for Disease Control and Prevention, 179 million people get sick due to foodborne pathogens and 6,186 die each year in United States [3]. Table 1 shows some of the important pathogenic bacteria and the diseases that they cause.

Anaerobic spore formers (clostridium and related genera) are a significant concern for foodborne illness due to toxin production. They can be toxigenic, neurotoxigenic or spoilage bacteria. *Clostridium botulinum* and *Clostridium tetani* are neurotoxigenic species. While *Clostridium perfringens* is a prominent foodborne pathogen, the second largest cause of food poisoning in the USA, where it

causes nearly 1,000,000 cases per annum with net financial load of US$382 million [4, 5]. It is also responsible for diarrhea, avian enteritis necroticans, fulminant disease, clostridial myonecrosis and enterotoxemia due to production of variety of toxins with diverse characteristics [6].

Over the past 20 years, it has been reported that diagnostic difficulties can hinder the rapid detection and identification of anaerobes on a species level. Cultivation of strict anaerobes in microbiology laboratory can be challenging as it demands highly equipped systems with strict anaerobiosis and reduced culture media, owing to the fact that oxygen is toxic for most of the anaerobes. Moreover, most anaerobic species are slow growing (can take up to 14 days to grow) and many are inactive for certain biochemical tests. Beside classical methods; including selective media, evaluation of rapid tests (such as nitrate disks, spot indole, growth in 20% bile containing media, etc), commercial kits are also being used for their detection. In spite of the capability and accuracy of these commercial kits, some clinically important anaerobic pathogens such as *Prevotella nanceiencis*, *Bacteroides nordii*, *Bacteroides cellulosilyticus*, *Bacteroides dorei* and many others are commonly either misidentified not identified at all [7, 8, 9]. Updated culture based as well as molecular based techniques such as 16S rDNA sequencing and QRT-PCR has enabled rapid detection and identification of many anaerobes. Various *Bacteroide* species have been detected from clinical samples by use of QRT-PCR [10, 11].

In this article, we review recent techniques, both culture based and molecular, that can be used to comparatively rapid detection of such important anaerobes.

Culture-dependent techniques

To culture strict anaerobes, techniques capable of generating rapidly a low oxygen level (<0.5%) atmosphere are suitable [12]. Anaerobic chambers are being used for generation of such anaerobic atmosphere, however, it can be expensive for most laboratories. Generation of anaerobic atmosphere by chemical compounds [such as sodium borohydride ($NaBH_4$), sodium bicarbonate-citric acid] is more applicable as compared to bulky anaerobic chamber [13]. Such chemical compounds have been utilized in the development of a disposable Quick anaero-system for culturing the strict anaerobes. The whole system consists of three components. First one is a

disposable anaerobic gas pack which was developed to maintain absolute anaerobic atmosphere. This component is further subdivided into 2 subunits. First part is equipped with silica (SiO_2) and $NaBH_4$ tablets. The other part produces CO_2, generated by the reaction of sodium bicarbonate with citric acid. These two parts are connected with a narrow-tipped (10ml) plastic tube which is also used for pouring of tap water. Second component is a disposable culture envelope, a sealer and a reusable rack. Third component is catalyst unit which utilizes 10g alumina pellets coated with 0.5% palladium, kept below the roof of the rack. Working of this gas container is based on the generation of volatile hydride (SiH_4), produced by the reaction of SiO_2 and $NaBH_4$ tablets with water. Efficacy of this quick anaero–system was evaluated by culturing 12 anaerobes in both the Quick anaero-system and BD GasPak EZ Anaerobe System. By comparing the growth of the anaerobes in both the systems, it was estimated that 2 out of 12 and 9 out of 12 anaerobes (cultured on LB- and Blood-agar plates, respectively) showed better growth in Quick anaero-system [14].

For the isolation of anaerobes from rumen, a new medium was used by Kenters et al. [15] to assess the concentrations of inorganic components of rumen, so that it may mimic to the chemical environment of rumen. Major components of this bicarbonate-buffered mineral media are KH_2PO_4, $(NH_4)_2SO_4$, KCl, $NaHCO_3$, L-cysteine·HCl·H2O, resazurin solution and trace element solution SL10. Media preparation is done in O_2-free 100% CO_2 atmosphere. After the collection of rumen contents from animals, rumen fluid, substrates and Vitamin 10 concentrate are prepared as described by Kenters et al. [15]. Rumen samples are prepared for the cultivation and the cells are harvested after the incubation. The method has proved to be a successful tool for the cultivation of *Firmicutes, Bacteroidetes,* and *Spirochaetes*, as confirmed by 16S rDNA comparative analysis.

Oxygen toxicity is a major problem for anaerobes which can be avoided by the use of antioxidant molecules. In a research work by La Scola et al. [16] the efficacy of antioxidant molecules was evaluated by cultivating the obligate anaerobes in aerobic atmosphere. Six clinical anaerobes *Fusobacterium necrophorum, Finegoldia magna, Prevotella nigrescens, Solobactreium moorei, Atopobium vaginae* and *Ruminococcus* were cultivated in Schaedler media supplemented with antioxidants (such as ascorbic acid and glutathione). For all the tubes, supplemented with antioxidants, the growth was observed within 0.3cm of the surface area, but there was no growth in control plates (without ascorbic acid). Thus the use of antioxidants is proved to be a successful method for culturing these clinical

anaerobic isolates in aerobic atmosphere. Moreover, this media also allows the growth of aerobic bacteria. Utilization of antioxidants can be regarded as an easy approach for cultivation of anaerobes with a regular incubator in aerobic environment [16].

Replacement of the atmospheric oxygen with O_2-free gases along with the usage of reducing agents in culture media are useful steps to further facilitate the cultivation of anaerobes. A six well plate method was developed by Nakamura et al. [17] which works together with the AnaeroPack System [18]. This method was proved to be a better technique for culturing the strict anaerobes like methanogens, sulfate reducing bacteria and hydrogen-producing syntrophs. The method was examined by inoculation of anaerobes in both aerobic and anaerobic conditions. Methanogens and sulfate reducing bacteria were used as test organisms. Sterilized W-gellan media (supplemented with reductants) was poured into each well and was inoculated with the culture dilutions (with the ratio of 13:0.1). The plates were covered with lid and kept in nylon bag equipped with two catalyst sachets (AnaeroPouch). For anaerobic inoculation, the whole method was also done in anaerobic chamber. The bag was sealed after replacing its atmosphere with H_2/CO_2 or N_2/CO_2. For comparison, role tube method was also performed as described by Hungate [19] with slight modifications. While comparing the results, it was found that for 2 isolates *Methanoculleu bourgensis* and *Desulfovibrio vulgaris*, CFU values were the same for all three methods, But for *Methanothermobacter thermautotrophicus*, six-well plate method with anaerobic inoculation gave the highest CFU value [17].

Obligate anaerobes residing in oral cavity play an important role in transmissible subcutaneous infections by producing ammonia, hydrogen sulfide and other cytotoxic substances. Development of modified trap method with *in-vivo* incubation (mini-trap method) by Sizova et al. [20] enabled the isolation of *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Gemella*, *Prevotella*, *Campylobacter* and *Veillonella* species. A custom built miniature trap (Hi-Tech Manufacturing) consisting of three steel plates, containing 72 through-holes, was used. Plugs of 0.1µl were formed in through holes by placing the central plate in 1% molten agar supplemented with basic anaerobic media (BM). After solidification, precut 1.0µm pore size polycarbonate membranes (GE Water & Process Technologies, Burlington, MA) were pressed against the plate by two side plates and tightened with screws. This mini-trap was introduced into a precut window and fixed in the upper lingual side adjacent to the gum in the oral cavity, with superglue. After 48 hours incubation, the apparatus

was transferred into anaerobic glove box and agar plugs were placed in basic anaerobic media. Microbial cells were collected, dried and were examined under Leica DMLB microscope [20].

Culture-independent techniques

Advanced molecular techniques such as target specific probes and Catalyzed Reporter Deposition- Fluorescent In Situ Hybridization (CARD)-FISH has enabled the detection of a variety of bacteria both aerobic and anaerobic, especially the unculturable ones [21]. DGGE/TGGE, TRFLP, DNA microarrays, direct sequencing of 16S rRNA amplicons and microbiome shotgun sequencing are found to be robust and high throughput quantitative techniques for identification and characterization of a wide range of gut microbes [22]. Although qPCR is a rapid method for phylogenetic identification, but rolling circle amplification (RCA), Loop Mediated Isothermal Amplification (LAMP), Nucleic Acid Sequence-Based Amplification (NASBA) and Strand Displacement Amplification (SDA) have proved to be novel methods for nucleic acid amplification under isothermal conditions, providing better efficiency as compared to traditional PCR as described by Zhao et al. [23]. These methods were found to be better for identification of food associated pathogens, such as *Listeria monocytogenes*, *Staphyloccocus aureus*, *Shigella* spp., *Escherichia coli*, *Streptococcus pneumonia*, *Salmonellae*, *Vibrio parahaemolyticus*, *Chlamydia pneumonia*, *Aspergillus fumigatus* and *Mycobacterium tuberculosis* [23].

Recent advances in polyclonal and monoclonal antibody production increased the sensitivity and specificity of immunological assays, resulting in quick identification of foodborne pathogens and toxins [24] [25]. Enzyme-Linked Immunosorbent Assays (ELISA), Lateral Flow Immunoassays and Immunomagnetic Separation Assays are found to be rapid immunological techniques for detection of many foodborne pathogens such as *Salmonella* spp, *E. coli* as well as for botulinum toxins and enterotoxins [23].

Nagy [26] introduced a successful modification of the Matrix-Assisted Laser Desorption/ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) for the identification, typing and antibiotic resistance determination of anaerobes. Mass spectrometry (MS) evaluate the mass: charge ratio of proteins and other compounds. The first effort to apply this technique for the characterization of whole microbes was made in 1975 [26]. Relatively large biomolecules were analyzed in 1980s, due to development in soft-ionization

MALDI-TOF MS. This technique allows the differentiation of bacteria on a specie level due to less influence of culturing conditions [26-28]. Compared with the conventional methods, MALDI-TOF MS devices are simple to operate, and thus can be used by non-MS specialists in microbiological laboratories [29]. Samples are collected by placing the desired colony on target plate with help of sterile pipette tip. After air drying, a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid is applied. Dried samples are covered with matrix solution and dried. Computer software compares the results with reference database which contains data of a variety of clinically important isolates [30 31].

With the advancements in MS- databases, identification of different anaerobes such as *Bacteroides*, *Clostridia*, *Porphyromonas* and *Prevotella* genera have become more rapid. It also allows the differentiation of closely related species, which is barely achievable by phenotypic techniques. Discrimination power of MALDI-TOF MS has also been confirmed by correct identification of 25 clinical and environmental *Clostridium* isolates [7].

Clostridium difficile infection (CDI) is one of the most important nosocomial infection, causing economic burden on health care system worldwide, and is considered as a serious public health threat by the U.S. Center for Disease Control and Prevention [12, 32]. Limitations in laboratory diagnostic capacity has made CDI identification a difficult task in developing countries. A workflow has been proposed by Cheng et al. [33] which shows 100% sensitivity and 92.8% specificity, compared to culture dependent methods. Fecal samples were collected and were tested for glutamate dehydrogenase (GDH) and *C. difficile* toxin A & B (CDAB) by commercial VIDAS kits (bioMérieux). GDH is produced by all strains of *C. difficile*. Use of these commercial kits is an automated and easy approach for rapid detection of *C. difficile*. Use of GDH assay for screening of the suspected CDI fecal samples is the first step of this work flow. GDH positive specimens are then tested CDAB for toxin detection. Negative CDAB results can be referred for molecular detection of toxin genes. This new workflow with a combination of different testing methods is found to be a successful technique which can improve the diagnosis rate from 8.2% to 19.2% with reasonable cost (from US$8 to US $15.6) [33].

Microbial source tracking methods (MST) are also useful tools to track down the source of the contaminant bacteria. Use of host specific PCR is a popular approach of MST. It has been used for determination of fecal pollution, as it detects the genetic markers of fecal microbes specific to a specific host [34].

Many commercial kits, both culture- and molecular based, are also available for rapid detection of clinically important bacteria. Different companies have made effort to launch a variety of commercial products, which work either on culture based or molecular based approach for detection and identification of clinically important anaerobes. Some advanced commercial products including culture Media, Gas generator system, reagent and detection kits have been enlisted in table 2.

This will help lessen the sufferings of the patients along with reduction in mortality rates.

Conclusion

It is evident that the rapid identification of the pathogens is crucial for the timely treatment of the patients and can prove lifesaving. With ever growing illness cases, especially with the spread of multi-drug resistance, it is of utmost concern that more rapid and efficient techniques to culture and detect anaerobes be introduced. Developments in scientific techniques can help reduce detection time of anaerobic pathogens. Likewise, molecular techniques can also be tweaked for the rapid detection of the infectious agents. Moreover, to culture the unculturable pathogens, genomic studies can be helpful to find out their culture requirements.

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Table 1. Some of the important pathogenic bacteria and the diseases that they cause.

S. No.	Pathogens	Diseases (Major manifestation)
1	*Bacillus anthracis*	Anthrax
2	*Bordetella pertussis*	Whooping cough
3	*Borrelia burgdorferi*	Lyme disease
4	*Brucella sp.*	Brucellosis
5	*Campylobacter jejuni*	Diarrhea
6	*Clostridium perfringens*	Gas gangrene
7	*Clostridium tetani*	Tetanus
8	*Escherichia coli (EHEC)*	Bloody diarrhea
9	*Haemophilus influenzae*	Meningitis, Pneumonia, Respiratory tract infections
10	*Helicobacter pylori*	Peptic ulcer
11	*Mycobacterium tuberculosis*	Tuberculosis
12	*Mycobacterium leprae*	Leprosy
13	*Neisseria gonorrhoeae*	Gonorrhea
14	*Neisseria meningitidis*	Meningitis, Sepsis
15	*Salmonella typhimurium*	Typhoid fever
16	*Shigella dysenteriae*	Shigellosis (dysentery, diarrhea)
17	*Staphylococcus aureus*	Skin infections, Meningitis, Pneumonia
18	*Streptococcus pneumoniae*	Pneumonia, Meningitis
19	*Vibrio cholerae*	Cholera
20	*Yersinia pestis*	Plague

Table 2. Some commercial systems to identify medically important anaerobes.

Sr. No	Product name	Catalog number	Product type	Company	Culture based /molecular based	Web link
1	Anaerocult® IS	116819 |	Gas generator system	Merck Millipore	Culture based	http://www.merckmillipore.com/INTL/en/search/-?SearchTerm=*&SingleResultDisplay=SFProductSearch&SearchContextCategoryUUIDs=guKb.qB.0X4AAAFAnhE.1Zwo
2	Anaerocult® A	113829 |	Reagent	Merck Millipore	Culture based	http://www.merckmillipore.com/INTL/en/search/-?SearchTerm=*&SingleResultDisplay=SFProductSearch&SearchContextCategoryUUIDs=guKb.qB.0X4AAAFAnhE.1Zwo

3	Foodproof® *Clostridium botulinum* Detection LyoKit	S 400 08	Detection kit	BIOTECON Diagnostics	Single multiplex real-time PCR reaction	http://www.bc-diagnostics.com/products/kits/real-time-pcr/foodborne-pathogens/foodproof-clostridium-botulinum-detection-lyokit/#detail
4	*C. diff*-Strip	C-1020	Dipstick	CORIS BIOCONCEPT	Molecular based (Ag detection)	http://www.corisbio.com/Products/Human-Field/Clostridium-difficile.php
5	*Clostridium* K-SeT	K-1220	Cassette	CORIS BIOCONCEPT	Molecular based (Ag detection)	http://www.corisbio.com/Products/Human-Field/Clostridium-difficile.php
6	VIT® *Clostridium perfringens*	01110029	Kit	Vermicon	Gene probe technology	http://www.vermicon.com/en/en/products/VIT_Clostridium_perfringens-488
7	Dehydrated and Ready-To-Use Culture Media	-	Culture Media	Merck Millipore	Culture based	https://www.merckmillipore.com/INTL/en/products/industrial-microbiology/pathogen-and-spoilage-testing/pathogen-detection/pathogen-detection-by-product/dehydrated-and-ready-to-use-culture-media/PI2b.qB.phQAAAFAeu5kiQpx,nav
8	Prime Pro qPCR reagent kit	TKIT07005M	Lyophilized kits	Techne	Molecular based	http://www.techne.com/product.asp?dsl=7085
9	Oxoid™ *Clostridium difficile* Test Kit	DR1107A	kit	Thermofisher	Latex agglutination test	https://www.thermofisher.com/order/catalog/product/DR1107A
10	*Clostridium Difficile* Toxin A and B ELISA Kit	ABIN1098188	kit	Antibodies-online	Sandwich ELISA	http://www.antibodies-online.com/kit/1098188/Clostridium+Difficile+Toxin+A+and+B+ELISA+Kit/
11	Cobas *C. diff* test	06768261190	kit	Roche	Molecular based	https://usdiagnostics.roche.com/en/products.html#/n/PARAM3961/pb/AND/c/ar/f/IVD
12	MicroBio µ3D system	-	Fully automated system	Microbio corporation	Equipped with anaerobic conditioning kit	http://www.microbio.co.jp/Eng/index.php?anaerobe_detection
13	Rapid Automated Bacterial Impedance Technique (RABIT)	R01073	direct and indirect impedance measurement system	Don Whitely Scientific	Measure the changing in metabolism of microbes	http://www.dwscientific.co.uk/rabit/
14	Biolog Microbial ID System	91391	Identification system	Biolog	Based on metabolism	http://www.biolog.com/products-static/microbial_identification_overview.php
15	Anaerobic Conditioning Kit	ANA ERO-MB2	Anaerobic cultivation plates	Microbio corporation	Culture based	http://www.microbio.co.jp/Eng/index.php?anaerobic_kit