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Article: **Advancements in Methods Used to Detect and Culture Medically Important Anaerobic Bacteria**

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Advancements in Methods Used to Detect and Culture Medically Important Anaerobic Bacteria

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Abstract

Anaerobic bacteria are among the most important bacteria involved in a number of diseases and infections. These bacteria also cause food borne illnesses. Due to their fastidious nature, culturing anaerobic bacteria is a difficult task. Moreover, anaerobic bacteria can take several days and weeks to grow in laboratory conditions. Besides, most bacteria simply cannot be cultured in laboratories using standard (anaerobic) cultivation techniques known so far. Difficulties in microbiological detection result in delayed diagnosis of the related diseases. Many patients suffer because of the fact that rapid identification is not only difficult, but in many cases, remains 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 diseases and for the safe supply of food. The current article reviews and discusses advanced techniques, both culture-dependent and culture-independent, that allow the rapid detection of important anaerobic bacteria. Advancements in culturing techniques have reduced the time required to grow anaerobic bacteria in laboratories. Whereas, advancements in molecular techniques have enabled the rapid detection of medically important anaerobes including *Clostridium*, *Bacteroides*, and many others.

1. Introduction

Anaerobes are the dominant members of normal human microbiota, predominantly residing on the mucosal membrane 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 numbers 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 culturable anaerobes. However, several other species are uncultivable through conventional laboratory techniques known so far. The breakdown of mucosal barrier can lead to the contamination of anaerobes in the sterile sites of the body, leading to severe infections [1] and causeing 1–17% positive blood cultures [2]. Moreover, they

Table 1. Some Important Pathogenic Bacteria and the Diseases 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

are also involved in food borne infections, lung infections and brain abscesses. According to the Center for Disease Control and Prevention, 179 million people get sick and 6,186 die each year in the United States due to foodborne pathogens [3]. Table 1 shows some of the important pathogenic bacteria and the diseases they cause.

Toxins produced by anaerobic spore formers (*Clostridium* and related genera) are a significant cause of foodborne illnesses. They can be toxigenic, neurotoxigenic or spoilage bacteria. *Clostridium botulinum* and *Clostridium tetani* are neurotoxigenic species. *Clostridium perfringens* is a prominent foodborne pathogen and the second largest cause of food poisoning in the USA, where it causes nearly 1,000,000 cases per annum with the net financial load of US$382 million [4, 5]. It is also responsible for causing diarrhea, avian enteritis necroticans, fulminant disease, *clostridial myonecrosis* and enterotoxemia due to the production of a 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 at species level. The cultivation of strict anaerobes in a microbiology laboratory can be challenging as it demands highly equipped systems with a strict anaerobiosis and a 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. Besides classical methods including selective media and evaluation through rapid tests (such as nitrate disks, spot indole, growth in 20% bile containing

media), commercial kits are also used for their detection. Despite 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 or not identified at all [7, 8, 9]. Updated culture based and molecular based techniques such as 16S rDNA sequencing and QRT-PCR allow the rapid detection and identification of many such anaerobes. Various *Bacteroide* species have been detected from clinical samples using QRT-PCR [10, 11].

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

2. Culture-dependent Techniques

To culture strict anaerobes, techniques capable of rapidly generating a low oxygen (<0.5%) atmosphere are suitable [12]. Anaerobic chambers are used for the generation of such an anaerobic atmosphere, however, it can be expensive for most laboratories. The generation of anaerobic atmosphere using chemical compounds [such as sodium borohydride ($NaBH_4$), sodium bicarbonate-citric acid] is more applicable as compared to using the bulky anaerobic chamber [13]. Such chemical compounds are utilized in the development of a disposable Quick anaero-system for culturing strict anaerobes. The whole system consists of three components. The first is a disposable anaerobic gas pack developed to maintain the absolute anaerobic atmosphere. This component is further subdivided into two subunits. The first is equipped with silica (SiO_2) and $NaBH_4$ tablets. The other 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 also used for pouring tap water. The second component consists of a disposable culture envelope, a sealer and a reusable rack. The third component comprises a catalyst unit which utilizes 10g alumina pellets coated with 0.5% palladium, kept below the roof of the rack. The working of the 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. The efficacy of this system was evaluated by culturing 12 anaerobes in both the Quick anaero-system and the BD GasPak EZ Anaerobe System. While comparing the growth of 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 the Quick anaero-system [14]. For the isolation of anaerobes from rumen, a new medium was used by Kenters et al. [15] to assess the concentration of the inorganic components of rumen, so that it may mimic 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 concentrates are prepared as described by Kenters et al. [15]. Rumen samples are prepared for cultivation and cells are harvested after 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 an aerobic atmosphere. Six clinical anaerobes *Fusobacterium necrophorum*, *Finegoldia magna*, *Prevotella nigrescens*, *Solobactreium moorei*, *Atopobium vaginae* and *Ruminococcus* were cultivated in the Schaedler media supplemented with antioxidants (such as ascorbic acid and glutathione). For all the tubes supplemented with antioxidants growth was observed within 0.3cm of the surface area, although there was no growth in control plates (without ascorbic acid). Thus, the use of antioxidants proved to be a successful method for culturing these clinical anaerobic isolates in an aerobic atmosphere. Moreover, this media also allows the growth of aerobic bacteria. The utilization of antioxidants can be regarded as an easy approach for the cultivation of anaerobes with a regular incubator in an aerobic environment [16].

The replacement of atmospheric oxygen with O_2-free gases along with the use 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 has proved to be an excellent technique for culturing strict anaerobes including methanogens, sulfate reducing bacteria and hydrogen-producing syntrophs by the inoculation of anaerobes in both aerobic and anaerobic conditions. For this purpose, methanogens and sulfate reducing bacteria were used as test organisms. Sterilized W-gellan media (supplemented with reductants) was poured into each well inoculated with culture dilutions (with the ratio of 13:0.1). The plates were covered with lid and kept in a nylon bag equipped with two catalyst sachets (AnaeroPouch). For anaerobic inoculation, the whole method was also executed in an 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 two isolates *Methanoculleu bourgensis* and *Desulfovibrio vulgaris*, CFU values were the same for all three methods. However, for *Methanothermobacter thermautotrophicus*, six-well plate method with anaerobic inoculation gave the highest CFU value [17].

Obligate anaerobes residing in the oral cavity play an important role in transmissible subcutaneous infections by producing ammonia, hydrogen sulfide and other cytotoxic substances. The development of modified trap method with *in vivo* incubation (mini-trap method) by Sizova et al. [20] has made possible 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 using two side plates and tightened with screws. This mini-trap was introduced into a precut window and fixed with superglue in the upper lingual side adjacent to the gum in the oral cavity. After 48 hours of incubation, the apparatus was transferred into an anaerobic glove box and agar plugs were placed in the basic anaerobic media. Microbial cells were collected, dried and examined under Leica DMLB microscope [20].

3. Culture-independent Techniques

Advanced molecular techniques such as target specific probes and Catalyzed

Reporter Deposition- Fluorescent In Situ Hybridization (CARD)-FISH allow 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 were found to be robust and high throughput quantitative techniques used for the identification and characterization of a wide range of gut microbes [22]. Although qPCR is a rapid method for phylogenetic identification, still Rolling Circle Amplification (RCA), Loop Mediated Isothermal Amplification (LAMP), Nucleic Acid Sequence-Based Amplification (NASBA) and Strand Displacement Amplification (SDA) proved to be novel methods for nucleic acid amplification under isothermal conditions, providing better efficiency as compared to the traditional PCR as described by Zhao et al. These methods were found to be better for the 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 have 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 rapid immunological techniques used for the detection of many foodborne pathogens such as *Salmonella* spp, *E. coli*, as well as for botulinum toxins and enterotoxins [23].

A study introduced the successful modification of the Matrix-Assisted Laser Desorption/ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) for the identification, typing and determination of the antibiotic resistance of anaerobes. Mass Spectrometry (MS) evaluates the mass to charge ratio of proteins and other compounds. The first effort to apply this technique for the characterization of the whole microbe was made in 1975 [7]. Relatively large biomolecules were analyzed in 1980s due to the developments in soft-ionization MALDI-TOF MS. This technique allows for the differentiation of bacteria at a specie level due to the limited influence of culturing conditions [26, 27]. Compared with the conventional methods, MALDI-TOF MS devices are simple to operate and can be used by non-MS specialists in microbiological laboratories. Samples are collected by placing the desired colony on the target plate with the 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. Computer software compares the results with the reference database which contains the data of a variety of clinically important isolates [28, 29].

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

Clostridium difficile infection (CDI) is among the most important nosocomial infections. It causes economic burden on

Table 2. Some Commercial Systems Used 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-

Sr. No	Product name	Catalog number	Product type	Company	Culture based /molecular based	Web link
						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	ANAERO-MB2	Anaerobic cultivation plates	Microbio corporation	Culture based	http://www.microbio.co.jp/Eng/index.php?anaerobic_kit

the health care system worldwide and is considered as a serious public health threat by the U.S. Center for Disease Control and Prevention [12, 30]. Limitations in laboratory diagnostic capacity has made CDI identification a difficult task in developing countries. A workflow has been proposed by [31], which shows 100% sensitivity and 92.8% specificity compared to culture dependent methods. Fecal samples were collected and 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*. The use of these commercial kits is an automated and easy approach for the rapid detection of *C. difficile*. The use of GDH assay for the 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 to for the molecular detection of toxin genes. This new workflow with a combination of different testing methods was found to be a successful technique which can improve the diagnosis rate from 8.2% to 19.2% at reasonable cost (from US$8 to US $15.6) [32].

Microbial Source Tracking (MST) methods are also a useful tool to track down the source of the contaminant bacteria. The use of host specific PCR is a popular approach of MST. It has been used to determine fecal pollution as it detects the genetic markers of fecal microbes associated with a specific host [32].

Many commercial kits, both culture and molecular based, are also available for the rapid detection of clinically important bacteria. Different companies have attempted to launch a variety of commercial products, which work either on a culture based or a molecular based approach for the detection and identification of clinically important anaerobes. Some advanced commercial products including culture media, gas generator system, reagent and detection kits are enlisted in Table 2.

4. Conclusion

It is evident that the rapid identification of pathogens is crucial for the timely treatment of patients and can prove lifesaving. With ever growing cases of illnesses, 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 the detection time of anaerobic pathogens. It will also ensure the reduction of mortality rate. Likewise, molecular techniques can also be tweaked for the rapid detection of infectious agents. Moreover, to culture the unculturable pathogens, genomic studies can be helpful to find out their culture requirements.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Bennett JE, Dolin R, Blaser MJ. *Mandell, douglas, and bennett's principles and practice of infectious diseases*: vol. 2. Elsevier Health Sciences; 2014.

[2] Brook I. The role of anaerobic bacteria in bacteremia. *Anaerobe*. 2010;16(3):183-189. https://doi.org/10.1016/j.anaerobe.2009.12.001

[3] Scallan E, Hoekstra RM, Angulo FJ, et al. Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis*. 2011;17(1):7-15. https://doi.org/10.3201/eid1701.P11101

[4] Doyle CJ, Gleeson D, Jordan K, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. Anaerobic sporeformers and their significance

with respect to milk and dairy products. *Int J Food Microbiol*. 2015;197:77-87. https://doi.org/10.1016/j.ijfoodmicro.2014.12.022

[5] Scharff RL. Economic burden from health losses due to foodborne illness in the United States. *J Food Prot*. 2012;75(1):123-131. https://doi.org/10.4315/0362-028X.JFP-11-058

[6] Uzal FA, Freedman JC, Shrestha A, et al. Towards an understanding of the role of Clostridium perfringens toxins in human and animal disease. *Future Microbiol*. 2014 Mar;9(3):361-377. https://doi.org/10.2217/fmb.13.168

[7] Nagy E. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: a new possibility for the identification and typing of anaerobic bacteria. *Future Microbiol*. 2014;9(2):217-33. https://doi.org/10.2217/fmb.13.150

[8] Hernández-Macedo ML, Barancelli GV, Contreras-Castillo CJ. Microbial deterioration of vacuum-packaged chilled beef cuts and techniques for microbiota detection and characterization: a review. *Braz J Microbiol*. 2011;42:1-1. https://doi.org/10.1590/S1517-83822011000100001

[9] Frank D, Zhang Y, Li Y, et al. Shelf life extension of vacuum packaged chilled beef in the Chinese supply chain. A feasibility study. *Meat Sci*. 2019;153:135-143. https://doi.org/10.1016/j.meatsci.2019.03.006

[10] Tong J, Liu C, Summanen P, Xu H, Finegold SM. Application of quantitative real-time PCR for rapid identification of Bacteroides fragilis group and related organisms in human wound samples. *Anaerobe*. 2011;17(2):64-68. https://doi.org/10.1016/j.anaerobe.2011.03.004

[11] Niestępski S, Harnisz M, Korzeniewska E, Osińska A. Markers Specific to Bacteroides fragilis Group Bacteria as Indicators of Anthropogenic Pollution of Surface Waters. *Int J Environ Res Public Health*. 2020;17(19):7137. https://doi.org/10.3390/ijerph17197137

[12] Jones AM, Kuijper EJ, Wilcox MH. Clostridium difficile: a European perspective. *J Infect*. 2013;66(2):115-28. https://doi.org/10.1016/j.jinf.2012.10.019

[13] Imhof A, Heinzer I. Continuous monitoring of oxygen concentrations in several systems for cultivation of anaerobic bacteria. *J Clin Microbiol*. 1996;34(7):1646-8. https://doi.org/10.1128/jcm.34.7.1646-1648.1996

[14] Yang NW, Kim JM, Choi GJ, Jang SJ. Development and evaluation of the quick anaero-system-a new disposable anaerobic culture system. *Korean J Lab Med*. 2010;30(2):133-7. https://doi.org/10.3343/kjlm.2010.30.2.133

[15] Kenters N, Henderson G, Jeyanathan J, Kittelmann S, Janssen PH. Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *J Microbiol Methods*. 2011;84(1):52-60. https://doi.org/10.1016/j.mimet.2010.10.011

[16] La Scola B, Khelaifia S, Lagier JC, Raoult D. Aerobic culture of anaerobic bacteria using antioxidants: a preliminary report. *Euro J Clin Microbiol Infect Dis*. 2014;33(10):1781-3.

https://doi.org/10.1007/s10096-014-2137-4

[17] Nakamura K, Tamaki H, Kang MS, et al. A six-well plate method: less laborious and effective method for cultivation of obligate anaerobic microorganisms, *Microbes Environ.* 2011;26:301-306. https://doi.org/10.1264/jsme2.ME11120

[18] Summanen PH, McTeague M, Väisänen ML, Strong CA, Finegold SM. Comparison of recovery of anaerobic bacteria using the Anoxomat®, anaerobic chamber, and GasPak® jar systems. *Anaerobe*. 1999;5(1):5-9. https://doi.org/10.1006/anae.1999.0184

[19] Hungate RE. Chapter IV A roll tube method for cultivation of strict anaerobes. In*Methods in microbiology.* 1969 (Vol. 3, pp. 117-132). Academic Press.

[20] Sizova MV, Hohmann T, Hazen A, Paster BJ, Halem SR, Murphy CM, Panikov NS, Epstein SS. New approaches for isolation of previously uncultivated oral bacteria. *Appl Environ Microbiol*. 2012;78(1):194-203.

[21] Vartoukian SR, Palmer RM, Wade WG. Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol Lett*. 2010;309(1):1-7. https://doi.org/10.1111/j.1574-6968.2010.02000.x

[22] Fraher MH, O'toole PW, Quigley EM. Techniques used to characterize the gut microbiota: a guide for the clinician. *Nat Rev Gastroenterol Hepatol*. 2012;9(6):312-22. https://doi.org/10.1038/nrgastro.2012.44

[23] Zhao X, Lin CW, Wang J, Oh DH. Advances in rapid detection methods for foodborne pathogens. *J Microbiol Biotechnol*. 2014;24(3):297-312.

[24] Leonard P, Hearty S, Brennan J, et al. Advances in biosensors for detection of pathogens in food and water. *Enzyme Microbial Technol*. 2003;32(1):3-13. https://doi.org/10.1016/S0141-0229(02)00232-6

[25] Rubab M, Shahbaz HM, Olaimat AN, Oh DH. Biosensors for rapid and sensitive detection of Staphylococcus aureus in food. *Biosens Bioelectron*. 2018;105:49-57. https://doi.org/10.1016/j.bios.2018.01.023

[26] Holland RD, Wilkes JG, Rafii F, et al. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ ionization with time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom*. 1996;10(10):1227-32.

[27] Krishnamurthy T, Ross PL. Rapid identification of bacteria by direct matrix-assisted laser desorption/ ionization mass spectrometric analysis of whole cells. Rapid *Commun Mass Spectrom*. 1996; 10(15):1992-6.

[28] Seng P, Rolain JM, Fournier PE, La Scola B, Drancourt M, Raoult D. MALDI-TOF-mass spectrometry applications in clinical microbiology. *Future Microbiol*. 2010 Nov;5(11) :1733-54. https://doi.org/10.2217/fmb.10.127

[29] Kostrzewa M, Nagy E, Schröttner P, Pranada AB. How MALDI-TOF mass spectrometry can aid the diagnosis of hard-to-identify pathogenic bacteria–the rare and the unknown. *Expert Rev Mol Diagn*. 2019;19(8):667-82.

https://doi.org/10.1080/14737159.2019.1643238

[30] Grosse-Herrenthey A, Maier T, Gessler F, Schaumann R, Böhnel H, Kostrzewa M, Krüger M. Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization–time-of-flight mass spectrometry (MALDI–TOF MS). *Anaerobe*. 2008;14(4):242-9. https://doi.org/10.1016/j.anaerobe.2008.06.002

[31] Cheng JW, Xiao M, Kudinha T, et al. The role of glutamate dehydrogenase (GDH) testing assay in the diagnosis of Clostridium difficile infections: a high sensitive screening test and an essential step in the proposed laboratory diagnosis workflow for developing countries like China. *Plos one*. 2015;10(12):e0144604. https://doi.org/10.1371/journal.pone.0144604

[32] Harwood VJ, Staley C, Badgley BD, Borges K, Korajkic A. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS Microbiol Rev*. 2014;38(1):1-40. https://doi.org/10.1111/1574-6976.12031