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Critically Appraised Topic

A clinically orientated procedure for the workup of anaerobic bacteria in the era of MALDI-TOF: feasible or fiction?

Clinical bottom line

Currently there is no uniform Belgian laboratory approach concerning anaerobic microbiology practices. A variety in identification and reporting procedures regarding anaerobic bacteria was observed. Organizations like the ESCMID Study Group for Anaerobic Infections (ESGAI) are trying to harmonize European practices in anaerobic microbiology, centralizing the new matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) method in their workup schemes.

Our multi-center retrospective case study pointed out that antimicrobial therapy regarding anaerobic infections was mainly started empirically without further adaptations. Reporting anaerobic identification and anti-microbial susceptibility testing (AST) results timely and completely did not result in higher starting rates of anaerobic anti-microbial therapy. However clinicians did start significantly more anti-microbial agents based on anaerobic identification or AST results if results were reported timely, completely and with AST. Impact on patients outcome, duration of hospitalization or anti-microbial therapy of anaerobic culturing reports could not be demonstrated.

General introduction and research motives

Anaerobic bacteria are the most prevalent forms of life in the human body. While their pathogenic role was well established at the turn of the 20th century, they were, and still are often neglected. During the past decades, there has been a changing interest in anaerobic infections, based upon verification of their role as pathogens by recovery from infected sites, efficacy of antibiotic treatment, and elucidation of virulence factors. Debates are ongoing on how far laboratories should go in identifying anaerobic bacteria. It is unknown how Belgian laboratories organize their workup of anaerobic cultures and whether or not important differences exist in their practical approach. The introduction of MALDI-TOF also brought major changes in anaerobic microbiology. Using this new identification method, many anaerobic species appear for the first time on laboratory reports, sent to clinicians. Questions about clinical relevance, the need to report and fully identify all these different anaerobic species arise, especially in mixed anaerobic cultures. Currently there are no guidelines suggesting feasible workup schemes for anaerobic cultures, using the new MALDI-TOF identification method. On the other hand we do not know if anaerobic culture results influence clinician's decision making.

Questions

a) **Survey on the procedures for identification, susceptibility testing and reporting of anaerobic bacteria: observing the differences.**

b) **Clinical impact of anaerobic cultures: Multi-center retrospective case study of anaerobic cultures.**

Search terms

[A] MeSH Database (PubMed): MeSH term: "Clinical anaerobic microbiology", "Anaerobic bacteria and clinical relevance", "Workup anaerobic microbiology", "Anaerobic infections and clinical relevance", "Anaerobic bacteria and susceptibility", "Anaerobic infections and management", "Anaerobic bacteria and virulence", "Anaerobic bacteria and anti-microbial susceptibility testing", "Anaerobic infections and outcome"

[B] PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters

[C] UpToDate Online version 23.3 (2015): "Anaerobic infections", "Anaerobic microbiology"

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Appraisal

a) Survey on the procedures for identification, susceptibility testing and reporting of anaerobic bacteria: observing the differences

A survey on the practical approach of anaerobic microbiology was sent to 53 Belgian hospital laboratories. A response degree of 64% (34 laboratories) was achieved. The web-based survey consisted of fifteen questions. For each question we give an overview of the answers, followed by recommendations from guidelines and literature.

Question 1: From which samples do you perform anaerobic cultures?

1. Results

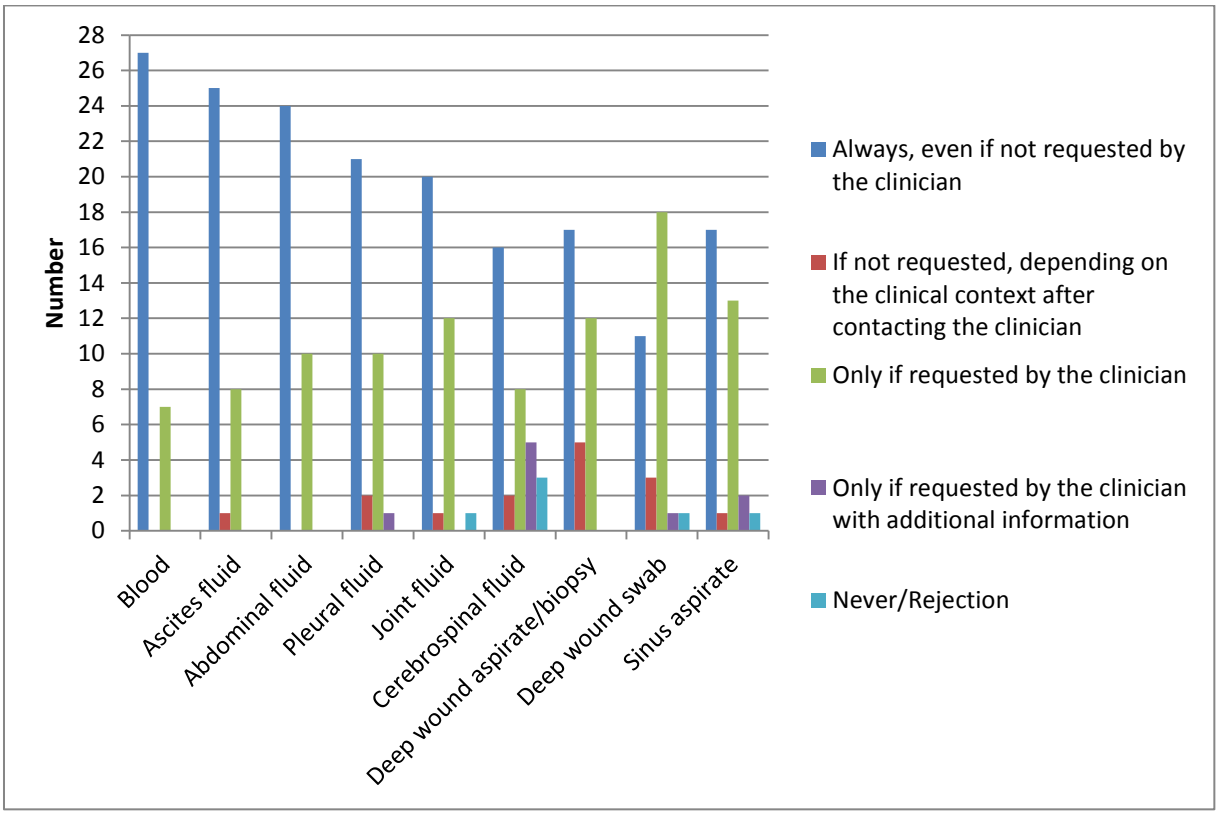


Figure 1: Generally accepted sample types for anaerobic culture (rejection ≤ 10%).

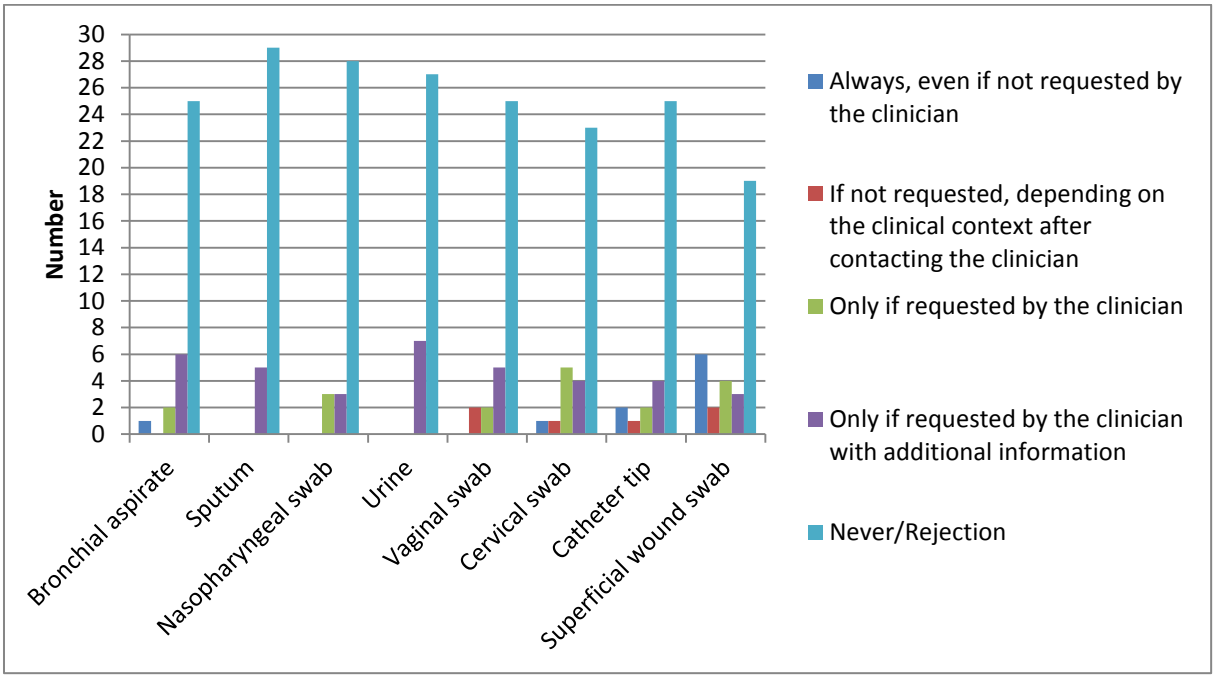


Figure 2: Generally rejected sample types for anaerobic culture (rejection ≥ 50%).

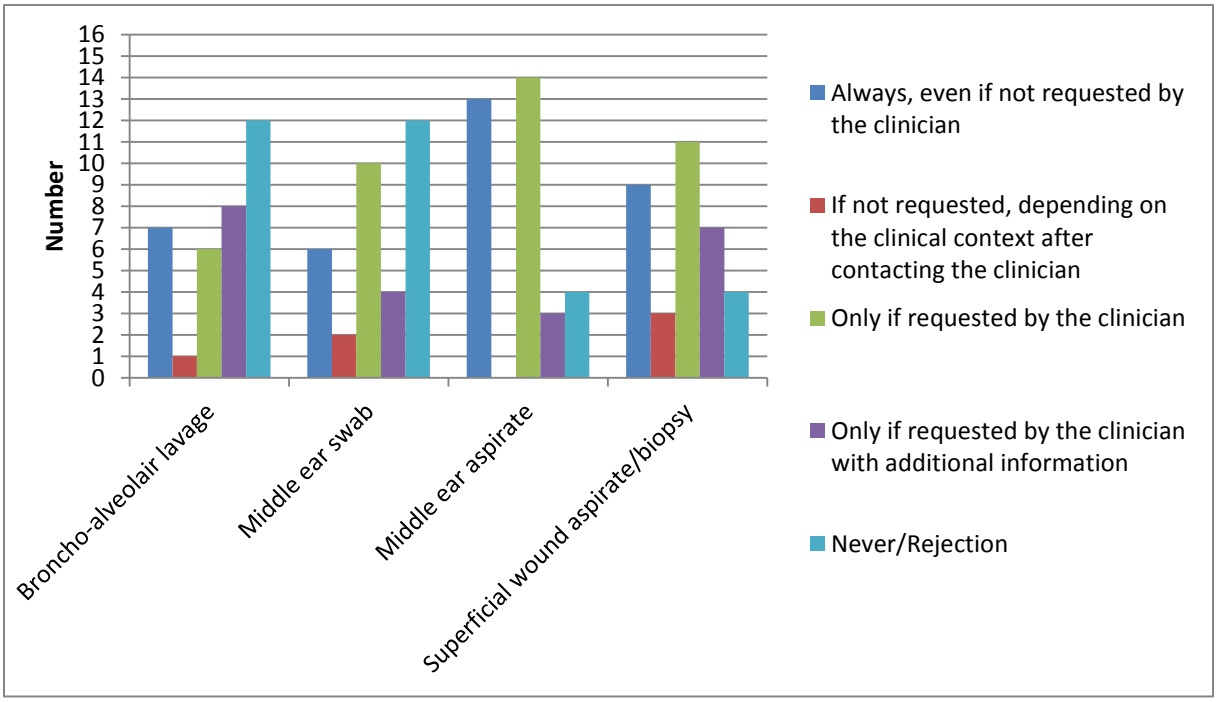


Figure 3: Different opinions exist for these sample types (10% < rejection > 50%).

All participating laboratories answered that culture of anaerobic bacteria is performed on certain sample types while other sample types are refused. All laboratories accept blood, deep wound aspirates/biopsies and most sterile fluids (pleural, ascites and abdominal) for anaerobic culture. Only three percent rejects sinus aspirates, deep wound swabs and joint fluids and nine percent rejects cerebrospinal fluids for culture of anaerobes. Only a minority accepts bronchial aspirates (26%), sputum (15%), nasopharyngeal swabs (18%), urine (21%), vaginal (26%) and cervical (32%) swabs, catheter tips (26%) and superficial wound swabs (44%). There are different opinions concerning rejection or acceptance of broncho-alveolar lavages, middle ear swabs or aspirates and superficial wound aspirates or biopsies. These sample will be discussed in detail.

2. Guidelines and literature

Two questions are important in deciding whether a sample should be rejected or accepted (Extract 1). Is the sample received in an appropriate recipient (correct sample type)? Is this sample originating from a relevant anatomical body site were anaerobic bacteria could have an important role in the ongoing infection?

Anaerobes	
Specimen for anaerobes not received in appropriate container	Notify physician or charge nurse and request properly handled specimen. If physician insists specimen be processed, refer to supervisory personnel or comment in laboratory record and in report form that inappropriate transport may have influenced recovery of significant anaerobic bacteria.
Anaerobic cultures requested on autopsy material; bronch wash; decubitus ulcer material (not punch biopsy of tissue beneath eschar); drain; drain site; environment; exudate; feces; gastric washing (other than newborn); midstream or catheterized urine; mouth, nose, or prostatic secretions; sputum material on swabs from ileostomy or colostomy; fistual or intestinal contents; throat or vaginal secretions	Inform physician or charge nurse that, as detailed in laboratory manual, these specimens are not cultured for anaerobic bacteria, since these anatomic sites harbor anaerobes normally and usually. If physician insists, refer him/her to supervisory personnel.

Extract 1: Rejection criteria for anaerobic microbiological specimens (*1*).

Aspirates collected with needle and syringe, chirurgical obtained tissues and biopsies are considered most suitable specimens for culturing anaerobic bacteria (Table 1). The use of swabs is discouraged. Only if no suitable sample type can be obtained (e.g. animal bite wounds involving a small puncture and abscess), swabs can be accepted. Swabs absorb small sample volumes and many organisms tend to adhere to the fibers, reducing the probability that pathogenic and relevant anaerobic species are cultured. (*1-3*). Furthermore, swabbing wounds, ulcers or mucous membranes increases the recovery of non-relevant normal microbiota (*1, 3, 4*). Microbiologists should give a remark on the anaerobic result if clinicians insist on performing anaerobic culture from inappropriate sample types (*1*). If inappropriate specimens cannot be replaced (e.g. brain abscess, fully drained abscess), they should be processed anyway with a comment on the laboratory report, warning for incorrect collection or transportation (*2*). Precious samples (e.g. cerebrospinal fluid (CSF), joint and other sterile body fluids obtained by aspiration, tissues and/or biopsies) should never be discarded without culturing before discussion with the clinician (*5*).

Table 1: Acceptable specimen types for anaerobic culture (*1*).

Site	Acceptable specimens	Unacceptable specimens
Head and neck	Abscess aspirate obtained by needle and syringe after surface decontamination Biopsy material surgically obtained	Throat or nasopharyngeal swabs Gingival swabs Superficial material collected with swabs
Lungs	Transtracheal aspirate Material from percutaneous lung puncture Biopsy material surgically obtained Bronchoscopic specimen obtained by protected brush Thoracotomy specimen	Expectorated sputum Induced sputum Endotracheal aspirate Bronchoscopic specimens not specially collected
Central nervous system	Abscess aspirate obtained by needle and syringe Biopsy material surgically obtained	Aerobic swabs
Abdomen	Peritoneal fluid obtained by needle and syringe Abscess aspirate obtained by needle and syringe Bile Biopsy material surgically obtained	Aerobic swabs
Urinary tract	Suprapubic aspirate	Voided urine Catheterized urine
Female genital tract	Culdoscopy specimens Endometrial aspirate obtained by suction or protected collector Abscess aspirate obtained by needle and syringe Biopsy material surgically obtained IUD[a] for *Actinomyces* species	Vaginal or cervical swabs
Bone and joint	Aspirate obtained by needle and syringe Biopsy material surgically obtained	Superficial material collected with swabs
Soft tissue	Aspirate obtained by needle and syringe Biopsy material surgically obtained Aspirate from sinus tract obtained by needle and small plastic catheter Deep aspirate of open-wound margin obtained through decontaminated skin Deep aspirate of surface ulcer obtained through decontaminated skin	Superficial material collected from skin surface or edges of wound

[a] IUD, intrauterine device. Culture of an IUD for *Actinomyces* is controversial. *Actinomyces* spp. are normal organisms of the genitourinary tract microbiota, and their isolation may not always represent infection. If cultures are done, results should always be correlated with pathological tissue findings (2, 3, 9, 13).

Garcia specifies which samples should be rejected based on anatomical origin (Extract 1). These samples are taken from body sites, always harboring endogenous non-relevant anaerobic bacteria.

Concerning pulmonary samples, Garcia, Wadsworth and M56-A Clinical and Laboratory Standards Institute (CLSI) guidelines clearly state that bronchial washings including broncho-alveolar lavages and all other respiratory samples, not collected through a double-lumen catheter are totally inappropriate for anaerobic culture. The only pulmonary samples appropriate for anaerobic culture, are transtracheal aspirates, material from percutaneous lung punctures, surgically obtained lung biopsies and protected brush bronchoscopic samples (*1-3*).

Garcia, UK National Health Services (NHS) and Versalovic state that tympanocentesis through an intact ear drum and culturing middle ear fluid is a valuable tool for definitive diagnosis, to guide

therapy or to evaluate therapy failure in otitis media (*1, 6, 7*). However, diagnosis is usually made on clinical grounds and tympanocentesis should be reserved for complicated, recurrent or chronic persistent middle ear infections. These guidelines agree that middle ear fluids should be considered as normally sterile and anaerobic culture should always be performed. Garcia does not mention swabs for diagnosing otitis media (*1*). A NHS guideline, concerning ear infections, states that middle ear swabs are not useful in investigating otitis media unless there is eardrum perforation. While anaerobic bacteria are rarely involved in acute otitis media, anaerobes can be cultured from middle ear effusions in up to 25% of the patients with chronic otitis media (*6*). Thus anaerobic culture should only be performed on swabs from patients with chronic otitis media and eardrum perforation.

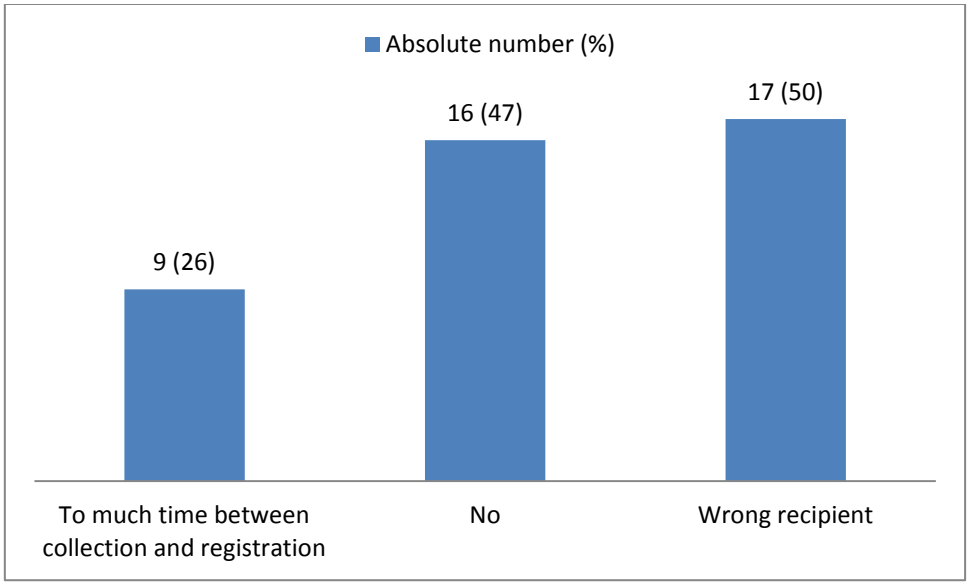
Superficial swabs taken from wounds, decubitus or other deep ulcers are generally considered as inappropriate for culture of both aerobic and anaerobic bacteria due to lack of concordance between bacteria at the surface and etiological bacteria in the depth (*1-4*). This statement was confirmed in two recently published studies using 16S rRNA and pyrosequencing on samples from decubitus and other ulcers (*8, 9*). The study results suggest that traditional culturing methods may be extremely biased as they select for organisms which grow rapidly in pure culture such as staphylococci. Culturing failed to identify major pathogens, especially anaerobes. Anaerobic microbiology should be performed on superficial biopsy or aspirated specimens after decontamination of the area (*2-4*). Garcia does not agree with this statement and says that anaerobic culture is only required for aspirates or biopsies of wounds and abscesses extending deeper than the dermis. Garcia specifies relevant deep abscess and wound sites requiring anaerobic culturing (Table 2)(*1*).

Table 2: Relevant types of deep and superficial abscesses and wounds (*1*).

Specimen types	Site or source	Comments
Abscess		
Superficial	Boils, furuncles, infected cysts, skin abscesses, superficial surgical wounds	Defined as an infected space that may drain through the skin but does not extend deeper than the dermis; aerobic culture only required.
Deep	Any site, including deep tissues; usually related to secondary infection of a deep wound, contusion, or hematoma	Defined as a closed infected space that extends deeper than the dermis into deep tissues whose cavity may be encapsulated; aerobic and anaerobic cultures required.
Wound		
Superficial	Abrasion, cut, laceration, or ulcer (any site), plus associated skin diseases (impetigo, folliculitis, cellulites) or burns	Defined as a wound in the skin that does not extend deeper than the dermis; aerobic culture only required
Deep	Typically applies to deep surgical wounds that go across a mucosal surface (e.g., abdominal, pelvic, or chest), bite wounds, deep traumatic wounds (e.g., gunshots, stabs, punctures) third-degree burns due to electrocution	Defined as a wound that penetrates deeper than the dermis of the skin or is located in deep tissues

Question 2: Do you use other rejection criteria besides sample type for performing anaerobic culture?

1. **Results**



In our survey eighteen laboratories (53%) use additional rejection criteria besides sample type. Seventeen laboratories (50%) reject samples in an unsuitable recipient. Nine (26%) laboratories reject samples when too much time has passed between collection and registration (time not specified).

2. **Guidelines and literature**

Oxygen is toxic for obligate anaerobic bacteria. This is why clinical specimens are ideally transported to the laboratory as quickly as possible using an oxygen-free, pre-reduced anaerobically sterilized (PRAS) transport tube or vial (*1-3*). Anaerobes vary in the conditions they require for survival. In accordance with their oxygen sensitivity, some organisms are classified as "moderately sensitive" (*Bacteroides fragilis*, *Prevotella oralis*, *Prevotella melaninogenica*, *Fusobacterium nucleatum* and *Clostridium perfringens*) or as "extremely sensitive" (some *Bacteroides fragilis* strains, *Peptostreptococcus spp.*) for oxygen (*10, 11*).

The maximum transportation time to the laboratory depends on sample volume, recipient and sample type (Table 3) (*1*). The survival of anaerobic bacteria is better in large aspirated volumes (> 2 mL) or big lumps of tissue. Anaerobic bacteria also survive longer in pus or tissue and less in swabs. The use of specific anaerobic collection/transport media can prolong the transportation time, raising the yield of anaerobic culture (Table 4).

Table 3: Suggested transport times for certain specimen volumes and collection methods (*1*).

Specimen type	Optimal time for transport to laboratory	Additional comment(s)
Aspirated material		
Very small vol (<1.0 ml)	≤10 min	Transport small vol of aspirated material in anaerobic transport vial whenever possible for best possible results.
Small vol (~1.0 ml)	≤30 min	
Large vol (>2.0 ml)	≤2–3 h	Transport large vol of purulent material; large pieces of tissue; or aspirated material, tissue, biopsy material, or curettings in an anaerobic transport medium or container.
In anaerobic transport device	≤2–3 h	These specimens can generally be accepted for anaerobic culture with good results even after a delay of 8–24 h. Include comment regarding transport delay in report when these cultures are processed.
Tissue or biopsy material		
In sterile container	≤30 min	
In anaerobic bag or transport device	≤2–3 h	
Anaerobic swabs		
In tube with moist anaerobic atmosphere	≤1 h	
In anaerobic transport medium	≤2–3 h	

Table 4: Anaerobic specimen transport devices (*1*).

Specimen type	Transport system	Commercially available systems[a]
Aspirated material	Vial or tube with anaerobic atmosphere and agar base with indicator system	Port-A-Cul tube or vial (BD) Anaerobic Transport Medium (Anaerobe Systems) A.C.T. transport tube (Remel)
Tissue, biopsy material, or curettings	Bag systems that act by removing molecular oxygen or tube systems that permit material to be added	Bio-Bag (type A) (BD Biosciences) Anaerobic Transport System (Anaerobe Systems) A.C.T. transport tube (Remel) AnaeroGen (Oxoid) AnaeroPouch (Mitsubishi) Venturi Transystem (Copan) Port-A-Cul widemouthed jar (BD Biosciences)
Specimen collected on swabs	Tube with anaerobic atmosphere and agar base with indicator system, or tube with anaerobic atmosphere and reduced transport medium	Anaerobic Transport System (Anaerobe Systems) A.C.T. transport tube (Remel) Port-A-Cul (BD Biosciences) Venturi Transystem Vi-Pak Amies (Copan Diagnostics)

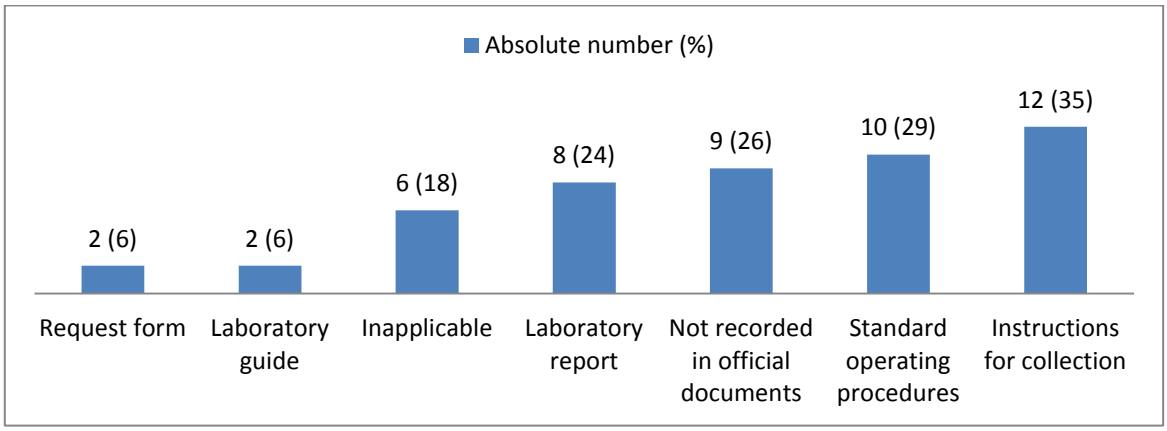
[a] Partial list of suppliers.

A major drawback of these specific media is the cost (e.g. €19,25 incl. VAT for one Port-A-Cul® Transport Jar with screw cap for tissues and biopsies). Cheaper alternatives are syringes and anaerobic blood culture bottles. Guidelines do not recognize these alternatives as good transportation media for anaerobic bacteria because they can cause spillage of the content, there is a potential danger of needle stick injuries and oxygen can diffuse into plastic syringes (*1-3*). If syringes are used, they should at least be capped and contain a considerable amount of aspirate (> 2 mL) with an airspace above the fluid level as small as possible and be kept upright to avoid mixing with air. These recommendations are not official.

Laboratories not using specific transport media should introduce rejection criteria based on transportation time. However the difficulty remains monitoring time between sampling and delivery in the laboratory.

Question 3: In which documents do you mention rejection criteria for anaerobic culture?

1. Results



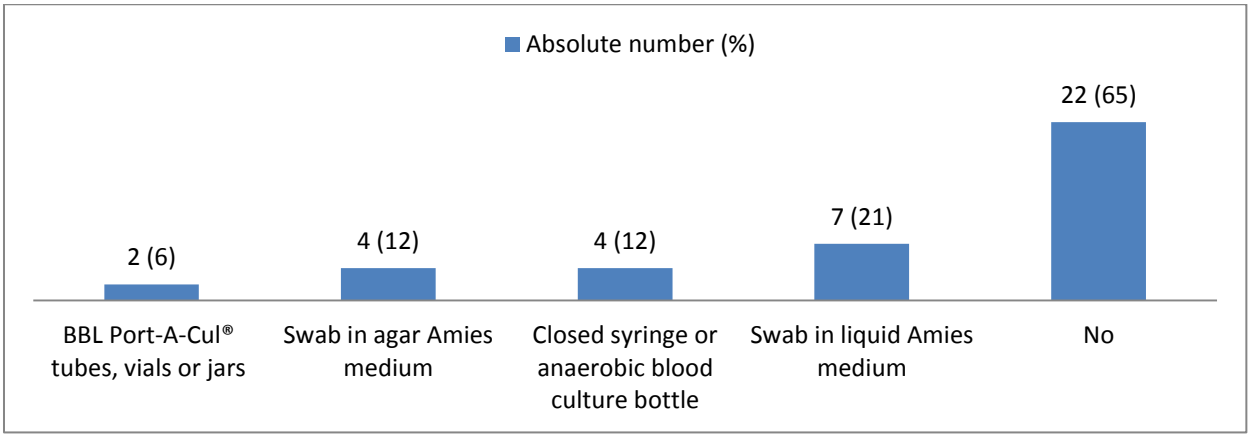
Only nineteen laboratories (56%) describe rejection criteria for performing anaerobic culture in official documents, included in the quality system. Most of these laboratories mention rejection criteria in the instructions for sample collection (35%) or in standard operating procedures for lab technicians (29%). Eight laboratories (24%) mention sample rejection or comment on inappropriate sampling on their reports for clinicians. Only a few laboratories mention rejection criteria on their request form (6%) or in their laboratory guide (6%), containing an overview of available tests.

2. Guidelines and literature

CLSI recommends written guidelines to clinicians for appropriate anaerobic specimens along with proper selection, collection and transport. When a specimen is rejected by the laboratory there should be a protocol to notify the clinician immediately of the rejection and the need for a specimen replacement and a comment on the suitability of the sample (*2*).

Question 4: Do you use specific anaerobic collection or transport media?

1. Results



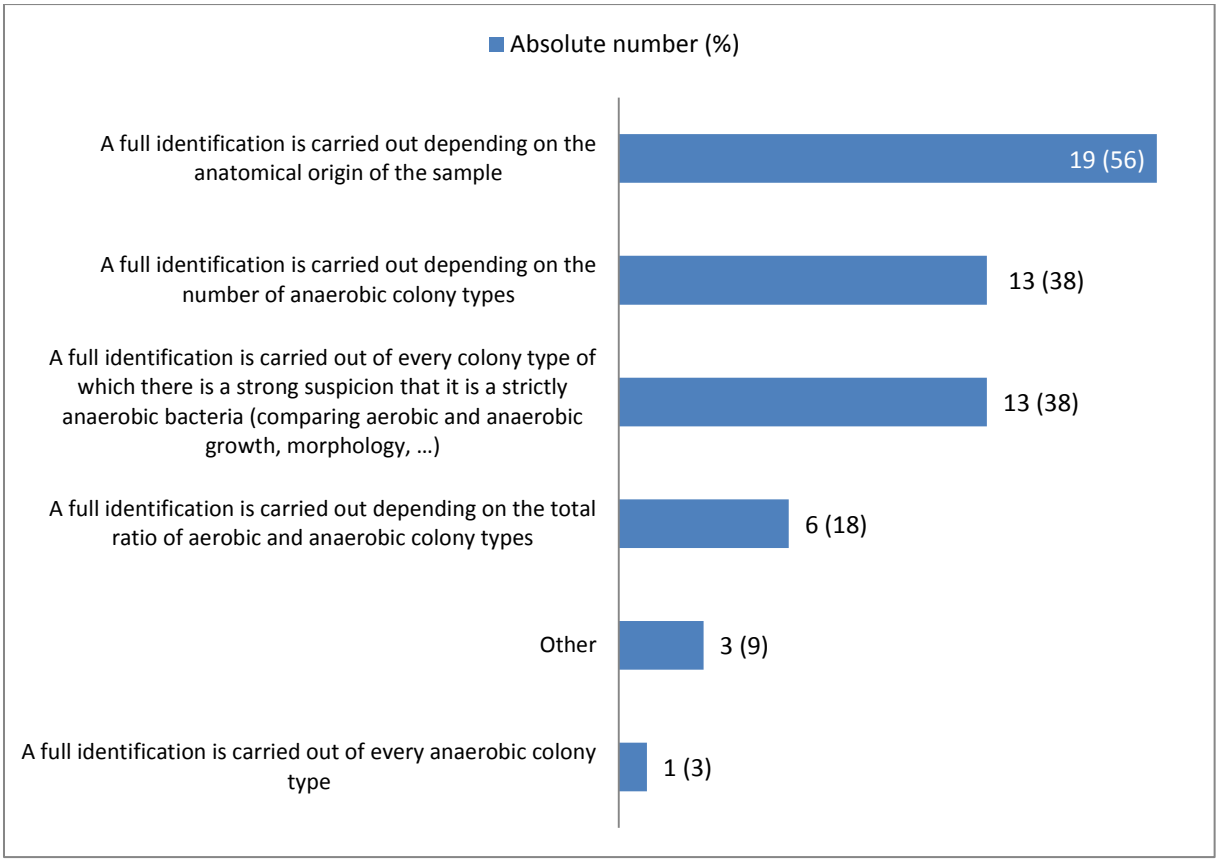
Most participating laboratories (65%) do not use specific anaerobic transport media for anaerobic cultures. Laboratories mainly use anaerobic swab systems (33%), a closed syringe or anaerobic blood culture bottle (12%). Only a few laboratories (6%) use specific tubes, vials or jars containing a pre-reduced transport medium with reducing agents. Liquid Amies medium is the most frequently used transport medium for anaerobic culture samples in this survey (21%).

2. Guidelines and literature

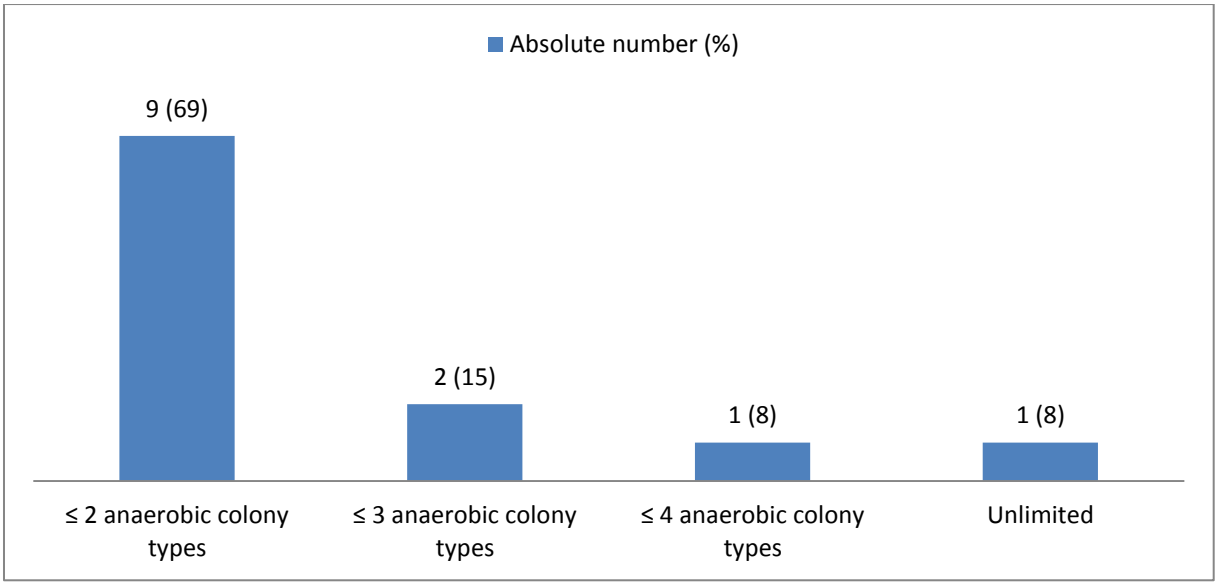
Although liquid Amies medium is not recognized as specific transport medium for anaerobic culture samples in official guidelines (*1, 3*), sufficient evidence shows that liquid and agar Amies medium are suitable for the transportation of swab samples containing aerobic and anaerobic bacteria (*12-16*). The CLSI M40-A document was used recently in two studies to evaluate the Copan e-Swab® liquid Amies swab for maintenance of bacterial viability (*15, 16*). Both studies concluded that the number of recovered CFU's decreases after six hours, and some aero-intolerant anaerobic species (*Peptostreptococcus anaerobius*, *Prevotella melaninogenica* and *Fusobacterium nucleatum*) could not be recovered after 24 hours. Aero-tolerant *Actinomyces spp.*, *Propionobacterium spp.*, some *Clostridium spp.* and *Bacteroides spp.* appeared to be more stable (> 24 hours). Laboratories using swabs in Amies medium should be cautious, since these studies indicate that fastidious and low inoculum anaerobic bacteria might be missed after longer storage and transport (> 4-6 hours) (*14-16*), especially if they are kept at room temperature (*16*). However Garcia and CLSI do recommend storage at room temperature (*1, 2*).

Question 5: When do you perform a full identification (species level) of cultured anaerobic colony types?

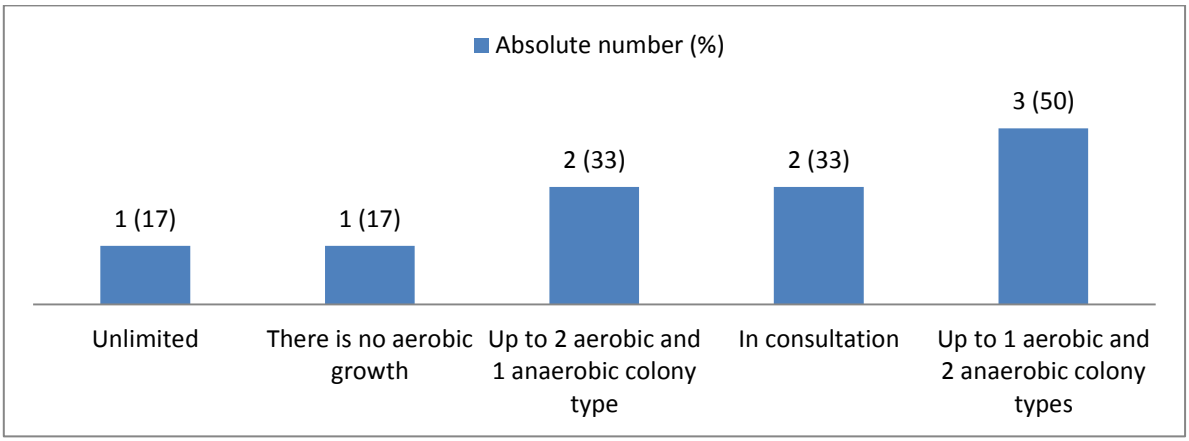
1. Results



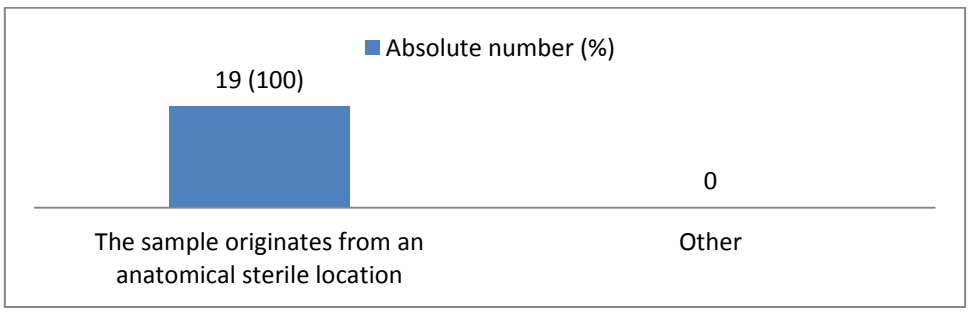
Additional question 5A for thirteen laboratories: *Up to how many anaerobic colony types would you fully identify in one sample?*



Additional question 5B for six laboratories: *Which identification ratio is used per sample if you take both aerobic and anaerobic colony types into account?*



Additional question 5C for nineteen laboratories: *Which anatomical locations give rise to a full identification of anaerobic colony types?*



In our survey all laboratories have facilities to culture and isolate anaerobic bacteria. Nineteen laboratories (56%) will perform a full identification of an anaerobic colony type depending on the anatomical origin of the sample. All these laboratories would fully identify anaerobic colony types

from samples originating from an anatomical sterile location. A full identification is carried out depending on the number of anaerobic colony types by thirteen laboratories (38%) of which the majority (69%) limits full identification up to two anaerobic colony types per sample. Thirteen laboratories (38%) perform a full identification of every colony type, strongly suspicious for being a strictly anaerobic bacteria and this by comparing aerobic and anaerobic growth on culture plates, typical morphology, odor,… . Six laboratories (18%) make a ratio between the number of aerobic and anaerobic colony types, three of these laboratories (50%) will fully identify up to one aerobic and two anaerobic colony types. Only one laboratory will identify every anaerobic colony type.

2. Guidelines and literature

There are ongoing discussions concerning how extensive laboratories should work out anaerobic cultures. When a laboratory determines the extent of anaerobe bacteriology that will be provided, it performs a risk assessment. The CLSI states that hospital laboratories not providing resources for isolation of anaerobic bacteria imply an extreme risk for potential patient harm (*2*). However they do not refer to relevant studies, supporting this statement.

Citron and Appelbaum state that samples from the central nervous system, ocular, serious pulmonary, liver abscesses and serious soft tissue infections (such as gas gangrene), osteomyelitis and infections in any normally sterile body site (anatomical location not in direct contact with a colonized mucosal surface) ask for a complete workup, independent of the number of cultivated bacteria (*17*). A Cumitech document dating from 1991 suggests that the extent of anaerobe identification depends on the clinical situation. Bacteremia, tubo-ovarion and lung abscesses, serious skin, soft tissue, bone and joint infections and every infection not responding to antimicrobial therapy require full identification of all anaerobic colony types (*18*).

If only anaerobic bacteria are grown from clinical samples , a complete identification is warranted (*1-3, 17*). If a mixed aerobic/anaerobic flora is likely to cause the infection, some believe full identification is not necessary (1, 16, 17). In these situations the extent of isolation and identification should be based on the diversity of growth. If five or more anaerobic colony types appear to be present (including facultative organisms), no clinical benefit in identifying individual isolates exists and mixed flora may be reported (*5*). Many laboratories limit identification of poly-microbial infections to less than four isolates (*2*). This approach may overlook the fact that all organisms grown from a relevant specimen may be involved in the infection and that some can be resistant for anti-microbial agents. Because many anaerobic infections from non-sterile sites are poly-microbial and bacterial constituents may act synergistically, identification of all colony types is advised by some experts (*2*). Only if a full identification is performed, information about virulence, resistance and primary site of infection can be judged correctly. The poly-microbial nature of abdominal, pelvic, skin and soft tissue abscesses is apparent in the majority of patients. The number of isolates varies between two and six (*19*). The average number of isolates is 3,6 in skin and soft tissue infections (2,6 anaerobes and one aerobe) per specimen (*20-22*), five in intra-abdominal infections (three anaerobes and two aerobes) per specimen (*23-27*) and four in pelvic infections (2,8 anaerobes and 1,2 aerobes) per specimen (*28, 29*). The results of these studies are however difficult to compare and reproduce because collection, transportation and cultivation methods differ among the studies. Also newer, more sensitive techniques like 16S rRNA or next generation sequencing should be used to

reveal the real microbiological flora of abscesses. For brain and dental abscesses these studies already exist (*30-32*), revealing more diverse anaerobic flora then conventional culturing methods.

Careful examination of the primary culture combined with good knowledge of colony morphology remains crucial even if advanced identification methods like MALDI-TOF are used. Anaerobic organism indications from primary culture plates include colony size, color, shape, density, hemolysis, pitting, pigmentation and smell. Garcia and CLSI give examples of typical clinically relevant anaerobic colony types (*1, 2*).

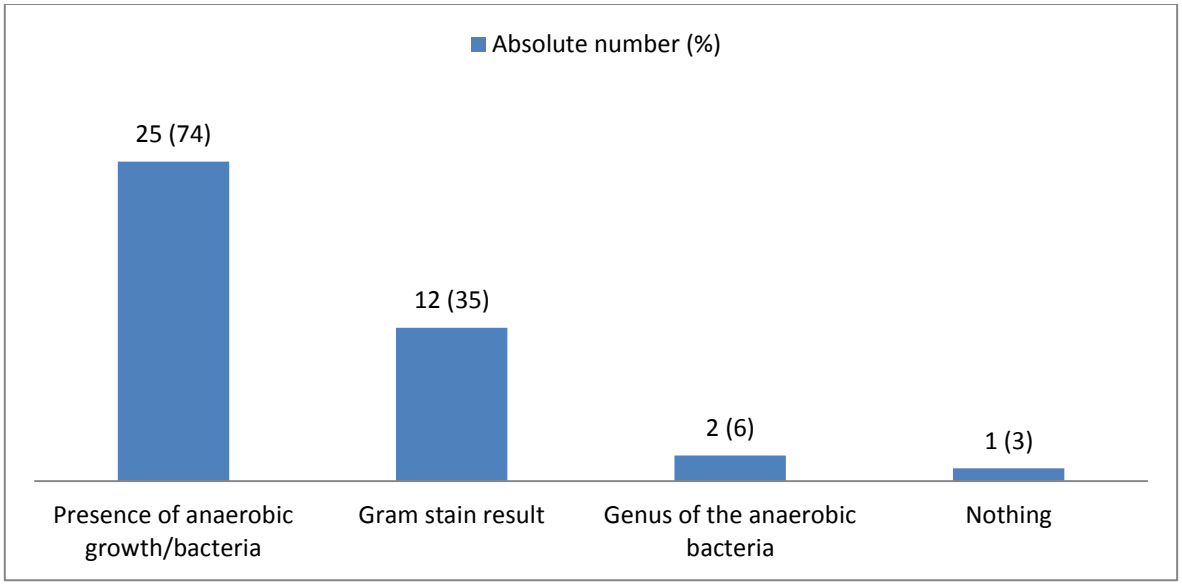
Table 5: Anaerobic organism clues from primary plates, and use of supplemental media (*1*).

Colony morphology	Possible identification	Supplemental medium
Agar pitting	*Bacteroides ureolyticus* group	
Black or tan pigmentation	*Porphyromonas* spp. or pigmented *Prevotella* spp.	EYA for lipase (*Prevotella intermedia*)
Brick red fluorescence	*Porphyromonas* spp. or pigmented *Prevotella* spp. (*Porphyromonas gingivalis* does not fluoresce)	EYA for lipase (*Prevotella intermedia*)
Chartreuse fluorescence (gram-negative rod)	*Fusobacterium* spp.	EYA for lipase
Chartreuse fluorescence (gram-positive rod)	*Clostridium difficile* or *Clostridium innocuum*	CCFA
Double zone of beta hemolysis	*Clostridium perfringens*	EYA for lecithinase
"Fried egg"	*Fusobacterium necrophorum, Fusobacterium varium*	EYA for lipase, BBE for bile growth
"Greening" of medium	*Fusobacterium* spp.	EYA for lipase
Large with irregular margin	*Clostridium* spp.	EYA for proteolytic activity
"Medusa-head"	*Clostridium septicum*	PEA
"Molar tooth"	*Actinomyces* spp.	
Pink to red colony (gram-positive rod)	*Actinomyces odontolyticus*	
Speckled or "bread-crumb"	*Fusobacterium nucleatum*	
Swarming growth	*Clostridium septicum, Clostridium sordellii Clostridium tetani*	PEA to prevent swarming

To conclude, guidelines and literature do not agree on how extensive laboratories should identify anaerobic growth in the MALDI-TOF era. For pure anaerobic growth and samples from anatomically sterile body sites consensus seems to exist to fully identify all anaerobic colony types. This is not the case for poly-microbial anaerobic growth and samples from non-sterile body sites. Suggested practical workup schemes of anaerobic cultures were conceived in the pre-MALDI-TOF era, using Gram stain, biochemical systems and or rapid disk and spot tests for the identification. MALDI-TOF gives an accurate and fast full identification of an anaerobic colony type without the need to perform aero-tolerance testing. Using MALDI-TOF, limited anaerobic workup based on the amount of different aerobic and/or anaerobic colony types seems no longer justified. New approaches and workup schemes using this fast and accurate identification method are needed. However we believe that the most effective protocols should be customized for each laboratory and should consider technical expertise, available resources, budget constraints and the clinical need.

Question 6: What is reported to the clinician if limited workup of an anaerobic culture is applied?

1. **Results**



Nearly all surveyed laboratories (97%) notify clinicians of anaerobic bacterial presence if no full identification is performed. Mostly by reporting the presence of anaerobic growth (74%) or Gram stain results (35%). Only a few laboratories (6%) will report the genus of anaerobic bacteria. Only one laboratory reports nothing if no full identification is performed.

2. **Guidelines and literature**

Guidelines concur that presence of anaerobic growth should always be notified or reported to clinicians, even if there is a limited workup. Not reporting the presence of anaerobic growth introduces an extreme risk for potential patient harm (*2*). Reporting Gram stain results or simple presence of anaerobic growth provides information that can guide antimicrobial therapy (*1-3*).

In an effort to simplify procedures and economize resources, flowcharts have been developed for limited workup relying on Gram stain, rapid disk and spot tests. The question remains whether these schemes are still useful in the MALDI-TOF era. These recommendations use a combination of four identification levels: reporting the presence of anaerobes without further identification, ruling out certain pathogens, identifying isolates to the genus level and identifying isolates to the species level.

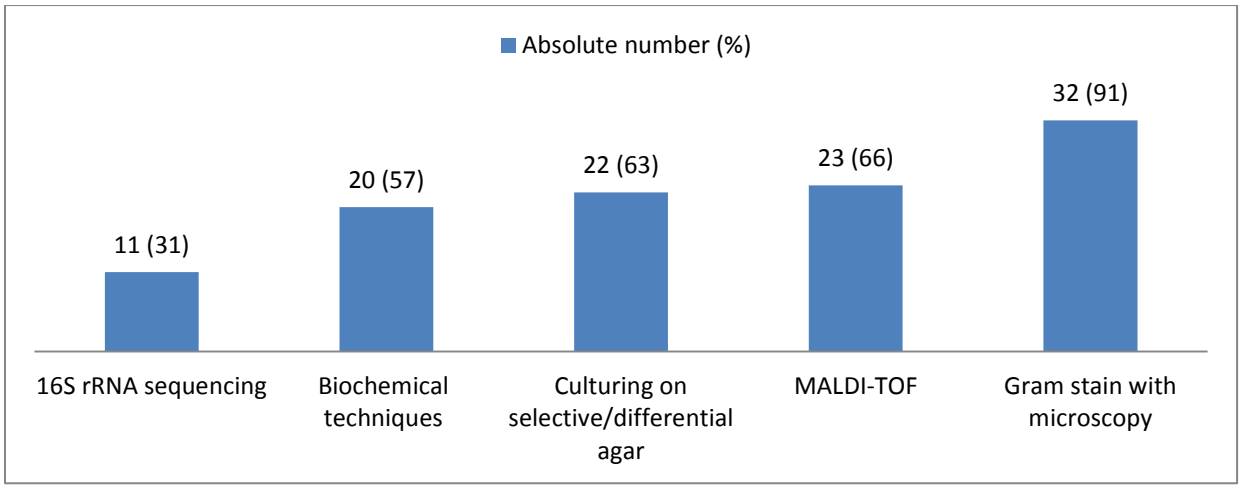
One scheme for identifying anaerobic cocci from non-sterile sites recommends reporting the presence of Gram negative or positive anaerobic cocci. If the isolate is a Gram negative bacillus, *Bacteroides fragilis* should be ruled out by lack of growth on a Bacteroides bile esculine plate. Likewise, if the isolate is an anaerobic sporulating Gram positive bacillus with double zone hemolysis on a blood agar, it should be fully identified to rule out *Clostridium perfringens*. The result should be reported as either "*Clostridium perfringens*" or "*Clostridium spp.*, not *Clostridium perfringens*". Anaerobic non-sporulating Gram positive bacilli can be reported as such without further identification (*1*).

A flowchart has also been developed for anaerobic isolates from sterile sites. In this scheme, anaerobic Gram negative and Gram positive cocci can be either reported as present or fully identified. Gram positive bacilli and Gram negative non-Fusobacterium like bacilli are identified to the species level, but Gram negative, indole-negative fusiform bacilli can be either fully reported or reported as "Fusobacterium-like" (*1*).

Baron and Citron also developed a low-cost identification scheme that identifies most anaerobic isolates to the genus level. In this scheme, Gram negative anaerobic cocci are all reported as *Veilonella spp.* and Gram positive anaerobic cocci are all reported as *Peptostreptococcus spp.*. However, Gram positive anaerobic cocci from deep soft tissue infections or those in pure culture should be identified to the species level. This is because *Finegoldia magna* is often cultured from these sites and is often resistant to clindamycin, an antibiotic that is frequently used to treat anaerobic infections. For anaerobic Gram negative and Gram positive bacilli, Baron and Citron developed flowcharts identifying organism to the genus level using spot and rapid tests along with colony appearance (*33*).

Question 7: Which identification methods are available for anaerobic bacteria in your laboratory?

1. **Results**



In this survey 66% of the laboratories are able to fully identify anaerobic bacteria using MALDI-TOF. Only 31% of the laboratories have access to 16S rRNA sequencing. Nearly all laboratories (91%) have Gram staining for presumptive identification of anaerobic bacteria. Many laboratories have selective/differential agars (63%) or biochemical identification techniques (57%).

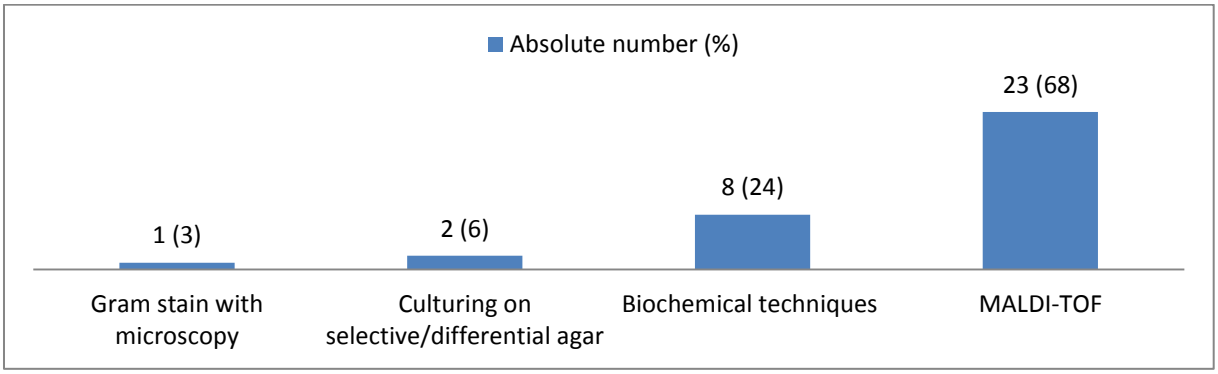
2. **Guidelines and literature**

Identification of anaerobic bacteria is performed in different stages. There is an initial screening and examination of the primary media for characteristic colonial growth. This screening can be supplemented with colony Gram stains and aero-tolerance testing if necessary. Next a presumptive identification using a combination of rapid disk, spot and biochemical tests can be performed. Some selective and differential media exist for presumptive identification of the *Bacteroides fragilis* group and Bilophia wadsworthia (Bacteroides bile esculine agar) and Clostridium spp. (Egg yolk agar). Finally, advanced identification methods for full identification to the species level can be used.

Conventional biochemical testing methods include API20A® (Biomérieux, Inc) and Minitek® (BD Biosciences). Rapid enzymatic systems such as the Anaerobe ANI card® and Rapid ID 32A® (Biomérieux, Inc), Rapid Anaerobe ID® (Dade Microscan, Inc), Crystal Anaerobe ID kit® (BD Biosciences), and Rapid ID-ANA® (Remel, Inc) eliminate the need for growth of isolates and differentiate many species that cannot be identified by biochemical testing. Other methods for full identification include gas liquid chromatography and two more recent technologies. MALDI-TOF techniques (Bruker MS®, VITEK MS®, Shimadzu MS®) and 16S rRNA gene sequencing of genetic markers become the new standards for identifications of anaerobic bacteria (*1-3*).

Question 8: Which identification method is used for the vast majority of anaerobic bacteria?

1. Results



In our survey MALDI-TOF is far out the most frequently used identification method for anaerobic bacteria (68%). Some laboratories use biochemical techniques (24%). Few use Gram staining with microscopy (3%) or culturing on selective/differential agars (6%). Nobody uses 16S rRNA for routine identification of anaerobic bacteria.

2. Guidelines and literature

Expensive and time-consuming 16S rRNA gene sequencing for identification of anaerobic bacteria is inapplicable in most routine laboratories, although it is considered as the "golden standard" for identification of anaerobic bacteria (*1-3*). Phenotypical methods have shown lack of specificity and many ambiguous or false anaerobic identifications (*34, 35*).

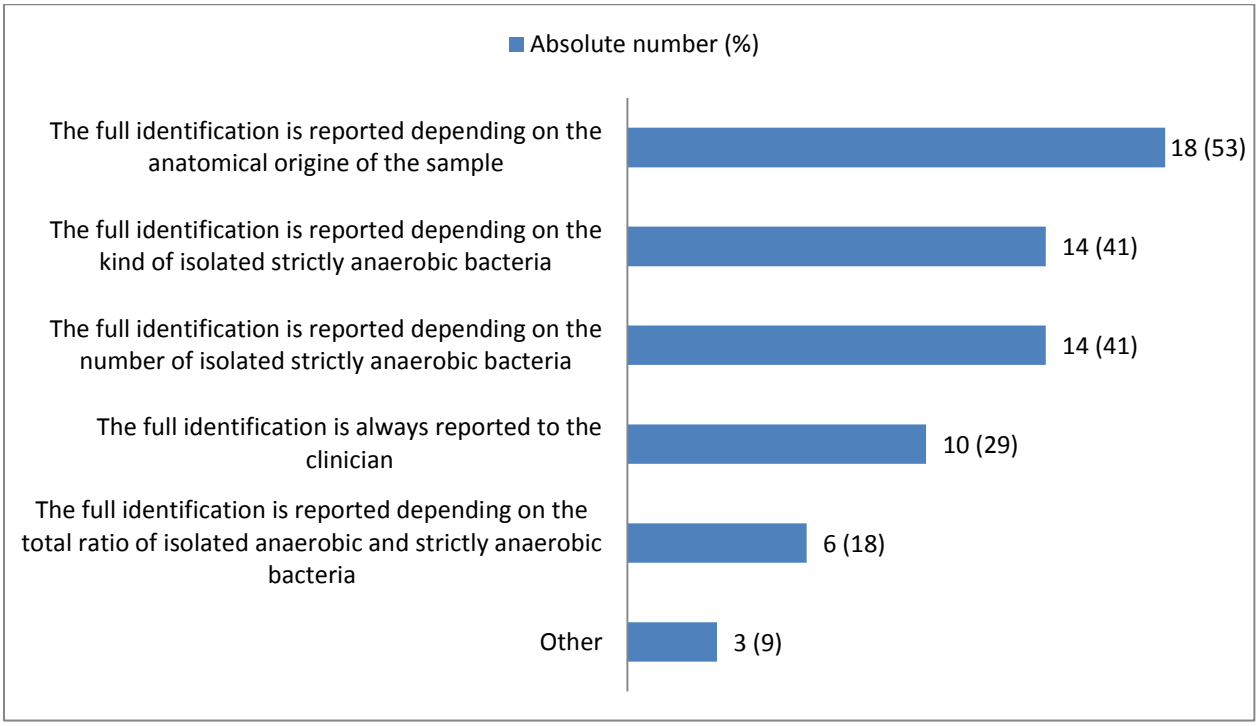
Different studies have demonstrated the accuracy and utility of MALDI-TOF for routine identification of anaerobic bacteria (*36*). Studies, using the Bruker Biotyper 3.0 system with a cutoff score of ≥2,0 and a direct spotting method, showed a correct species level identification rate ranging from 51% to 100% (*36, 37*). Obviously the quality and size of the database used for identification will influence the performance of MALDI-TOF. Nagy *et al.* analyzed 283 randomly selected anaerobic isolates obtained from clinically relevant materials with the Bruker Biotyper 3.0 using a full formic acid extraction. All anaerobic isolates were identified with standard biochemical tests. Discrepant or unidentified bacterial isolates were tested with 16S rRNA gene sequencing. About 88% of the isolates could be identified to species level by MALDI-TOF. From the 34 unidentified isolates only eight could be identified by 16S rRNA gene sequencing: 2x *Anaerococcus vaginalis*, 3x *Finegoldia magna*, 1x *Clostridium hathewayi*, 1x *Campylobacter ureolyticus* and 1x *Prevotella baroniae*. In 44 cases there was a discrepant result for MALDI-TOF and identification by phenotypic methods. MALDI-TOF results were correct and confirmed in all 44 cases using 16S rRNA gene sequencing. A review by Biswas *et al.*

demonstrated the accuracy of MALDI-TOF for the identification of bacteria that are difficult to culture, including many anaerobic bacteria (*38*). A CLSI guideline on MALDI-TOF is in preparation.

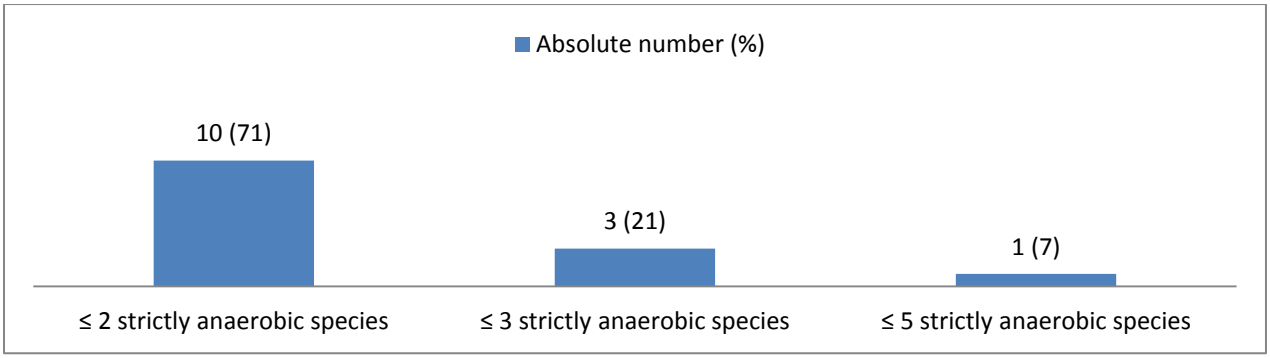
Laboratories using the MALDI-TOF technique should be aware of pre-analytical variables and importance of specimen preparation techniques. Hsu *et al.* found that direct formic acid overlay using a direct spotting method and a cutoff score of ≥ 1,7 could fully and accurately identify all of the 101 tested anaerobic clinical isolates after multiple sub-culturing. If a cutoff of 2,0 was used, 87% of the bacteria were identified correctly. Interestingly, after full formic acid extraction only one isolate (*Fusobacterium nucleatum*) could be identified additionally, compared with direct formic acid overlay. In contrast, two isolates (*Actinomyces* and *Gemella spp.*) that were correctly identified using direct formic acid overlay, could not be identified with full formic acid extraction. This study also shows that MALDI-TOF could identify anaerobes exposed to room air. With the direct formic acid overlay, the correct identification rate was 96% and 82% for anaerobes exposed to ambient air for one and five days respectively. These findings illustrate that MALDI-TOF allows accurate identification of anaerobes even when incubation conditions are suboptimal (*37*). This study did not use 16S rRNA sequencing but conventional biochemical testing as identifying comparator.

Question 9: When do you report a full identification (species level) of strictly anaerobic bacteria to the clinician?

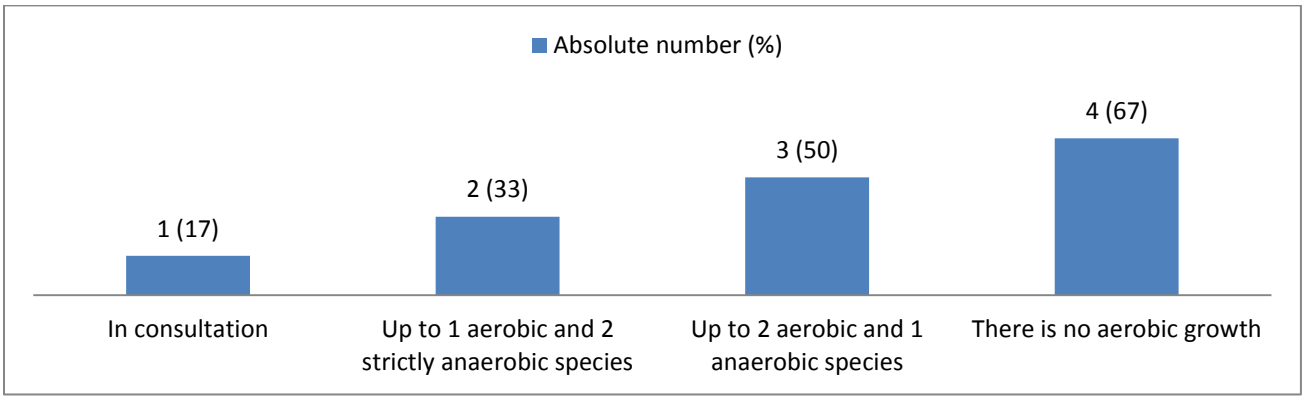
1. **Results**



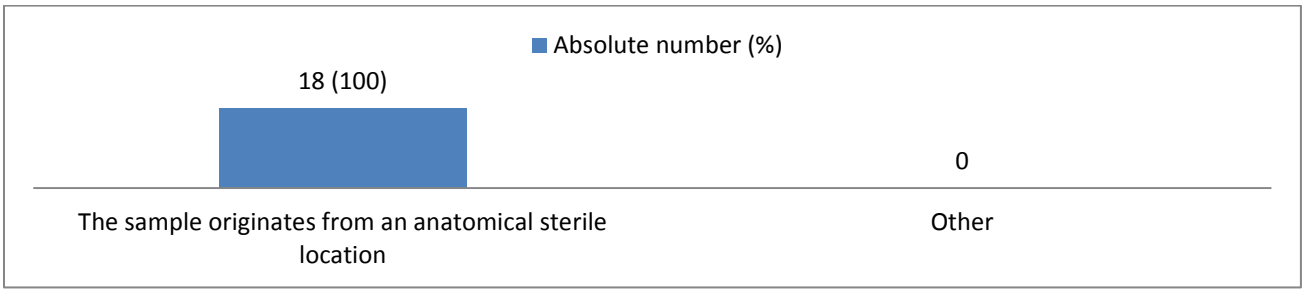
Additional question 9A for fourteen laboratories: *Up to how many strictly anaerobic bacteria would you report from one sample?*



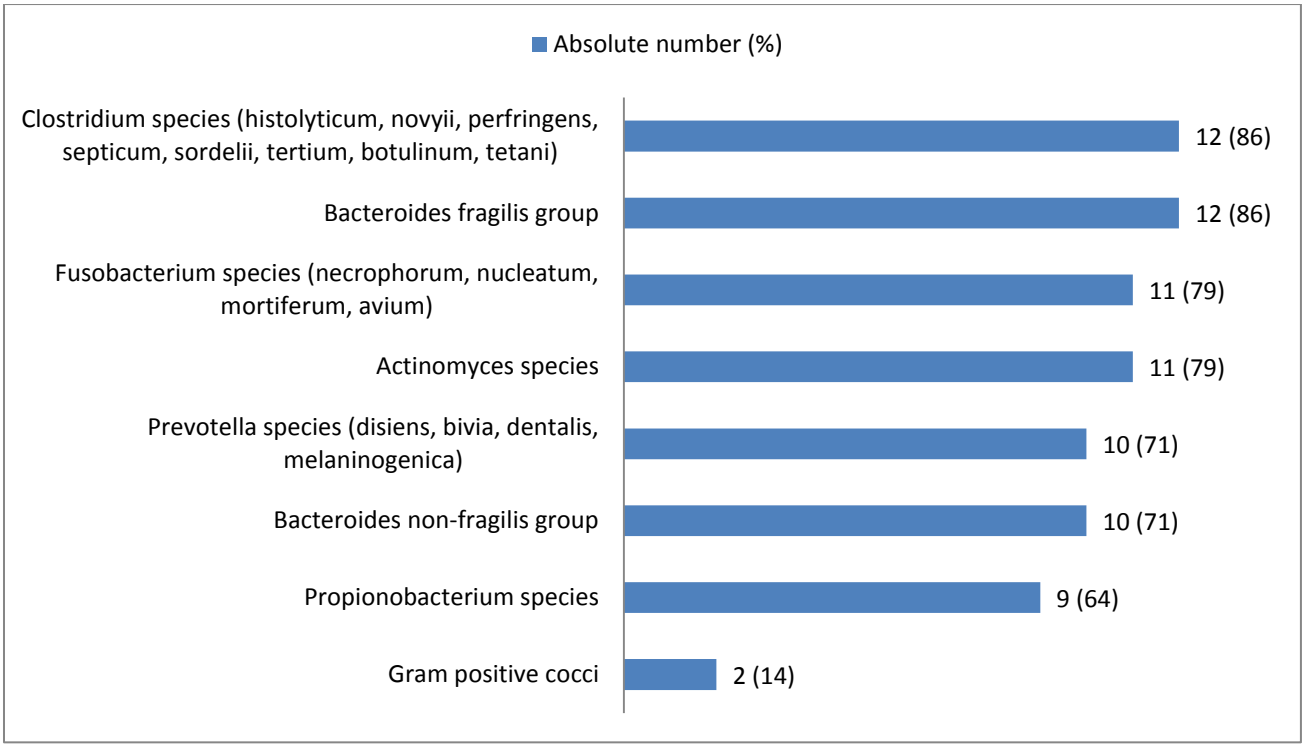
Additional question 9B for six laboratories: *Which ratio is used per sample if you take both aerobic and anaerobic species into account for reporting strictly anaerobic bacteria?*



Additional question 9C for eighteen laboratories: *Which anatomical locations give rise to reporting strictly anaerobic bacteria to the clinician?*



Additional question 9D for fourteen laboratories: *Which anaerobic species would you certainly report to the clinician?*



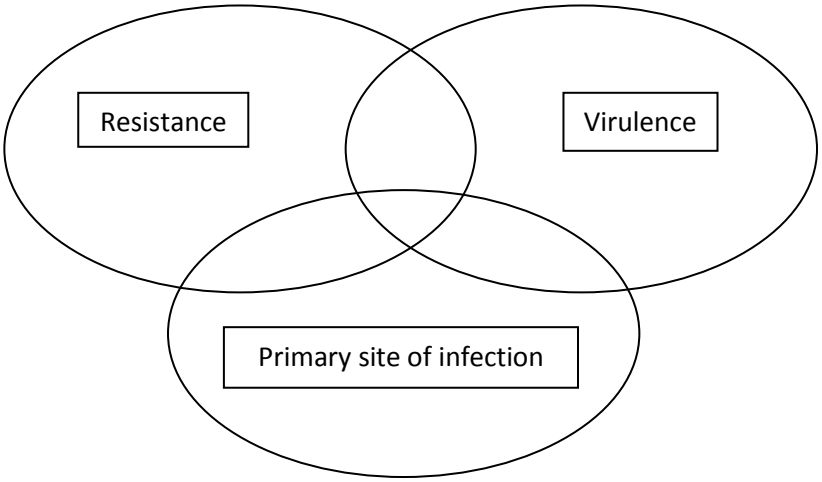
The majority of the participating laboratories (71%) does not always report isolated anaerobic bacteria. Local protocols for selective reportage are mainly based on the anatomic origin of the sample (53%), the kind of isolated strictly anaerobic bacteria (41%) or the number of identified strictly anaerobic bacteria (41%). All eighteen laboratories, selectively reporting anaerobes depending on anatomical origin, will always report species originating from sterile body sites. Laboratories, reporting species identification limited by the number of identified species, usually report a maximum of two strictly anaerobic species although some would report three species. Laboratories reporting species identification based on the kind of isolated anaerobic bacteria would certainly report species from the *Bacteroides fragilis* group (86%), histotoxic Clostridium spp. (86%), selected Fusobacterium spp. (79%) and Actinomyces spp. (79%), Prevotella (71%) and Bacteroides non-fagilis group species (71%). These laboratories are less eager to report Propionobacterium spp. (64%) or Gram positive cocci (14%).

2. Guidelines and literature

The use of MALDI-TOF for the identification of anaerobic bacteria creates new challenges for the microbiology laboratory. The amount of reported data and of unfamiliar organisms increases. Reportage of all this new and additional information can be misleading for clinicians, leading to under- or over-treatment. There are no validated standards for the reporting of anaerobic bacteria. Existing reporting strategies are based on knowledge of local and international experts and have not been clinically validated. Literature and guidelines do not give clear answers on how and what to report from anaerobic cultures.

After an extensive literature review only three reasons were withheld, justifying isolation, identification and reportage of anaerobic bacteria:

1. Information about potential primary site of infection
2. Information about potential resistance
3. Information about virulence



2.1 Which anaerobic bacteria give information about the primary site of infection?

- **A few specific clinical conditions or syndromes are caused by anaerobes:**

Clostridium difficile associated colitis is always caused by *Clostridium difficile*, isolated from faeces (1-3). Actinomycosis is caused by several *Actinomyces spp.*, isolated from tissue/biopsy or aspirated pus. The identification of Actinomyces in mucosa, where these bacteria are normal inhabitants, is mostly of no clinical significance in the absence of sulfur granules or a typical clinical syndrome, highlighting the importance of microbiological investigations in combination with histological analysis (*1, 39*). Spontaneous or traumatic clostridial myonecrosis is caused by histotoxic *Clostridium perfringens, histolyticum, novyi, septicum, fallax, bifermentas* or *sordellii*, isolated from tissue or biopsy samples (*1, 18, 40*). In Lemierre syndrome *Fusobacterium* is the predominant genus and *Fusobacterium necrophorum* is the most prevalent etiological bacteria. Other causative Fusobacteria include *Fusobacterium nucleatum, gonidiaforum* and *varium*. Occasionally, cases have been described by other bacteria (*Streptococcus, Proteus, Eikenella, Bacteroides, Prevotella* and *Peptostreptococcus spp.*) or by mixed cultures of anaerobes (*1, 41*). Samples for Lemierre syndrome diagnosis are blood cultures and aspirates from peritonsillar abscesses. Tetanus is caused by *Clostridium tetani*, cultured from wound tissue or aspirates. However, the diagnosis of tetanus is mainly based on the clinical presentation (1-3). Isolation of *Clostridium botulinum* from faeces, wound tissue or aspirates or suspected food is necessary for a definitive diagnosis of botulism but is mostly performed in specialized reference laboratories (1-3). Detection of any anaerobic bacteria in sterile body fluids (blood, cerebrospinal, pleural, ascites, joint fluid) is interpreted as infection of this body site and all isolates should be reported with full identification (*2, 17*).

- **Anaerobic infections and their isolates also provide a clue for underlying medical problems:**

Malignancy is often associated with the development of local or systematic anaerobic infections. Lung abscess can be indicative for underlying bronchogenic malignancy (*42*). Sepsis with *Clostridium*

spp. (especially *Clostridium septicum*) can be a sign of colon malignancy (*43*). Bacteremia with Gram negative anaerobic bacilli is also common in patients with solid tumors. Felner and Dowell reported that 23% of patients with *Bacteroides fragilis* group, *Fusobacterium spp.* or *Prevotella spp.* had malignancy as a predisposing factor (e.g. adenocarcinoma of the colon, uterine or cervical tumors) (*44*). Brain abscess can develop due to an underlying dental infection (*45*). Overall the main anatomic foci of anaerobic bacteremia were the gastrointestinal tract (49%), female genital tract (20%), throat (11%), skin and soft tissue (9%) and lower respiratory tract (6%) (*46*).

- **In particular for abscesses:**

Isolated anaerobic bacteria are a reflection of the endogenous flora near the infectious site, especially if there still is an anatomical connection but also for secluded abscesses (Figure 4) (*19*). Knowledge of the endogenous anaerobic flora is crucial for the interpretation of an anaerobic culture result. From skin and subcutaneous wounds and abscesses one can expect to isolate *Bacteroides fragilis* group (rectal area), *Prevotella* and *Porphyromonas spp.* (oral area). Deep head and neck abscesses mainly harvest *Prevotella, Porphyromonas, Fusobacterium* and *Peptostreptococcus spp.*. From the abdominal abscesses *Bacteroides fragilis* group, *Peptostreptococcus* and *Clostridium spp.* are the most frequent isolates anaerobic bacteria while in pelvic abscesses *Prevotella* and *Bacteroides spp.* are the dominant genera (Table 6 and 7) (*19, 40*).

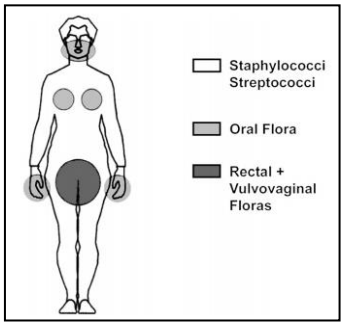


Figure 4: Distribution of organisms in abscesses, wounds, burn and decubitus ulcers (*40*).

Table 6: Incidence of various Gram positive anaerobes as indigenous flora in humans (*3*).

	Actinomyces spp.	*Bifidobacterium/ Lactobacillus spp.*	*Propionobacterium spp.*	*Eubacterium spp.* and related organisms	*Mobiluncus spp.*	*Clostridium spp.*	Gram positive cocci
Skin	0	0	2	+/-	0	0	1
Upper respiratory tract[1]	1	+/-	1	+/-	0	0	2
Mouth	2	1	+/-	2	0	+/-	2
Intestine	+/-	2	+/-	2	+/-	2	2
Genito-urinary tract[2]	+/-	1-2	+/-	+/-	+/-	+/-	2

Key: [1]Includes nasal passages, nasopharynx, oropharynx and tonsils. [2]Includes external genetalia, urethra, vagina and endocervix. 0, not found or rare; +/-, irregular; 1, usually present; 2, usually present in large numbers.

Table 7: Incidence of various Gram negative anaerobes as indigenous flora in humans (*3*).

	Bacteroides spp.	*Bilophila spp.*	*Fusobacterium spp.*	*Porphyromonas spp.*	*Prevotella spp.*	Other Gram negative bacilli	Gram negative cocci
Skin	0	0	0	0	0	0	0
Upper respiratory tract[1]	0	0	1	+/-	1	1	1
Mouth	0	+/-	2	1	2	1	1
Intestine	2	1	1	1	1	1	1
Genito-urinary tract[2]	+/-	+/-	1	+/-	1	1	1

Key: [1]Includes nasal passages, nasopharynx, oropharynx and tonsils. [2]Includes external genetalia, urethra, vagina and endocervix. 0, not found or rare; +/-, irregular; 1, usually present; 2, usually present in large numbers.

2.2 Which anaerobic bacteria have exceptional virulence?

The major virulence factors of anaerobes are summarized in Table 8. Nearly all studies investigating virulence of anaerobic bacteria were performed in mice. Only for anaerobic Gram negative bacilli, two studies tried to extrapolate mice model findings to human infections (*47-49*). These studies support the importance of encapsulated anaerobic organisms in acute and chronic infections. The predominance of encapsulated *Bacteroides, Prevotella* and *Porphyromonas spp.* (79-83% encapsulated), isolated from tonsillitis, orofacial abscesses and bloodstream infections, compared with their rate of encapsulation in the normal flora of the pharynx (35% encapsulated) and faeces (4% encapsulated) suggests greater virulence of these strains as compared to non-encapsulated strains (*47-49*). Complete eradication of experimental anaerobic Gram negative bacilli infections by metronidazole was not achieved when these organisms were encapsulated. Eradication was more efficacious for infections involving the non-encapsulated strains (*50*).

The pathogenicity of anaerobic cocci in human infections is not clear. Although most infections involving anaerobic cocci are poly-microbial, there are several reports of their isolation in pure culture (e.g. *Finegoldia magna, Peptostreptococcus anaerobius* and *asaccharolyticus, Peptococcus indolicus, Parvimonas micra, Anaerococcus vaginalis* and *Peptoniphilus harei*) which may suggest some species are more virulent then others. *Finegoldia magna* is the most virulent among Gram positive cocci and is most often isolated in pure culture from various infections. Anaerobic Gram negative cocci rarely seem to cause infections (*7*).

Virulence factors and human pathogenicity of non-sporulating Gram positive bacilli are not studied thoroughly. Non-sporulating Gram positive bacilli are usually isolated as part of a poly-microbial consortium from many different infections. Only for some *Actinomyces spp.* (*Actinomyces israelli, gerencseriae* and *graevenitzii*), isolated in pure culture from proven actinomycosis, evidence about virulence exists (*7*).

Table 8: Virulence factors of anaerobic bacteria.

Clinical important virulence factors	Effect, mechanism	Relevant for	Reference
Pili, lectin, fimbria	Adherence, colonization	*B. fragilis, P. melaninogenica, F. nucleatum, P. gingivalis, F. magna*	(*7, 51*)
Superoxide dismutase	Aero-tolerance	Most anaerobic bacteria	(*7, 51*)
Hyaluronidase, collagenase, fibrinolysis	Invasion of tissue	*F. magna* and other Gram positive cocci, anaerobic Gram negative bacilli, *Clostridium spp.*	(*7, 51*)
Immunoglobulin proteases	Evasion or inactivation of cellular or humoral immunity	*B. fragilis* and other anaerobic Gram negative bacteria	(*51*)
Mutual enhancement of growth	Synergy (mechanism unclear)	Especially *P. aeruginosa* or *S. aureus* with anaerobic cocci or Gram negative bacilli	(*52*)
Capsule formation	Higher virulence (mechanism unclear)	*Bacteroides, Prevotella, Porphyromonas, Fusobacterium, Clostridium spp., F. magna* and other anaerobic Gram positive cocci	(*53*)
Toxins	Neuro-, histo- or cytotoxic	Many *Clostridium spp.*	(*7, 18*)

2.3 Which anaerobic bacteria can be resistant to anti-microbial agents?

CLSI recommends periodic monitoring of regional and institutional resistance trends of clinically relevant anaerobic bacteria to guide empirical therapy (*54*). Wybo *et al.* recently published the fourth Belgian multicenter survey of antibiotic susceptibility of anaerobic bacteria (*55*). They collected 403 strictly anaerobic clinical isolates and determined their minimal inhibiting concentration (MIC) for various antibiotics using the E-test methodology. Both European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI breakpoints were used for interpretation of MIC values.

Table 9: Susceptibility of anaerobic bacteria in Belgium (*55*).

	Susceptibility of tested strains (%)							
Anaerobic spp.	**PENI[1]**	**FOX[2]**	**CLINDA[3]**	**MOXI[4]**	**AMC[5]**	**PTZ[6]**	**METR[7]**	**MERO[8]**
Bacteroides and *Parabacteroides spp.*	3	56	58	62	87	85	100	92
Fusobacterium spp.	81	100	81	71	100	100	100	100
Prevotella spp. and other anaerobic Gram negative bacilli	35	100	69	77	100	98	96	100
Clostridium spp.	71	90	82	66	100	95	100	100
Non-sporulating Gram positive bacilli	80	95	85	93	100	90	0	100
Anaerobic cocci	88	99	83	81	97	96	99	99

[1]Penicillin; [2]Cefoxetin; [3]Clindamycin; [4]Moxifloxacin; [5]Amoxicillin-clavulanic acid; [6]Piperacillin-tazobactam; [7]Metronidazol; [8]Meropenem

This Belgian survey concludes that resistance is observed in all anaerobic species but especially in *Bacteroides* and *Parabacteroides spp.*. Amoxicillin-clavulanic acid, piperacillin-tazobactam, meropenem and metronidazole remain very active anti-anaerobic microbial agents and are suitable for empirical use. Anaerobic bacteria show increasing resistance to penicillin, clindamycin and moxifloxacin so empirical use of these agents is discouraged. These national findings are comparable to our *Bacteroides fragilis* resistance profiles (Table 9).

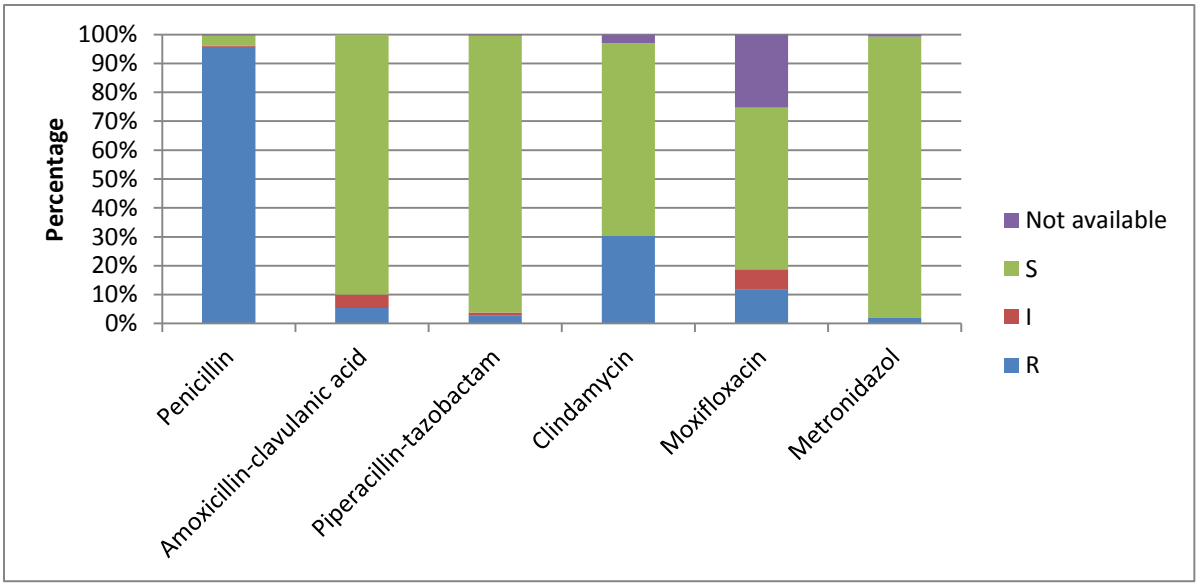


Figure 5: Resistance profile of 261 *Bacteroides fragilis* isolates from the Jessa hospital (01/01/2012 - 12/05/2015).

Resistance mechanisms in anaerobic bacteria are studied thoroughly and summarized in a recent review article (*56*). Resistance for β-lactam antibiotics was associated with changes in penicillin-binding proteins, presence of β-lactamase genes and loss of porins. Ampicillin resistance was often linked to the *cepA gene* in European *Bacteroides* and *Parabacteroides spp.*. Chromosomal cephalosporinase hyperinduction in *Bacteroides* and *Parabacteroides spp.* combined with a loss of porins results in amoxicillin-clavulanic acid or piperacillin-tazobactam resistance. Class B metallo-β-lactamases encoded by the *cfiA gene* cause resistance to carbapenems. Presence of the *cfiA gene* not necessarily results in resistance to carbapenems. While 9,4% of the European *Bacteroides fragilis* strains contained the *cfiA gene*, < 10% showed imipenem resistance *(56)*. Clindamycin resistance is linked to *erm genes*. Metronidazole resistance is linked with the presence of *nim genes* in *Bacteroides, Parabacteroides* and *Prevotella spp.*. In the Belgian survey, *nim genes* were detected in only 2,8% of the *Bacteroides* and *Parabacteroides spp.* (*55*). Quinolone resistance can result from mutations in *gyrA* and *parC genes* and efflux mechanisms such as *bexA gene* encoded efflux pumps in *Bacteroides spp.*.

Anaerobic species, ribotype, country, hospital center, antibiotic consumption and specimen were recently identified as possible contributing resistance factors of anaerobic bacteria (*56*). Interestingly, *Parabacteroides* and Bacteroides non-fragilis species (e.g. *Parabacteroides distasonis, Bacteroides theaiotaomicron, Bacteroides vulgates* and *Bacteroides ovatus*) showed higher resistance rates to antibiotics compared with those of *Bacteroides fragilis* (*56*). This finding suggests that identification to species level of *Bacteroides spp.* can be clinically important as different species can exhibit different resistance to certain antibiotics.

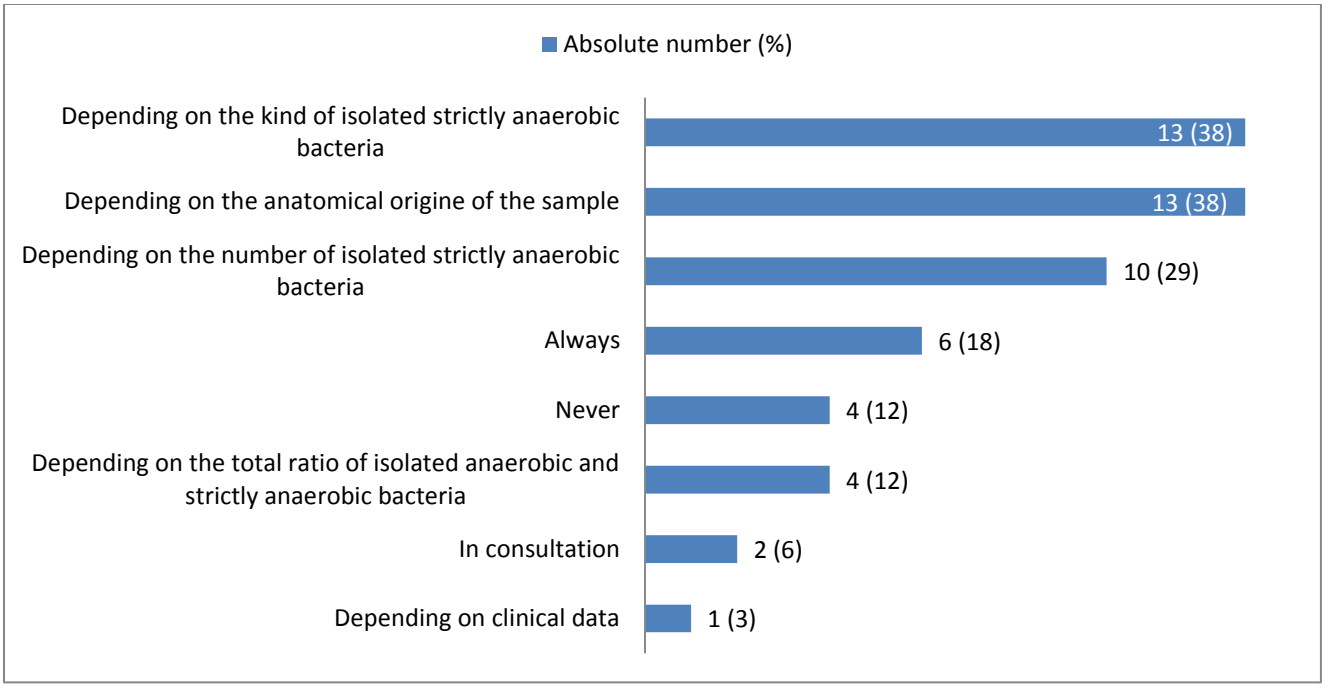
Table 10: Overview of anaerobic bacteria that should be identified to the species level and the rationale for reporting.

		Rationale for reporting these anaerobic bacteria		
Anaerobic organisms that should be identified to the species level	**Isolated from**	**Information primary site of infection, underlying medical problem or serious infection**	**Virulence**	**Unpredictable susceptibility (< 90% susceptible for ACA[1], PT[2] or MZ[3])**
Clostridium difficile	Faeces	CDAD[4]	Toxin, capsule, ribotype	No
Actinomyces spp.	Tissue, biopsy	Actinomycosis		No (intrinsically resistant for MZ[3])
Clostridium histolyticum, novyi, septicum, fallax, perfringens, bifermentas, sordellii	Tissue, biopsy	Clostridial myonecrosis **Serious infection!**	Toxin, capsule	No
Fusobacterium spp. (necrophorum, nucleatum, gonidiaforum, varium)	Blood, peritonsillar abscesses	Lemierre syndrome **Serious infection!**	Capsule	No
Clostridium tetani, botulinum	Tissue, biopsy	Tetanus, botulism **Serious infection!**	Toxin	No
Gram negative bacilli	Blood	Colon, uterine, cervical tumor? GI[5] infection?	Capsule	Some species
Clostridium septicum		Gastrointestinal malignancy?	Toxin, capsule	No
Other anaerobic bacteria		Contamination?		
All anaerobic bacteria	Lung abscess aspirate	Bronchogenic malignancy? **Serious infection!**		Some species
All anaerobic bacteria	Tubo-ovarian, liver, joint, graft, ocular infections or abscesses, sterile body sites, endocarditis, bone, brain abscess or other CNS[6] infection	**Serious infection!**		Some species
		Dental infection?		
All anaerobic bacteria isolated in pure culture	Relevant samples		Higher virulence (?)	Some species
Bacteroides fragilis group	Relevant samples	Rectal area or abdominal origin	Capsule	Yes
Bacteroides non-fragilis group (*P. distasonis, B. theaiotaomicron, B. vulgates* and *B. ovatus*)	Relevant samples	Rectal area or abdominal origin	Capsule	Yes (more resistant then *Bacteroides fragilis* group!)
Finegoldia magna	Relevant samples		Capsule	No

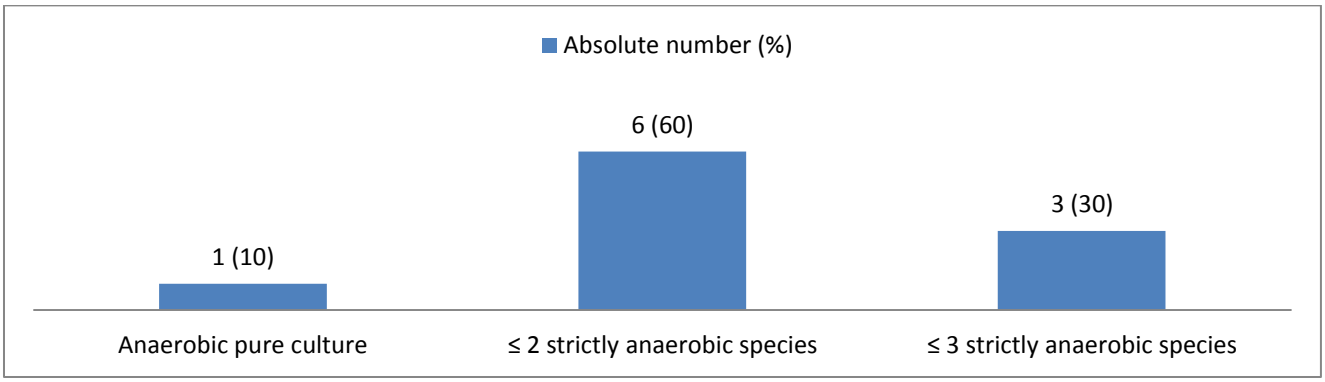
[1]Amoxicillin-clavulanic acid; [2]Piperacillin-tazobactam; [3]Metronidazol; [4]*Clostridium difficile* associated diarrhea; [5]Gastrointestinal; [6]Central nervous system.

Question 10: When do you perform an antimicrobial susceptibility test for strictly anaerobic bacteria?

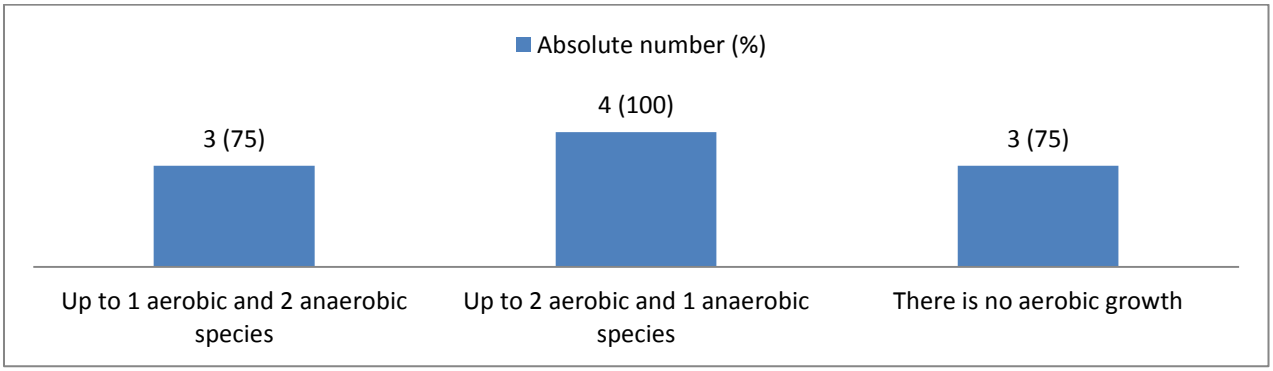
1. **Results**



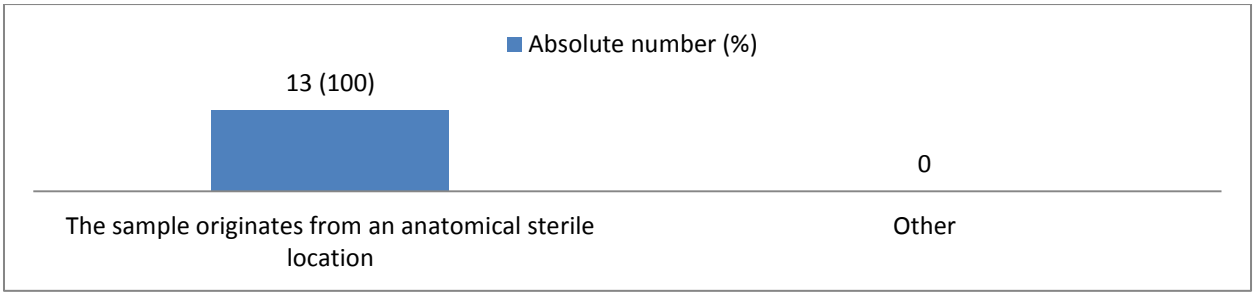
Additional question 10A for ten laboratories: *Up to how many strictly anaerobic bacteria would you subject to antimicrobial susceptibility testing in one sample?*



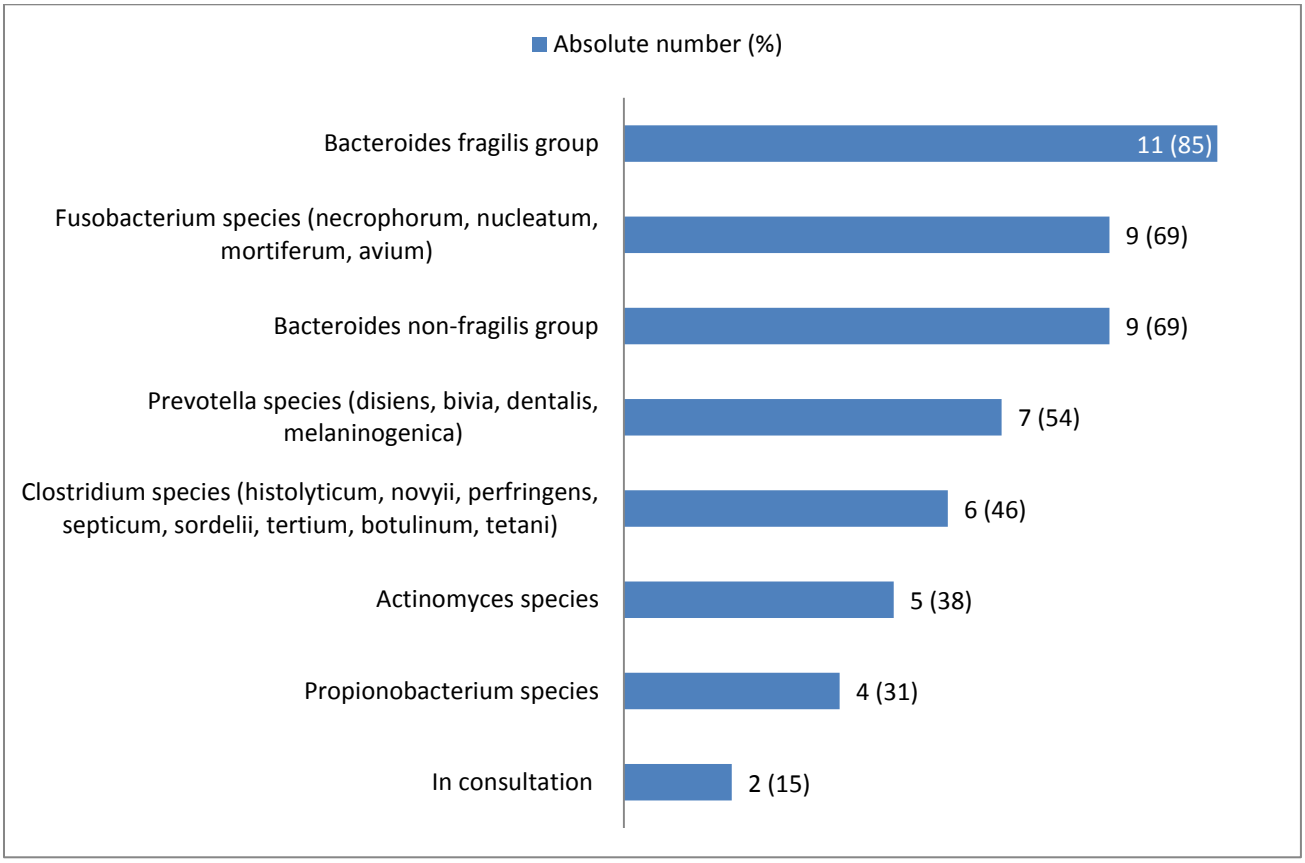
Additional question 10B for four laboratories: *Which ratio is used for one sample if you take both aerobic and anaerobic species into account for testing susceptibility of strictly anaerobic bacteria?*



Additional question 10C for thirteen laboratories: *Which anatomical locations give rise to susceptibility testing of strictly anaerobic bacteria?*



Additional question 10D for thirteen laboratories: *From which strictly anaerobic species would you certainly test susceptibility?*



In our survey four laboratories (12%) never perform AST for anaerobic bacteria. Only six laboratories (18%) will always perform AST for anaerobic bacteria. The other 24 laboratories (70%) perform AST only for certain anaerobic bacteria from certain clinical samples. In Belgian laboratories, the decision whether AST is performed, is mainly based on the kind of anaerobic bacteria that is isolated, the anatomical origin of the sample and the number of isolated anaerobic bacteria. Most laboratories would certainly test susceptibility of isolates belonging to the *Bacteroides fragilis* and non-fragilis group, *Fusobacterium, Prevotella* and *Clostridium spp.* and less of *Actinomyces, Propionobacterium spp.* and anaerobic cocci. AST is mainly performed on samples originating from sterile body sites. Most laboratories limit AST to three different anaerobic isolates in one sample.

2. Guidelines and literature

AST of anaerobic bacteria is controversial. Goldstein conducted a survey of anaerobic culture and susceptibility testing in 2008. In his survey 54% of the hospital labs had access to AST techniques but only 28% performed them in-house. Susceptibility testing was performed for blood isolates (100% of the laboratories), sterile body fluids (85-100% of the laboratories), surgical wounds (67-70% of the laboratories), other wounds (50-67% of the laboratories) and upon request (40% of the laboratories) and this for either all isolates or selected isolates. The survey does not specify which isolates were tested (*57*).

Four major indications for susceptibility testing of anaerobic isolates are listed by the CLSI, in which the choice of an antimicrobial agent may be critical (*54, 58*). Some indications are however difficult to implement in the routine workflow of a clinical laboratory:

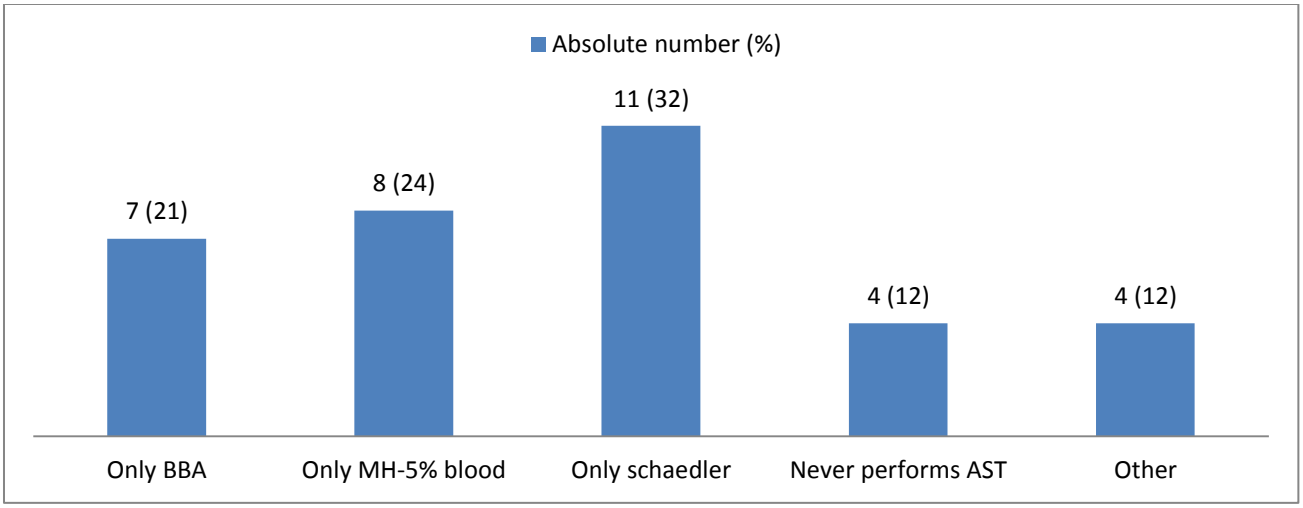
1. **Known resistance of a particular organism or species:** *Bacteroides fragilis* and non-fragilis group should be tested because of the unpredictable susceptibility (*55, 56*).
2. **Persistence of the infection despite adequate treatment with an appropriate therapeutic regimen:** It is unlikely that this strategy is manageable for clinical laboratories, since all clinical isolates should be stored for possible future AST. In case of persistent infection, clinicians should collect new relevant samples.
3. **Difficulty in making empirical decisions based on precedent cultures:** Clinicians may have difficulties in deciding which antibiotic is suitable for empiric use because of resistant anaerobic bacteria from precedent cultures. In this case anaerobic isolates from current cultures should be tested for susceptibility.
4. **Confirmation of appropriate therapy for severe infections or for those that may require long-term therapy:** Specific infections requiring AST of all anaerobic isolates include brain abscess, endocarditis, osteomyelitis, joint infection, infection of prosthetic devices, vascular grafts and bacteremia. Also isolates from normally sterile body sites should be tested unless they are believed to be contaminants.

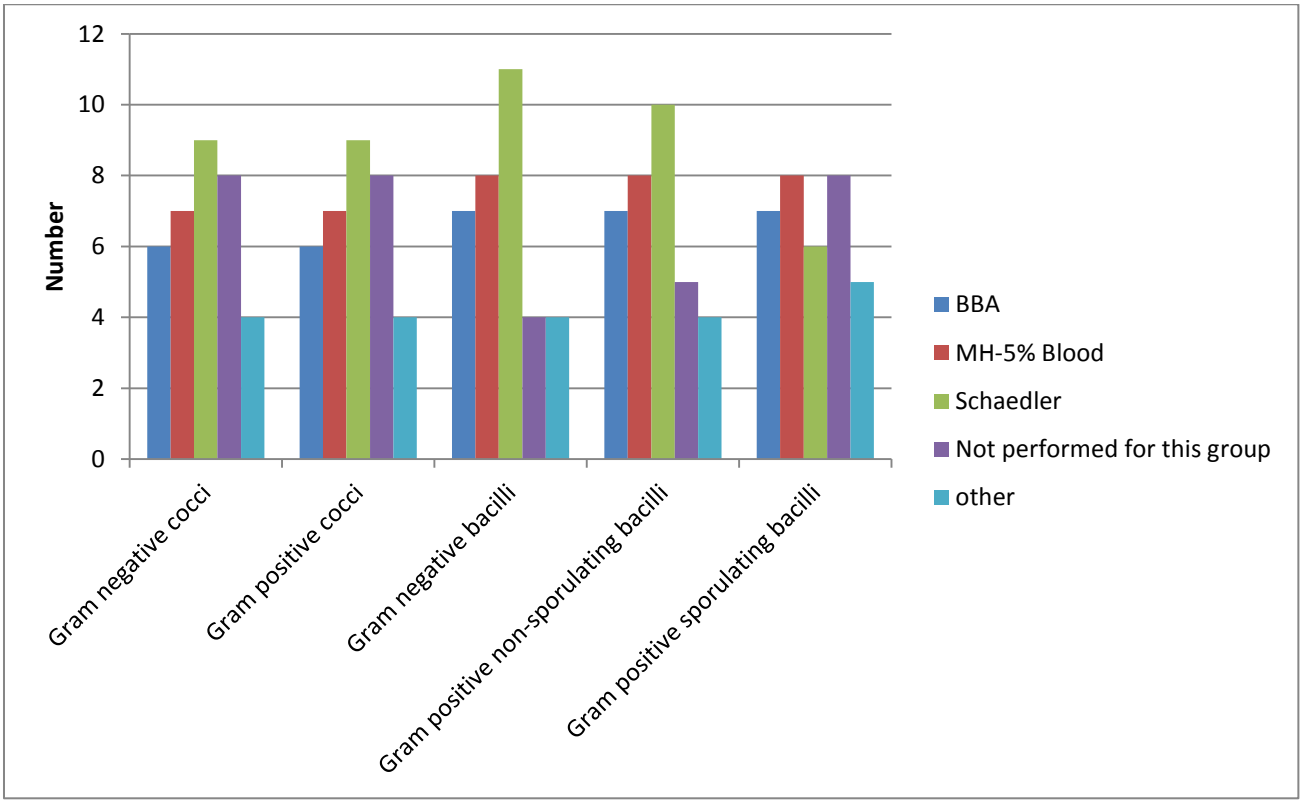
Brook *et al.* and Schuetz *et al.* recognize the four major indications of the CLSI for anaerobic AST and also believes that AST should be performed if anaerobic culture yields only one anaerobic isolate. They also suggest that highly virulent strains like *Prevotella, Fusobacterium, Clostridium, Bilophila* and *Sutterella spp.* should be tested (*59, 60*).

It is difficult to establish a correlation between *in vitro* susceptibility and clinical or bacteriological response for anaerobic infections (*59, 61*). Individuals may recover without antibiotics or surgery, others get better because of adequate drainage. In some instances eradication of the aerobic component of a poly-microbial infection seems sufficient (*59*). Several retrospective studies reported a correlation between resistance of anaerobic pathogens and poor clinical outcome (*62-64*). Other studies show that inappropriate therapy can affect the clinical outcome of the patient (*65-70*) in anaerobic bacteremia.

Question 11: On which agar do you perform susceptibility testing for strictly anaerobic bacteria?

1. **Results**





Among the questioned laboratories 32% uses Schaedler agar, 24% uses Mueller-Hinton agar (MH) with 5% blood (not specified) and 21% uses Brucella blood agar (BBA). The Schaedler agar is mostly used for AST of anaerobic Gram negative bacilli.

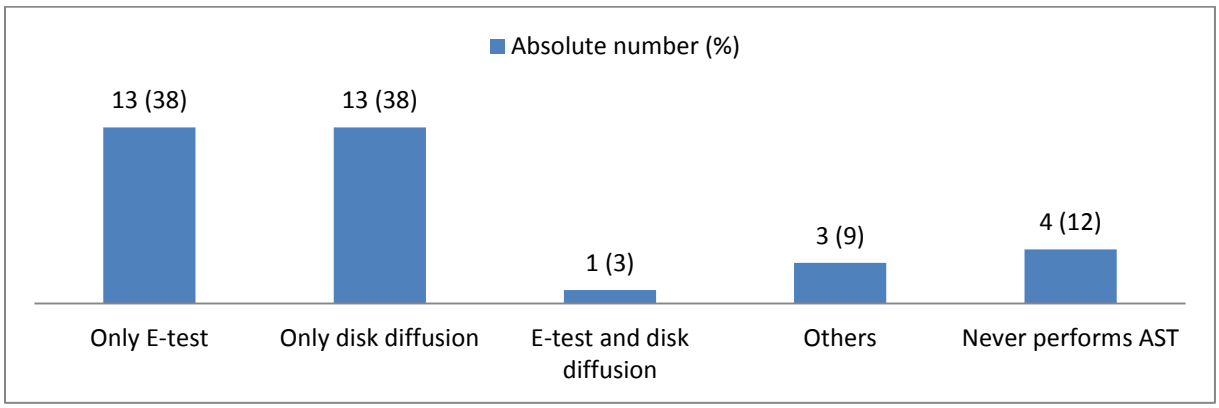
2. **Guidelines and literature**

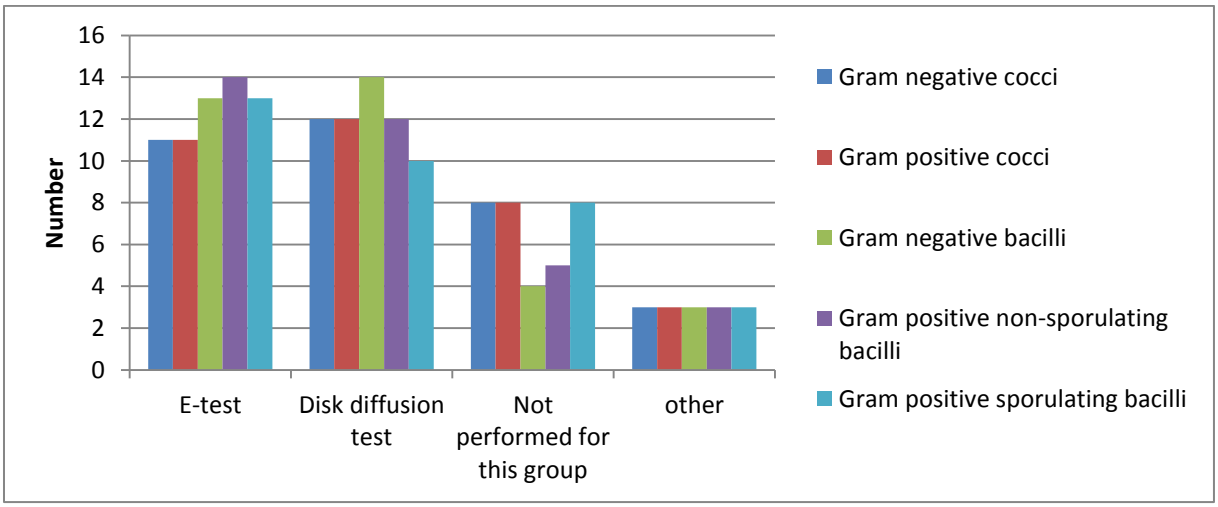
In a study of 2002 by Roe *et al.* growth characteristics of five different media were compared. The results supported the use of supplemented BBA with laked sheep blood as equal to or better than other media tested in the growth of all anaerobes (*71*).

In a second study, this group investigated whether the choice of media affected susceptibility results. Results for Wilkins-Chalgren agar with and without laked sheep blood were compared with supplemented BBA. In summary, a high degree of correlation was found when MIC values on Wilkins-Chalgren were compared with those of the other media for the organisms that would grow on both media. The investigators concluded that supplemented BBA provides results equivalent to the Wilkins-Chalgren reference standard while supporting the growth of fastidious organisms for susceptibility testing (*72*). Based on this study CLSI recommends the use of supplemented BBA for anaerobic AST (*54, 58*).

Question 12: Which method is used for testing susceptibility of strictly anaerobic bacteria?

1. Results





Most Laboratories perform anaerobic AST using E-test (38%) or disk diffusion (38%). Only one laboratory uses both methods. No laboratory uses the CLSI recommended agar or broth dilution method. There are no striking differences in AST methodology between the different anaerobic bacteria.

2. Guidelines and literature

In the survey of Goldstein 65% of the hospital laboratories who performed anaerobic AST used the E-test method. None of the labs used the agar dilution method (*57*). In our survey only 43% of the laboratories performing anaerobic AST use the E-test method.

Schuetz *et al.* discuss different anaerobic AST methods summarizing their advantages and disadvantages (Table 11) (*60*). CLSI only describes the reference agar dilution method and the alternative broth microdilution method for *Bacteroides fragilis* group organisms (*54*). This CLSI reference standard is not intended for testing single isolates. It should be considered as a standard against which other methods can be compared. CLSI emphasizes that other techniques, such as E-test, may be used as long as equivalence to agar or broth dilution is established (*54*). EUCAST states that disk diffusion criteria for anaerobic AST have not yet been defined and a MIC-method should be used. If a commercial MIC-method is used, the manufacturer's instructions should be followed (*73*).

Table 11: Advantages and disadvantages of anaerobic AST methods (*60*).

Method	Advantages	Disadvantages
Agar dilution	• Reference method against which other methods are compared	• Labor-intensive • Expertise required for performance • Considerable time is involved in setting up testing • Not amenable/appropriate for testing small numbers of organisms
Broth microdilution	• Multiple antimicrobials (≥10) can be tested per isolate • Commercial panels are available • Laboratories can customize their own panel of antibiotics to test	• Currently recommended by CLSI only for *Bacteroides fragilis* group organisms • Shelf life of frozen panels may be limited • Poor growth by some strains has been shown
MIC gradient diffusion method	• Ease of use • Precise MIC value is obtained • Convenient for testing of a few individual isolates • Multiple drugs can be tested at one time	• Expensive for surveillance purposes • Metronidazole resistance can be overestimated if anaerobiosis is inadequate
Rapid β-lactamase disk testing	• Rapid (at most 30 min to results)	• Only tests for β-lactamases • Can be used on a limited number of bacterial species • Negative result must be followed up with an MIC test for accurate prediction of β-lactam susceptibility

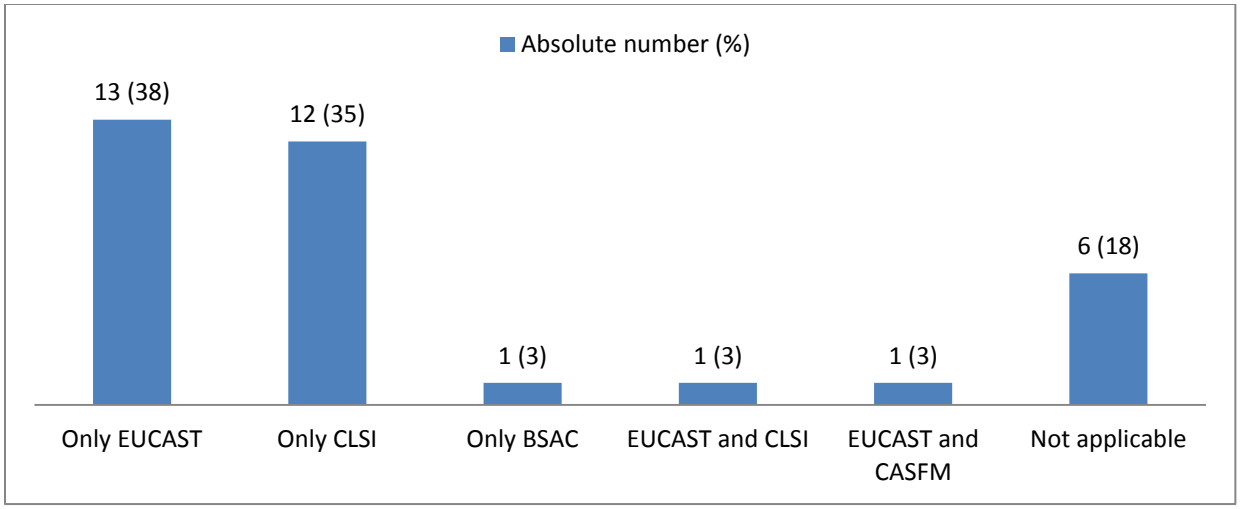
Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

The E-test method seems reliable for anaerobic AST (*74-76*). All publications describe a good correlation with the reference agar dilution method. However the gradient diffusion method can overestimate metronidazole resistance if incubation conditions are not adequate (*77*).

Recently Nagy *et al.* published their results of the development of an EUCAST disk diffusion method for AST of *Bacteroides fragilis* group isolates. Clinical isolates of the *Bacteroides fragilis* group were tested for amoxicillin-clavulanic acid (20/10 µg/disc), piperacillin-tazobactam (30/6 µg/disc), cefoxitin (30 µg/disc) imipenem-cilastatin (10 µg/disc), meropenem (10 µg/disc), clindamycin (10 µg/disc), tigecycline (15 µg/disc), metronidazole (5 µg/disc), moxifloxacin (5 µg/disc) using the disk diffusion method. BBA supplemented with haemin and vitamin K1 was inoculated with a 1 McFarland suspension and incubated at 37 °C in an anaerobic atmosphere for 24 hours. Zone diameters were compared with susceptibility results from agar dilution. In case of discrepant results MICs were determined by gradient testing and compared with the inhibition zones on the same plate. The agreement between zone diameters and agar dilution for imipenem, metronidazole, clindamycin, moxifloxacin and tigecyclin was good. There was an overlap of the results from both methods for amoxicillin-clavulanic acid and piperacillin-tazobactam intermediate and susceptible strains. The authors conclude that isolates with an inhibition zone < 23 mm for amoxicillin-clavulanic acid and < 25 mm for piperacillin-tazobactam should be retested by a MIC determination method. For cefoxitin resistant population could be separated with an inhibition zone < 17 mm, intermediate and susceptible isolates overlap (*78*).

Question 13: Which breakpoints do you use for the interpretation of anaerobic susceptibility testing?

1. **Results**



In our survey 44% of the laboratories uses EUCAST breakpoints, 38% use CLSI breakpoints, 3% use CASFM guidelines and 3% use BSAC breakpoints and diameters. This question is not applicable for six laboratories. Only two laboratories use more than one guideline for AST interpretation.

2. **Guidelines and literature**

The most recent 'Comité de l'Antibiogramme de la Société Français de Microbiologie' (CASFM) guideline does not mention breakpoints or diameters for anaerobic bacteria. It only mentions intrinsic resistance patterns for relevant anaerobic bacteria (*79*). The 'British Society for Antimicrobial Chemotherapy' (BSAC) guideline for AST continues harmonization with the EUCAST breakpoints. Their tables for anaerobes have been expanded to include MIC breakpoints that have been determined by EUCAST. BSAC offers zone diameters for some antibiotics only for *Bacteroides fragilis, Bacteroides thetaiotaomicron* and *Clostridium perfringens*. BSAC specifies that resistance by disc diffusion tests should be confirmed by MIC determination (*80*). The most striking difference is the lower EUCAST breakpoint (≤ 8/4) for piperacillin-tazobactam compared to CLSI (≤ 32/4) (*54, 58, 73*). CLSI defines breakpoints for moxifloxacin, while EUCAST states there is insufficient evidence justifying the use of newer fluoroquinolones for anaerobic infections (*54, 58, 73*). For tigecyclin only 'United Food and Drug Administration' (FDA) breakpoints exist (S ≤ 4; 8 = I; R ≥ 16) (*81*). EUCAST, CLSI and BSAC do not suggest tigecyclin breakpoints or zone diameters for anaerobic AST.

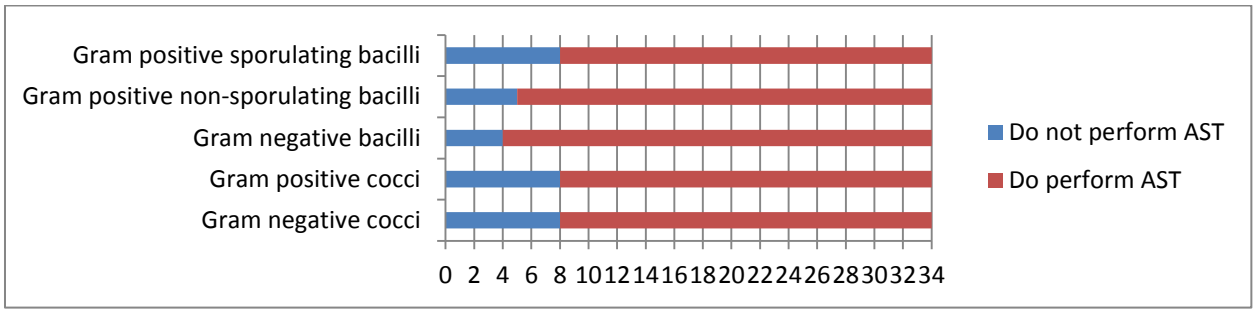
Table 12: Comparison of BSAC, CLSI and EUCAST breakpoints and zone diameters for anaerobic AST (*58, 73, 80*).

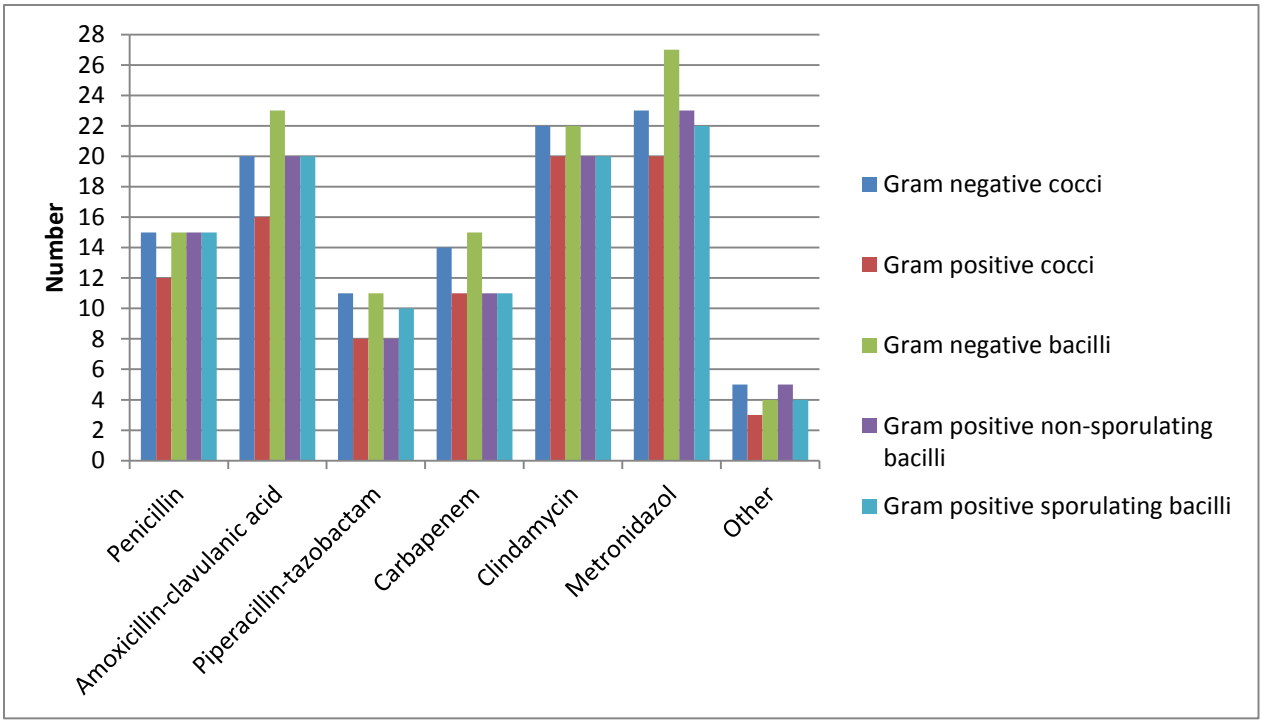
		MIC breakpoint (mg/L)				Zone diameter (mm)		
Guideline	**Antibiotic**	R	I	S	**Disc content (µg)**	R ≤	I	S ≥
BSAC	Penicillin	> 0,5	-	≤ 0,25	1 unit[1]	22	-	23
CLSI		≥ 2	1	≤ 0,5	-	-	-	-
EUCAST		> 0,5	-	≤ 0,25	-	-	-	-
BSAC	Amoxicillin-clavulanic acid	> 8/2	8/2	≤ 4/2	30[2]	20	21-28	29
CLSI		≥ 16/8	8/4	≤ 4/2	-	-	-	-
EUCAST		> 8/2	-	≤ 4/2	-	-	-	-
BSAC	Meropenem	> 8	4-8	≤ 2	10[3]	18	19-25	26
CLSI		≥ 16	8	≤ 4	-	-	-	-
EUCAST		> 8	-	≤ 2	-	-	-	-
BSAC	Clindamycin	> 4	-	≤ 4	2[3]	9	-	10
CLSI		≥ 8	4	≤ 2	-	-	-	-
EUCAST		> 4	-	≤ 4	-	-	-	-
BSAC	Metronidazole	> 4	-	≤ 4	5[3]	17	-	18
CLSI		≥ 32	16	≤ 8	-	-	-	-
EUCAST		> 4	-	≤ 4	-	-	-	-
BSAC	Piperacillin-tazobactam	> 16/4	16/4	≤ 8/4	75/10[2]	26	-	27
CLSI		≥ 128/4	64/4	≤ 32/4	-	-	-	-
EUCAST		> 16/4	-	≤ 8/4	-	-	-	-
BSAC	Moxifloxacin	-	-	-	-	-	-	-
CLSI		≥ 8	4	≤ 2	-	-	-	-
EUCAST		-	-	-	-	-	-	-
BSAC	Vancomycin	> 2	-	≤ 2	-	-	-	-
CLSI		-	-	-	-	-	-	-
EUCAST		> 2	-	≤ 2	-	-	-	-

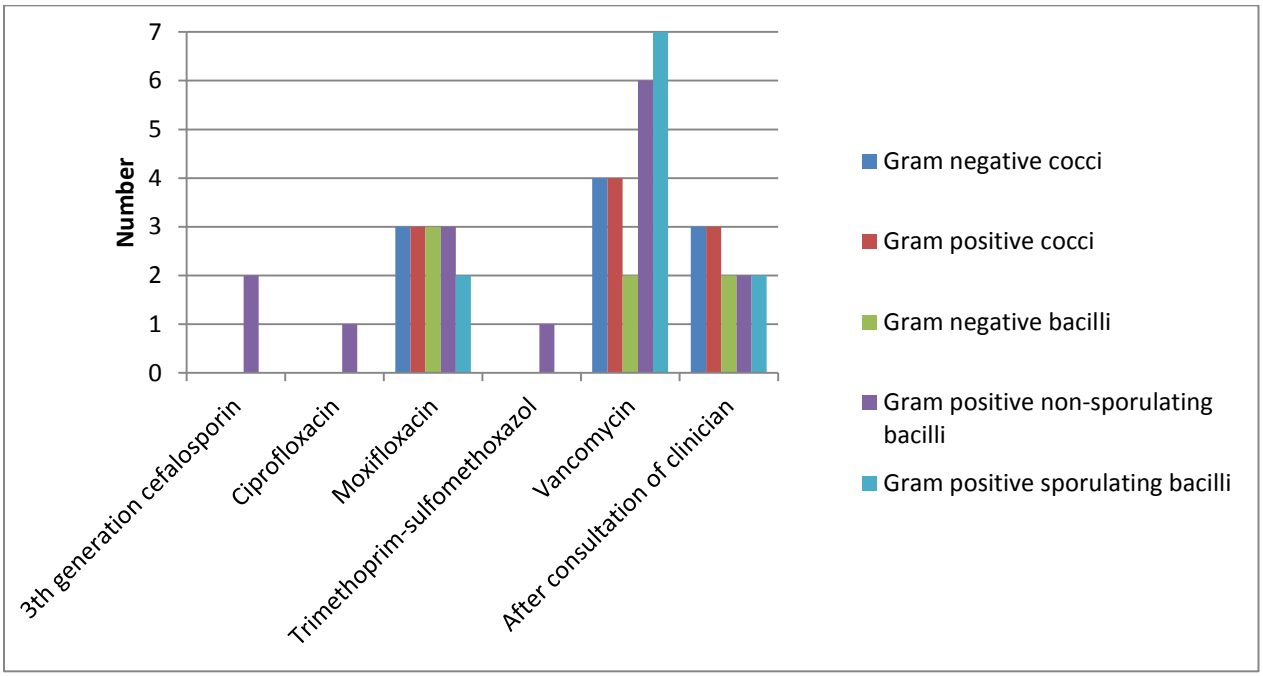
[1]Only for *Clostridium perfringens*; [2]Only for *Bacteroides fragilis*; [3]Only for *Bacteroides fragilis*, *thetaiotaomicron* and *Clostridium perfringens.*

Question 14: Which anti-microbial agents do you test for anaerobic bacteria?

1. Results







In our survey amoxicillin-clavulanic acid (62-77%), metronidazole (79-90%) and clindamycin (69-85%) are the most tested agents. Only a few laboratories perform susceptibility testing of 3th generation cephalosporins, ciprofloxacin, moxifloxacin or trimethoprim-sulfomethoxazol. No striking differences were observed between Gram positive and negative cocci, Gram negative bacilli and Gram positive sporulation or non-sporulating bacilli. AST is most frequently performed for Gram negative bacilli (88%). Vancomycin is tested mainly for Gram positive sporulating and non-sporulating bacilli.

2. Guidelines and literature

In a similar survey the most frequently tested anti-microbial agents were penicillin (83%), metronidazole (89%), clindamycin (83%) and ampicillin-sulbactam (67%) (*57*).

Table 13: Suggested grouping of antimicrobial agents to be considered for routine AST on anaerobic organisms (*54, 58*).

	***Bacteroides fragilis* Group and Other Gram-Negative Anaerobes**	**Gram-Positive Anaerobes[b]**
Group A Primary Test and Report	Amoxicillin-clavulanic acid Ampicillin-sulbactam Piperacillin-tazobactam Ticarcillin-clavulanic acid	Ampicillin[a] Penicillin[a]
		Amoxicillin-clavulanic acid Ampicillin-sulbactam Piperacillin-tazobactam Ticarcillin-clavulanic acid
	Clindamycin	
	Doripenem Ertapenem Imipenem Meropenem	Clindamycin
	Metronidazole	**Doripenem** Ertapenem Imipenem Meropenem
		Metronidazole
Group C Supplemental Report Selectively	Penicillin[a] Ampicillin[a]	Ceftizoxime Ceftriaxone
	Ceftizoxime Ceftriaxone	Cefotetan Cefoxitin
	Chloramphenicol	
	Cefotetan Cefoxitin	Piperacillin Ticarcillin
		Tetracycline
		Moxifloxacin
	Piperacillin Ticarcillin	
	Moxifloxacin	

CLSI classifies anaerobic anti-microbial agents in different groups. Group A agents are considered appropriate for routine AST and reporting for the specific organism groups. The listing of drugs in a single box designates clusters of agents for which interpretative results (R, I, S) and clinical efficacy are similar. Therefore only one of the agents within each box needs to be selected for testing. Group B antibiotics may warrant for primary testing but they should be reported selectively such as when the organism is resistant to agents of the same class, as in Group A. Other indications for reporting the result might include a selected specimen source (e.g. a 3th generation cephalosporin for Gram negative bacilli from cerebrospinal fluid), a poly-microbial infection, infection involving multiple sites, cases of patient allergy, intolerance or failure to respond to an agent of Group A or for purposes of infection control. Group C include supplemental antimicrobial agents that should be tested in reference laboratories on strains resistant to agents from Group A; for treatment of patients allergic to agents from Group A; for treatment of unusual strains or for reporting to reference laboratories as an epidemiological aid (*58*).

Because CLSI does not mention whether piperacillin-tazobactam or amoxicillin-clavulanic acid should be tested, susceptibility exchangeability for these anti-microbial agents was verified for 260 *Bacteroides fragilis* strains, isolated in our laboratory from clinical samples (Table 14). There was a categorical agreement of 91% between both agents. If we would have tested piperacillin-tazobactam and extrapolated amoxicillin-clavulanic acid susceptibility, false susceptibility for amoxicillin-

clavulanic acid would be assumed in 3% of *Bacteroides fragilis* isolates. Testing amoxicillin-clavulanic acid and extrapolating the results to piperacillin-tazobactam would result in false resistance for piperacillin-tazobactam in 3% of *Bacteroides fragilis* isolates. Phenotype frequencies from our *Bacteroides fragilis* population were similar to frequencies from literature (Table 15) (*82*). To conclude, our laboratory preferentially tests amoxicillin-clavulanic acid and extrapolates piperacillin-tazobactam susceptibility for *Bacteroides fragilis*. Piperacillin-tazobactam susceptibility of amoxicillin-clavulanic acid resistant strains can be tested additionally as some isolates can be susceptible for piperacillin-tazobactam. If piperacillin-tazobactam and amoxicillin-clavulanic acid resistance is observed in a *Bacteroides fragilis* isolate, presence of carbapenemase should be ruled out by testing carbapenem susceptibility.

Table 14: Amoxicillin-clavulanic acid and piperacillin-tazobactam susceptibility concordance in *Bacteroides fragilis isolates* (n=260) in the Jessa hospital.

	Piperacillin-tazobactam		
Amoxicillin-clavulanic acid	**R**	**I**	**S**
R	4	2	8
I	3	0	9
S	0	1	233

Table 15: β-lactam resistance phenotypes among *Bacteroides fragilis* in literature *(82)* and in the Jessa Hospital.

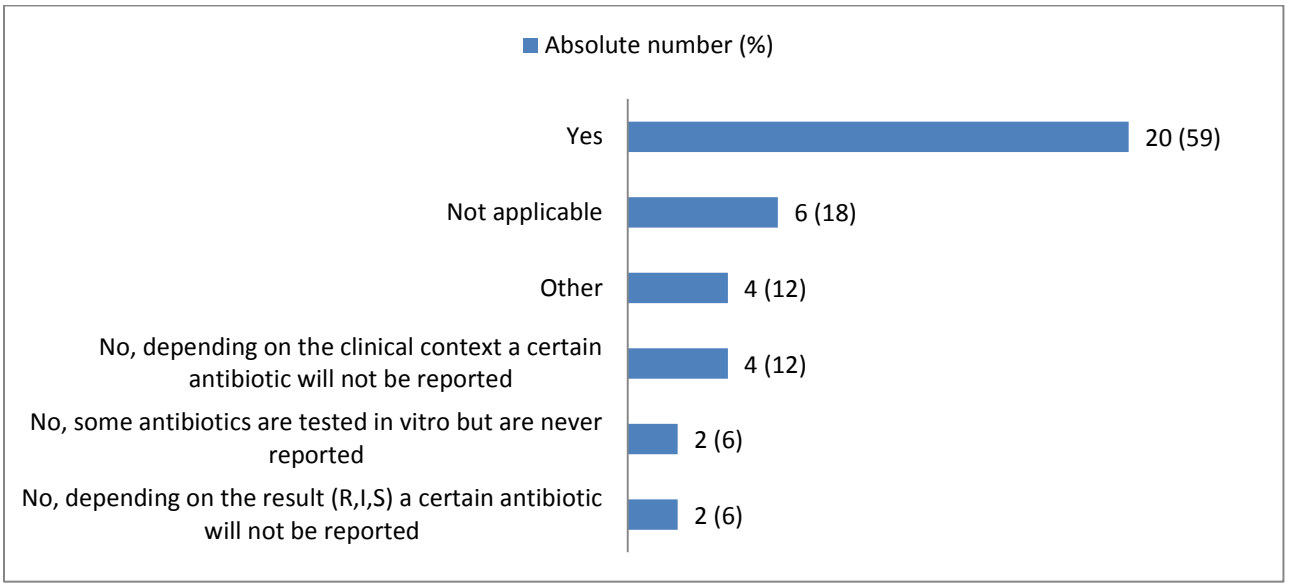
	Wild type: chromosomic β-lactamase	**β-lactamase hyperproduction + porin lack or silent carbapenemase**					**Carbapenemase (*cfiA gene*)[1]**
Amoxicillin-clavulanic acid	**S**	**S**	**I**	**R**	**R**	**I**	**R**
Piperacillin-tazobactam	**S**	**S**	**S**	**S**	**I**	**R**	**R**
Phenotype frequency literature (%)	80	9	5	3	3	/	2
Phenotype frequency Jessa (%)	89		3,5	3	1	1	1,5

[1]Only if the isolate is also resistant to a carbapenem.

CLSI states that β-lactamase positive strains should not be tested for penicillin, amoxicillin or ampicillin susceptibility and may be reported as resistant for these antibiotics. β-lactamase negative isolates must be tested for penicillin susceptibility because other bacterial mechanisms can give penicillin resistance. In Belgium ≥ 98% of the *Bacteroides spp.* and up to 70% of the *Prevotella spp.* are β-lactamase positive so testing β-lactamase and penicillin susceptibility for these species seems not useful. In *Fusobacterium spp., Clostridium spp.*, Gram positive non-sporulating bacilli and anaerobic cocci the prevalence of β-lactamase is low (0-9%). Therefore primary testing of β-lactamase and penicillin susceptibility for these species can be useful. Briefly, CLSI suggests penicillin susceptibility testing only for *Clostridium perfringens, septicum* and *sordelli*, who can be the singular cause of an infection. Other anaerobic infections mostly harvest mixed aerobic and anaerobic pathogens, including β-lactamase positive and negative strains (*54, 58*).

Question 15: Do you use non-selective reporting for tested anti-microbial agents?

1. **Results**



The majority (59%) of the participating laboratories reports all tested anti-anaerobic antibiotics. The remaining laboratories report susceptibility results based on either clinical context (12%) or final interpretation (R,I,S) (6%). Furthermore 6 % of the laboratories test some antibiotics that are never reported. For 6 laboratories this question is not applicable.

2. **Guidelines and literature**

In the previously mentioned CLSI guideline there are currently no antimicrobial agents for testing and reporting on anaerobic bacteria in group B (*54, 58*).

There are no other publications or guidelines mentioning selective reportage of antibiotics in anaerobic infections. There is no evidence justifying selective reportage.

A clinically orientated procedure for the workup of anaerobic bacteria using MALDI-TOF.

Sample quality and relevance remain most importantly for obtaining reliable and useful culture results:

- Reject samples from body sites harboring anaerobic bacteria normally and usually **(Extract 1)**
- Obtain relevant sample types (biopsy, tissue, aspirate or surgically obtained swab) **(Table 1)**
- Transport in adequate containers (anaerobic pre-reduced vials, tubes or bags or swab in Amies medium) **(Table 4)**
- Transport timely to laboratory **(Table 3)**

Rejection or acceptance of samples for anaerobic culture?

Identify all anaerobic colony types, strongly suspicious for being a strictly anaerobic bacteria: compare morphology of aerobic and anaerobic growth, typical odor, look out for typical anaerobic colony morphology **(Table 5)**.

When to identify anaerobic colony types?

Serious infection involving anaerobic bacteria?

Report ALL anaerobic organisms to species level:

- Central nervous system infection
- Sterile body site infection (ascites, pleural fluid, bloodstream)
- Tubo-ovarian infection
- Liver infection
- Joint or graft infection
- Ocular infection
- Endocarditis
- Osteomyelitis

Typical anaerobic clinical syndrome?

Report ALL pathognomonic anaerobic organisms to species level:

- CDAD[1] (*C. difficile*)
- Actinomycosis[2] (*Actinomyces spp.*)
- Myonecrosis (Histotoxic clostridia[3])
- Lemierre syndrome (*Fusobacterium spp.*)
- Tetanus (*C. tetani*)
- Botulism (*C. botulinum*)

Not a typical anaerobic clinical syndrome? No serious infection?

Report virulent anaerobic bacteria with(out) unpredictable susceptibility to species level:

- Gram negative anaerobic bacilli
- Any anaerobic bacteria isolated in pure culture
- *Finegoldia magna*
- Histotoxic clostridia[3]

Report other anaerobic bacteria simply as *"Other low-virulent anaerobic flora with predictable susceptibility present"*

Rationale for reporting fully identified anaerobic bacteria?

Perform AST of ALL anaerobic species

Perform AST of anaerobic Gram negative bacilli and bacteria isolated in pure culture

When to perform anaerobic AST?

[1]*Clostridium difficile* associated diarrhea; [2]Histological analyses should show sulfur granules; [3]*Clostridium histolyticum, novyi, septicum, fallax, bifermentas, sordelli, perfringens*

b) Clinical impact of anaerobic cultures: Multi-center retrospective case study of anaerobic cultures

Introduction

Although there seems indirect evidence, no studies directly proved clinical impact of anaerobic culture in *Bacteroides fragilis* bacteremia. In a case-control study of Redondo *et al.*, mortality was significantly higher in patients with anaerobic bacteremia (19%) compared to control patients without anaerobic bacteremia (9%) (*83*). Patients with anaerobic bacteremia were hospitalized on average 16 days longer then control patients (*83*). A prospective study of Nguyen *et al.* indicated that mortality was significant higher in patients with anaerobic bacteremia receiving inappropriate anti-microbial therapy (45%) compared to patients with anaerobic bacteremia receiving active treatment (16%) (*68*). In a retrospective study of Salonen *et al.*, mortality rate was 55% in patients with anaerobic bacteremia, not receiving appropriate anti-microbial therapy (*69*). In a case control study of Robert *et al.* mortality rate was 27%. In his study age, cancer and ineffective definitive anti-microbial therapy were associated with increased mortality rates. In this case-control study, 72 patients with anaerobic bacteremia were hospitalized longer and showed a trend toward increased mortality (*70*). Clinical impact of anaerobic culture is unclear for sample types other than blood cultures (body fluids, biopsies, abscess drainages,…) as no studies exist on this matter. We conducted a small multi-center retrospective case study, trying to answer the following investigational questions:

1. How long does it take to report identification and/or susceptibility results of anaerobic cultures to clinicians?
2. Is therapy regarding anaerobic infections affected by results of anaerobic cultures?
3. Is the outcome of patients with anaerobic infections affected by results of anaerobic cultures?

Methods

Seven hospital laboratories (Jessa Hasselt, Imelda Bonheiden, Heilig Hart Lier, Ziekenhuis Oost-Limburg Genk, One-Lieve-Vrouw Aalst, Sint-Lucas Gent, Sint-Augustinus Antwerpen) documented anaerobic culture results between February 16th and April 5th 2015. They were asked to include anaerobic culture results from relevant sample types (aspirates, sterile fluids, biopsies, drainages, surgically obtained deep swab,…). A sample could only be included if anaerobic bacteria were isolated for the first time from this patient, preventing that antimicrobial therapy was based on previous culture results. Anaerobic growth should at least be mentioned on the laboratory report, notifying the clinician of anaerobic presence. Anonymized reports and coded survey forms were collected from all hospitals and processed statistically using the t-test or χ^2-test (Analyse-it®, v2.22).

General results

We received 51 samples from 49 different patients. From one patient, three relevant samples were included as they were collected from the same anaerobic infection during the same surgical intervention. Anatomical origins and sample types are displayed in Figure 6.

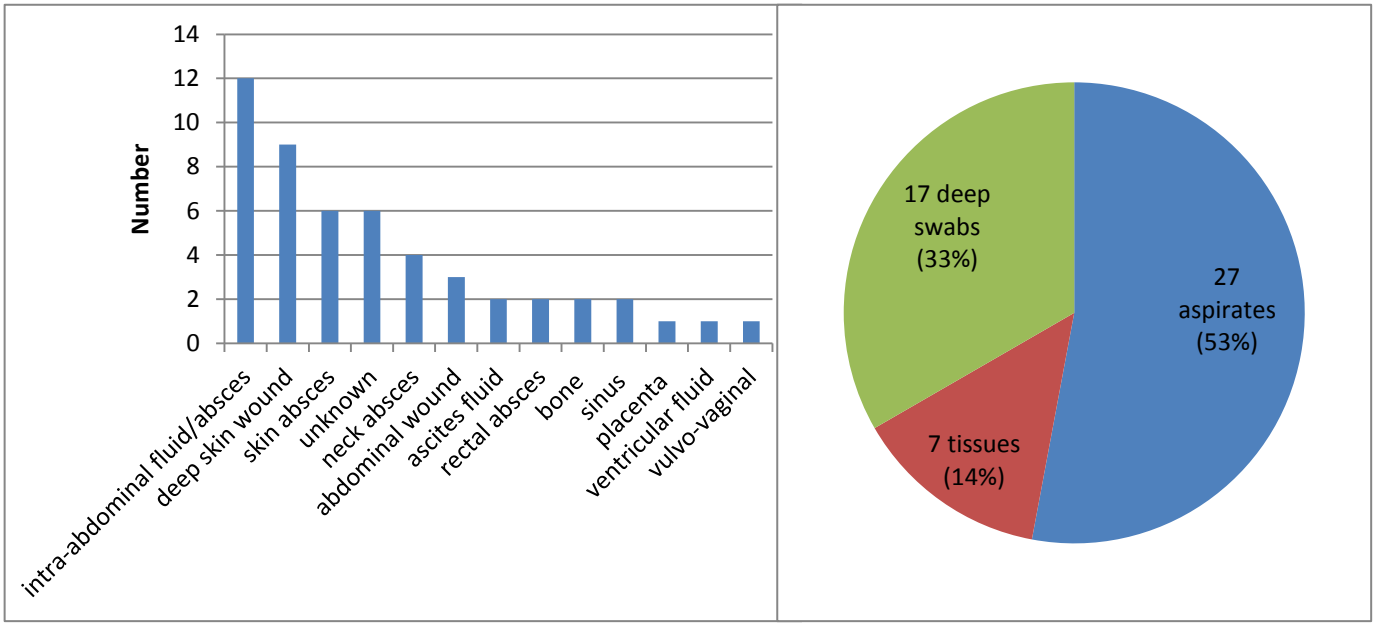


Figure 6: Sample types and anatomical origin of included samples (n=51).

Only four samples (8%) yielded a pure anaerobic growth, two with *Bacteroides fragilis* and two with *Propionobacterium acnes*. All other samples had mixed flora mostly (30%) in a 1:1 aerobic/anaerobic ratio (Figure 7). In total 93 aerobic and 63 anaerobic bacterial isolates were reported. *Escherichia coli* (21%) and *Bacteroides spp.* (43%) were the most prevalent bacteria (Figure 8 and 9).

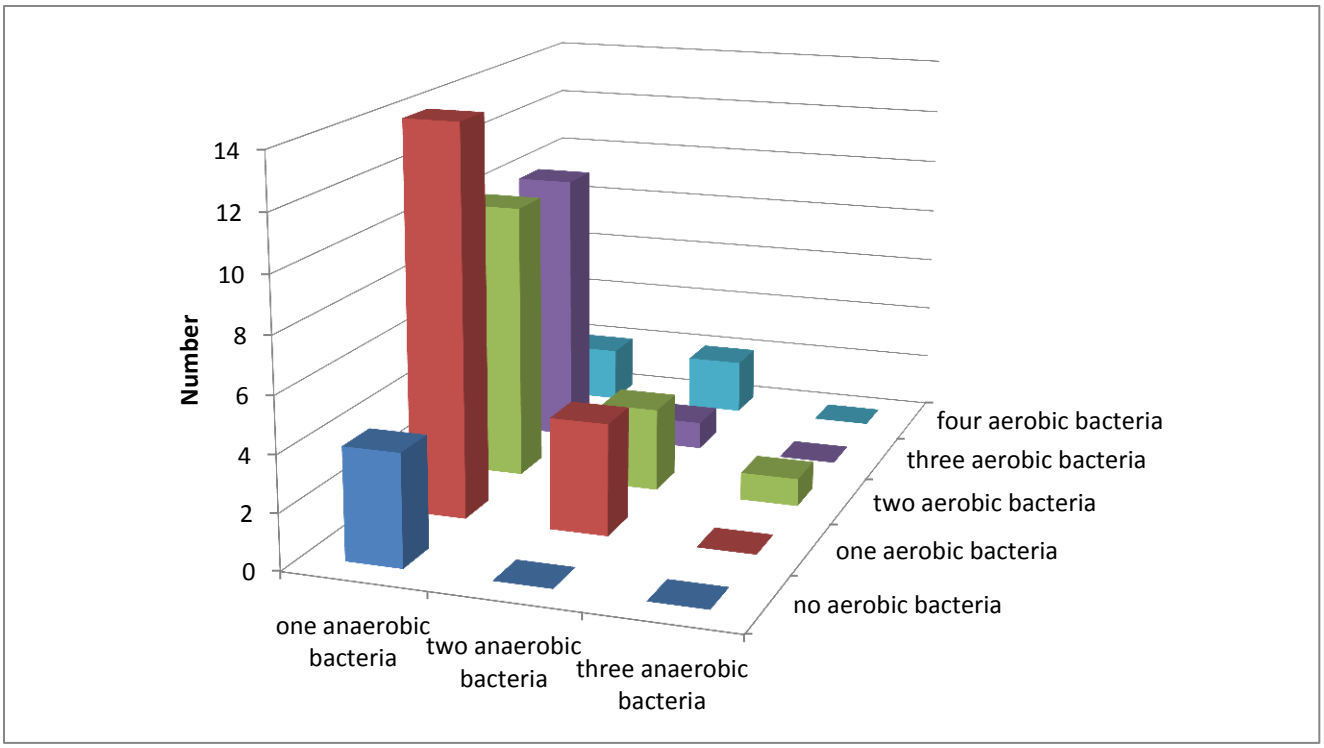


Figure 7: Number of reported aerobic and anaerobic bacteria per sample (n=49).

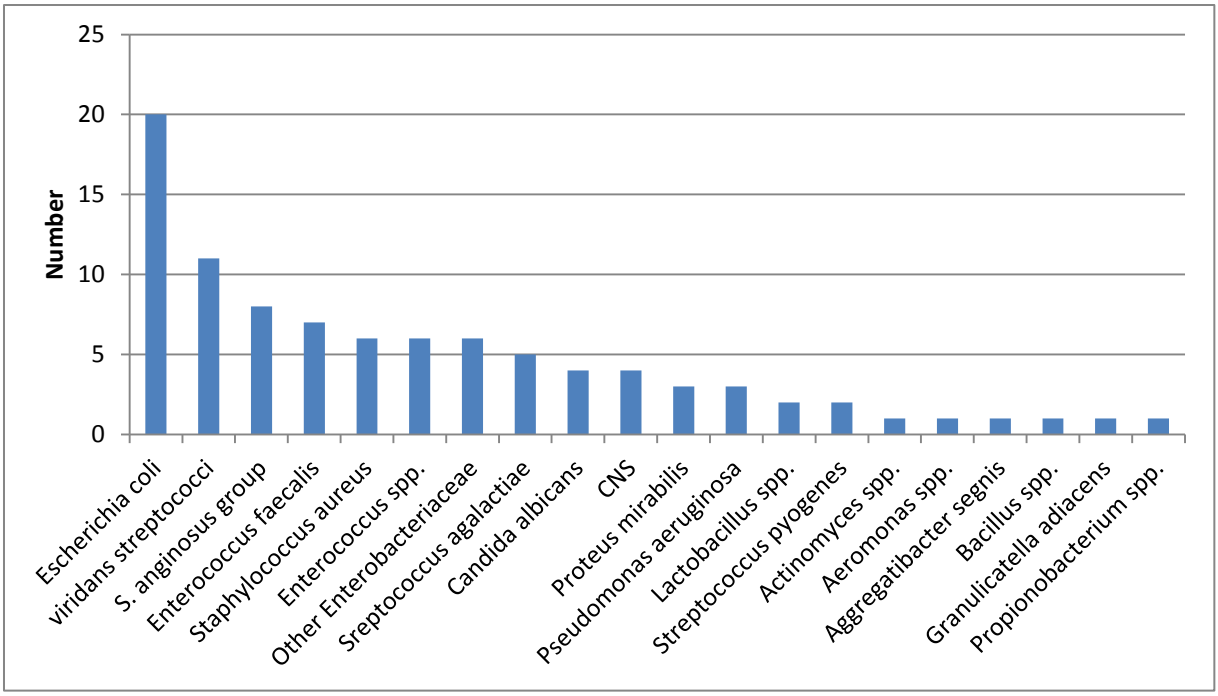


Figure 8: Isolated aerobic bacteria (n=93).

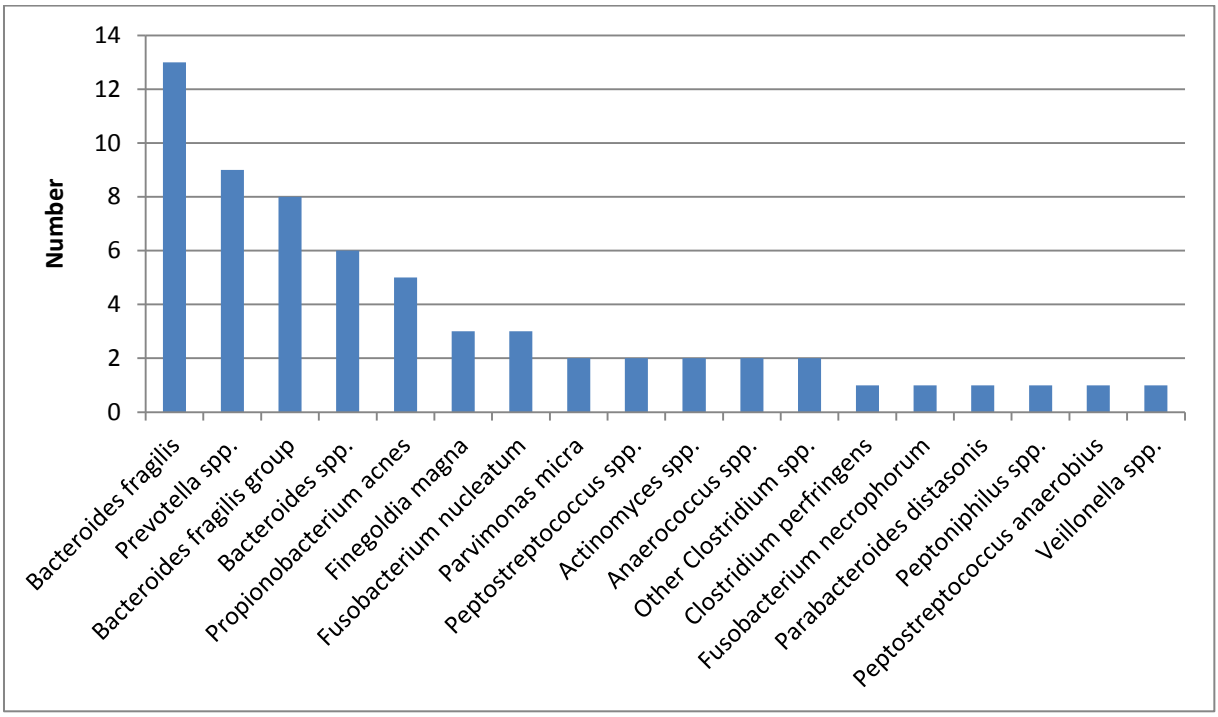


Figure 9: Isolated anaerobic bacteria (n=63).

Discussion of investigational questions

1. **How long does it take to report identification and/or susceptibility results of anaerobic cultures to clinicians?**

The average reporting time for aerobic bacterial identification and AST was respectively 1,5 and three days after sampling and 95% of the aerobic identifications and AST's was reported within three and four days, respectively. Seven identified aerobic isolates (7%) were not reported to the clinician. From 28 aerobic isolates (29%) AST was not performed (Figure 11).

For anaerobic bacteria the average reporting time for identification and AST was respectively three and five days after sampling and 95% of the identifications and AST's was reported within five and seven days, respectively. All identified anaerobic isolates were reported to the clinician and from 29 isolates (45%) AST was not performed (Figure 10).

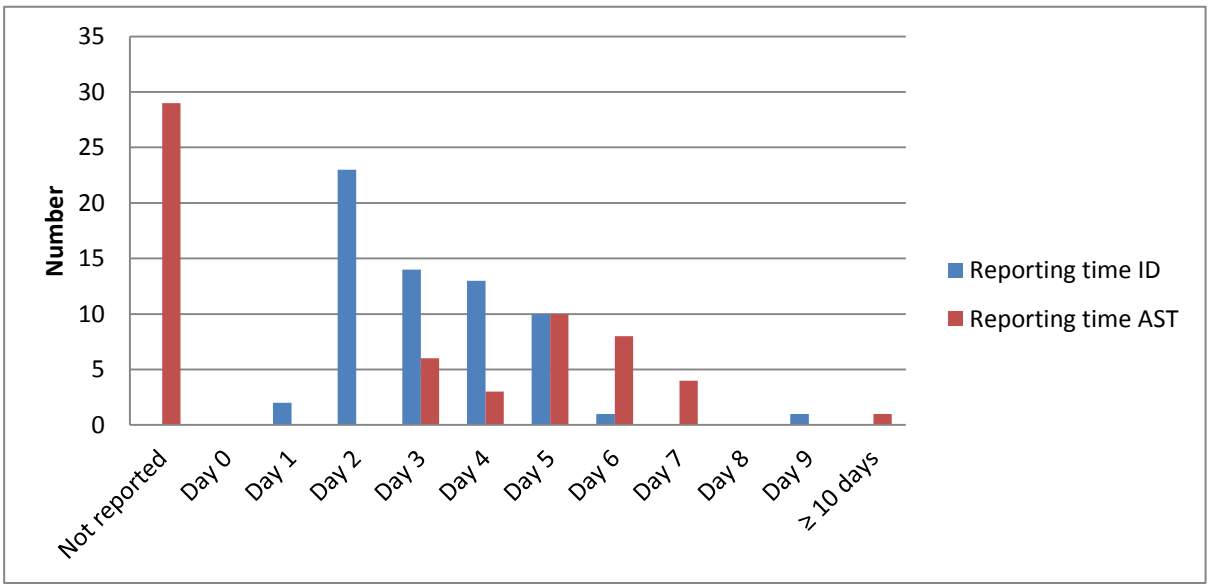


Figure 10: Reporting time of anaerobic bacteria (n=64).

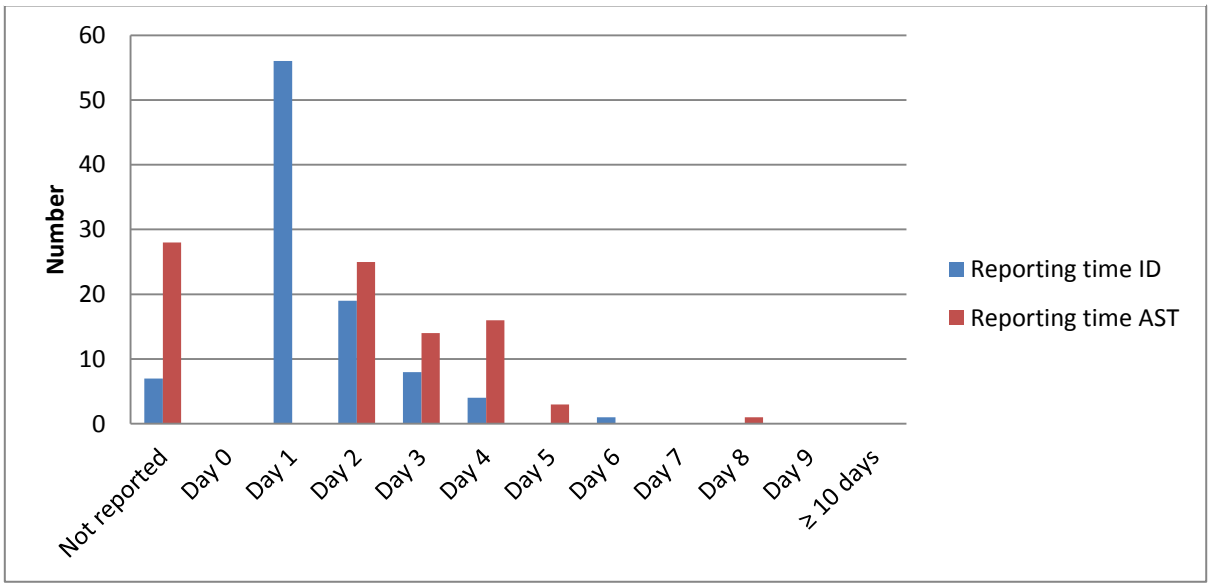


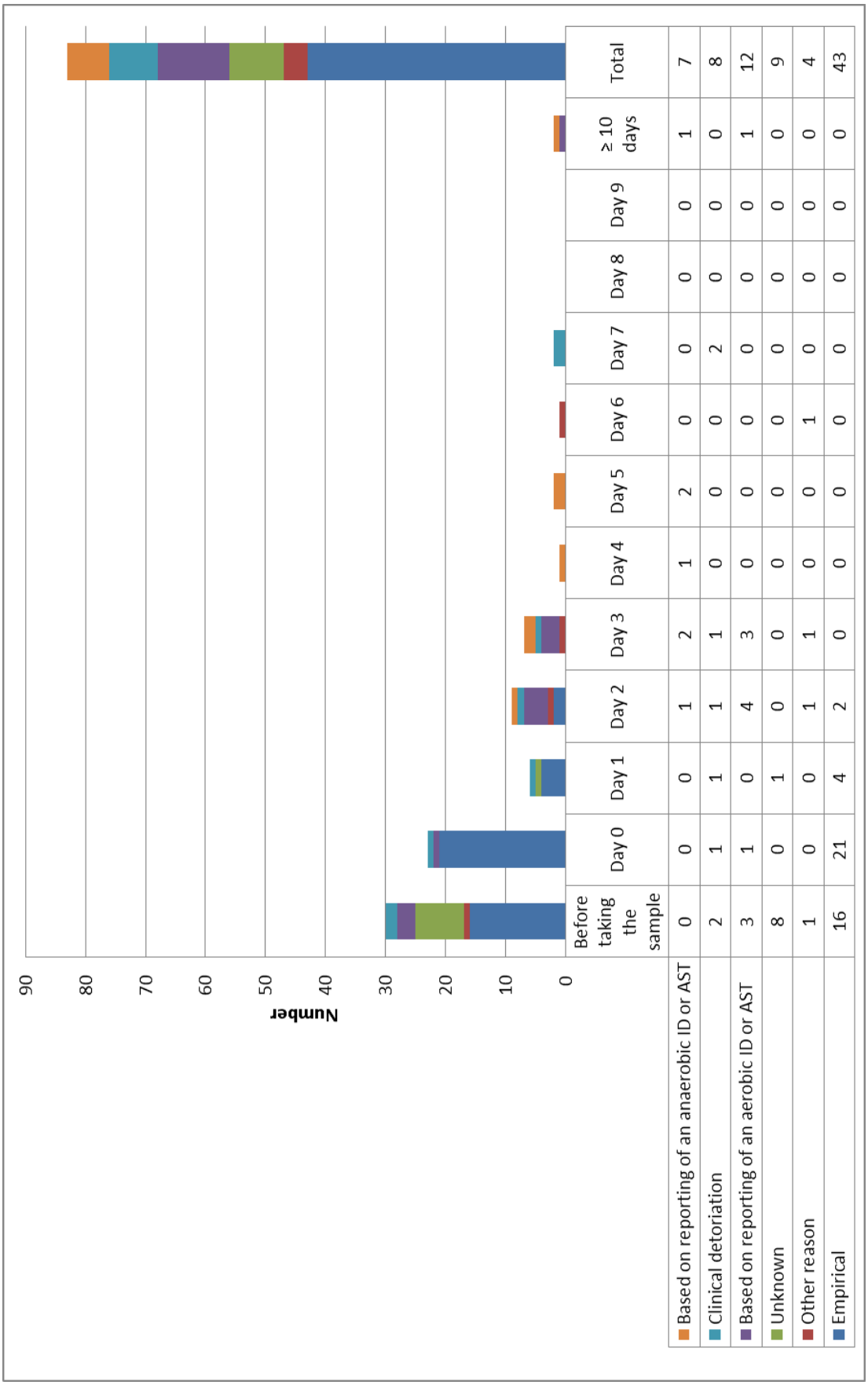
Figure 11: Reporting time of aerobic bacteria (n=95).

In conclusion, reporting aerobic identification and AST results, three and four days after sampling respectively was considered as 'late aerobic reportage'. For anaerobic bacteria reporting identification and AST results, five and seven days after sampling respectively was considered as 'late anaerobic reportage'. Overall reporting times for anaerobic cultures were longer then reporting times of aerobic cultures.

2. Is therapy regarding anaerobic infections affected by results of anaerobic cultures?

Used definitions:

- **'Unproven adequate anaerobic anti-microbial therapy':** No AST was performed but at least one anti-microbial agent with known anaerobic activity was administered empirically. Dosing and duration of therapy were correct *(84)*.
- **'Proven adequate anaerobic anti-microbial therapy':** AST of each anaerobic isolate was performed and at least one anti-microbial agent with proven susceptibility for each anaerobic isolate was administered. Dosing and duration of therapy was correct *(84)*.
- **'Proven inadequate anaerobic anti-microbial therapy':** AST of each anaerobic isolate was performed and an anti-microbial agent with proven resistance for each anaerobic isolate was administered.
- **'No anaerobic anti-microbial therapy':** No anti-microbial agent with proven or unproven anti-anaerobic activity was administered.
- **Anaerobic results 'reported timely':** Reporting anaerobic identification and AST results before day five and seven after sampling respectively was considered as timely.
- **Anaerobic results 'reported completely':** All isolated anaerobic bacteria were reported to the clinician.



	Before taking the sample	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	≥ 10 days	Total
Based on reporting of an anaerobic ID or AST	0	0	0	1	2	1	2	0	0	0	0	1	7
Clinical detoriation	2	1	1	1	1	0	0	0	2	0	0	0	8
Based on reporting of an aerobic ID or AST	3	1	0	4	3	0	0	0	0	0	0	1	12
Unknown	8	0	1	0	0	0	0	0	0	0	0	0	9
Other reason	1	0	0	1	1	0	0	1	0	0	0	0	4
Empirical	16	21	4	2	0	0	0	0	0	0	0	0	43

Figure 12: Reasons for starting or changing anti-microbial therapy in anaerobic infections (n=83).

From seven patients (14%) antimicrobial therapy is unknown. Four patients (8%) did not receive any antimicrobial therapy. In total there were 83 moments of starting or changing antimicrobial therapy in 38 different patients. Two patients received antibiotics with only aerobic activity. Reasons for starting or changing antimicrobial therapy are displayed in Figure 12.

Antimicrobial therapy was started or changed empirically 43 times (52%) in 29 different patients (76%), mainly before or on the day of sampling (day 0). In none of these 29 patients, antimicrobial therapy was changed afterwards. The number of empirically started antibiotics declines after the sampling day and becomes zero after day two because aerobic and anaerobic identification and AST results are reaching the clinicians by then. Only seven antimicrobial agents (8%) in seven different patients (18%) were started based on reportage of an anaerobic identification or AST result. Twelve antimicrobial agents (14%) were started in ten different patients (26%) based on reportage of an aerobic identification or AST result.

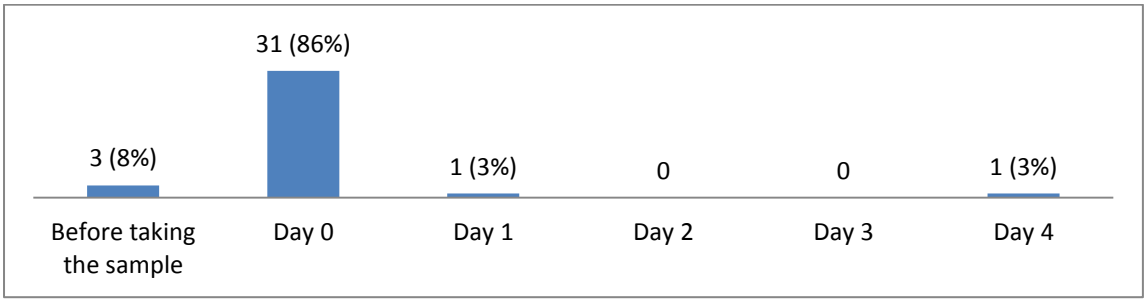
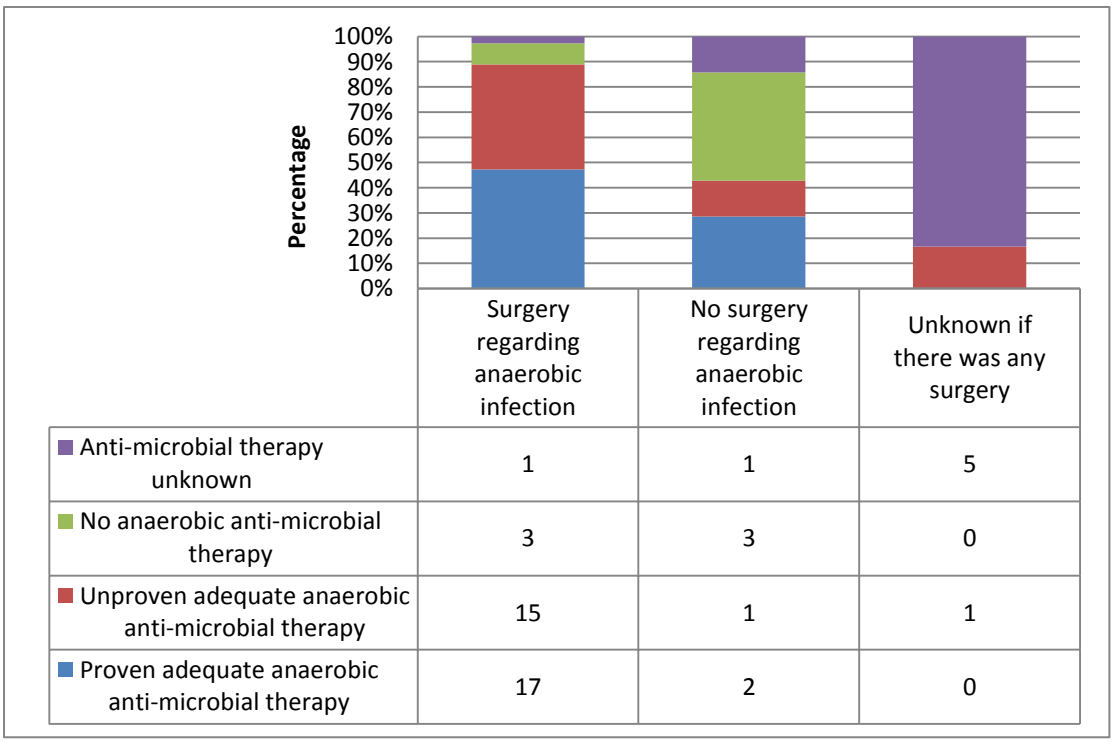


Figure 13: Timing of surgery (n=36).



	Surgery regarding anaerobic infection	No surgery regarding anaerobic infection	Unknown if there was any surgery
Anti-microbial therapy unknown	1	1	5
No anaerobic anti-microbial therapy	3	3	0
Unproven adequate anaerobic anti-microbial therapy	15	1	1
Proven adequate anaerobic anti-microbial therapy	17	2	0

Figure 14: Therapeutic interventions in anaerobic infections (n=49).

In our study 32 patients (65%) received adequate (proven or unproven) anaerobic anti-microbial therapy and underwent surgery regarding their anaerobic infection. Three patients (6%) received anaerobic anti-microbial therapy but did not underwent surgery. Three patients (6%) had no therapeutic intervention at all regarding their anaerobic infection (Figure 14). For most patients (86%) sampling was performed during a surgical intervention regarding the anaerobic infection (Figure 13).

Table 16: Way of reporting vs. starting or changing anti-microbial therapy (n=42)

	Anaerobic culturing results reported timely, completely and with AST results	Anaerobic culturing results not reported timely, completely or with AST results
Anaerobic anti-microbial therapy not adjusted based on anaerobic ID or AST	15	20
Anaerobic anti-microbial therapy adjusted based on anaerobic ID or AST	6	1

In patients whose anaerobic culturing results were reported timely, completely and with AST results, 28% of the clinicians adjusted antimicrobial therapy based on these results. In patients whose results were not reported timely, completely or with AST a significant lower number (5%; p=0,04) of clinicians adjusted antimicrobial therapy based anaerobic culture results (Table 16).

Table 17: Way of reporting vs. receiving anaerobic anti-microbial therapy (n=42)

	Anaerobic culturing results reported timely, completely and with AST results	Anaerobic culturing results not reported timely, completely or with AST results
Received anaerobic anti-microbial therapy	20	16
Did not receive anaerobic anti-microbial therapy	1	5

There was no significant proportional difference (p=0,08) between patients receiving anaerobic anti-microbial therapy, whose anaerobic culturing results were reported (95%) or were not reported (76%) timely, completely and with AST (Table 17).

We conclude that surgery in combination with anaerobic anti-microbial therapy was the most common therapeutic intervention in anaerobic infections in our survey. Antimicrobial therapy was mainly started empirically without further adaptations. Reporting anaerobic identification and AST results timely and completely did not correlate with higher starting rates of anaerobic anti-microbial therapy. An adjustment of antimicrobial therapy based on anaerobic culture results was observed in a minority of the anaerobic infections. However significantly more antimicrobial agents based on anaerobic identification or AST results were started if results were reported timely, completely and with AST.

3. **Is the outcome of patients with anaerobic infections affected by results of anaerobic cultures?**

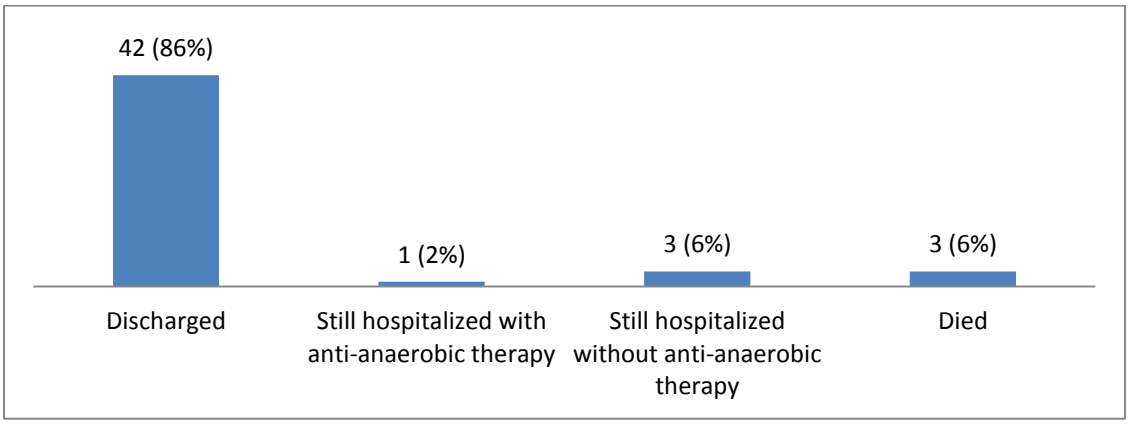


Figure 15: Patient outcome (n=49).

Table 18: Patient outcome vs. way of reporting anaerobic culture results (n=49).

	Anaerobic culturing results reported timely, completely and with AST results	Anaerobic culturing results not reported timely, completely or with AST results
Patient died	2	1
Patient survived	21	25

Table 19: Patient outcome vs. surgery regarding the anaerobic infection (n=43).

	Surgery regarding anaerobic infection	No surgery regarding anaerobic infection
Patient died	2	1
Patient survived	34	6

Table 20: Patient outcome vs. anaerobic anti-microbial therapy (n=42).

	Proven or unproven adequate anaerobic anti-microbial therapy	No anaerobic anti-microbial therapy
Patient died	2	1
Patient survived	34	5

In this study the mortality rate was 6%, which is less compared to reported mortality rates in anaerobic bacteremia (*68-70, 83*). Most patients (86%) were discharged (Figure 15). No significant difference (p=0,48) in outcome was observed between patients whose anaerobic culturing reports were (9%) or were not (4%) reported timely, completely and with AST. Fourteen percent of the patients not receiving surgery regarding the anaerobic infection died compared to 5% of the patients receiving surgery (p=0,41). Seventeen percent of the patients not receiving adequate anaerobic anti-microbial therapy regarding the anaerobic infection died compared to 5% of the patients receiving adequate anti-microbial therapy (p=0,33). The median duration of hospitalization was 8,5 days (1st

quartile 2,0 days; 3rd quartile 21,2 days). The median duration of anti-microbial therapy was 9,0 days (1st quartile 4,0 days; 3rd quartile 13,3 days). No significant differences in duration of hospitalization or antimicrobial therapy were observed for different patient groups (Table 21).

Table 21: Differences in average duration of hospitalization and anti-microbial therapy.

	Average duration of hospitalization (days)	*p-value t-test*	Average duration of anti-microbial therapy (days)	*p-value t-test*
Anaerobic culturing results reported timely, completely and with AST results	17,8	***0,18***	12,0	***0,95***
Anaerobic culturing results not reported timely, completely or with AST results	11,0		11,8	
Surgery regarding anaerobic infection	14,3	***0,54***	11,2	***0,08***
No surgery regarding anaerobic infection	9,6		3,8	
Proven adequate anaerobic anti-microbial therapy	19,3	***0,29***	11,6	***0,86***
Unproven adequate anaerobic anti-microbial therapy	12,8		12,2	

To conclude, impact on patients outcome, duration of hospitalization or anti-microbial therapy of anaerobic culture reporting practices could not be demonstrated. Although our sample size was too small and more patients should be included to gain statistical power, this small study observed a trend to higher mortality in patients not undergoing surgery or receiving adequate anaerobic anti-microbial therapy.

General conclusions

Currently there is no consensus regarding anaerobic microbiology practices. Different opinions were observed in identification and reporting procedures regarding anaerobic bacteria. Decisions on identification of anaerobic colony types is mainly based on anatomical origin of the sample, the number of anaerobic isolates and colony morphology. Reporting and performing AST of anaerobic bacteria is currently based on either anatomical origin of the sample, the number of anaerobic isolates or the kind of isolated anaerobic bacteria. From literature and guidelines only a few anaerobic species were withheld, requiring full identification and reporting with AST.

Our multi-center retrospective case study pointed out that surgery in combination with anaerobic anti-microbial therapy is the most common therapeutic intervention in anaerobic infections. Antimicrobial therapy was mainly started empirically without further adaptations. Reporting anaerobic identification and AST results timely and completely did not correlate with higher starting rates of anaerobic anti-microbial therapy. An adjustment of antimicrobial therapy based on anaerobic culture results was observed in a minority of the anaerobic infections. However

significantly more antimicrobial agents based on anaerobic identification or AST results were started if results were reported timely, completely and with AST. Impact on patients outcome, duration of hospitalization or anti-microbial therapy of anaerobic culturing reports could not be demonstrated. Although our sample size was too small, this study observed a trend to higher mortality in patients not undergoing surgery or receiving adequate anaerobic anti-microbial therapy. This study has some limitations as only patients with positive culture results are reviewed, so impact of a negative result on decisions regarding surgery or the need for continued antimicrobial therapy is not assessed. The study has no case-control setup with a small number of included patients.

To do/actions

Implementation of the clinically orientated workup for anaerobic cultures is considered. Lab technicians and clinicians should be consulted. New literature concerning anaerobic culture workup will be revised. More patients should be included in the retrospective case study in order to demonstrate impact on patient outcome.

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