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LABORATORY METHODS

Analytical Method

Identification of culturable bacteria

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VERSION HISTORY

DESCRIPTION OF CHANGES
This version has been reformatted using the new analytical methods template for authors. It provides an update of analytical techniques used to identify culturable bacteria in the IRSST laboratory, including microbial identification by mass spectrometry and by 16S rRNA gene sequencing. The MicroScan system and fatty acid profile analysis are no longer used by the IRSST for bacterial identification and have, accordingly, been removed from this version.

TARGET MICROBIAL AGENTS
Culturable facultative anaerobic and aerobic heterotrophic and mesophilic bacteria

APPLICATION

This method is used to identify culturable bacteria in air, surface, solid or liquid samples. Bacterial strains are isolated in a pure culture and then macroscopic and microscopic features of the bacterial colonies are observed and simple biochemical tests are performed. One or more of the following techniques may be used for the final identification: MALDI-TOF mass spectrometry, Sanger sequencing of the 16S rRNA gene or phenotyping of the strain using the Biolog system (biochemical tests).

LIMITATIONS AND INTERFERENCES

The performance of this method may be affected by a number of factors:

- Presence of growth conditions suitable for bacteria development
- Presence of confluent growth in culture media, making pure culture isolation difficult
- Presence of invasive microorganisms in culture media, making pure culture isolation difficult
- Maintenance of strain culturability during pure culture isolation
- Presence of target bacteria in database used

REAGENTS AND MATERIALS

- Gram staining kit
- Hydrogen peroxide 1%-5%
- Oxidase reagent
- Trypticase soy agar (TSA) or other media as needed

VITEK® MS

- Target slides: VITEK® MS-DS
- Matrix: VITEK® MS-CHCA
- Formic acid: VITEK® MS-FA

SeqStudio

- 16S Direct amplification primers
- BigDye Direct sequencing kit

Biolog

- GEN III microplates

EQUIPMENT

- Refrigerator (4°C ± 2°C)
- Incubator (37°C ± 2°C)
- Autoclave
- Biological safety cabinet
- Stereomicroscope
- Phase-contrast transmitted-light microscope with up to 100x magnification
- Micropipettes and sterile tips

VITEK® MS

- MALDI-TOF microbial identification system (*VITEK® MS, bioMérieux*)

SeqStudio

- Sequencer (*SeqStudio Genetic Analyzer, Applied Biosystems*)
- Thermocycler
- Sequence analysis software (*MicrobeBridge, Applied Biosystems)*

Biolog

- MicroLog M software

PREPARATION

Preliminary characterization

Step 1	Isolate in pure culture on TSA media all (or the specified number) of the colonies exhibiting different colonial morphologies on the initial Petri dish. Incubate at 37°C ± 2°C for 24 to 48 hours.
Step 2	For each isolate, observe and note macroscopic characteristics of bacterial colonies (size, colour, texture, media pigmentation, etc.).
Step 3	For each isolate, observe and note microscopic characteristics of bacterial cells. Prepare a wet mount to observe motility and a Gram stain to see shape, arrangement and Gram staining.
Step 4	On each isolate, perform a catalase test using 1%-5% hydrogen peroxide and an oxidase test using oxidase reagent.

Comments:

Other culture media can be used to facilitate identification of certain genera. The medium used for the initial culture generally dictates the culture medium used for the pure culture isolation.

Final identification

Step 5	Use the following analytical techniques to identify each isolate: **1.** Bacterial identification by MALDI-TOF mass spectrometry **2.** Bacterial identification by Sanger sequencing **3.** Bacterial identification by biochemical profiling The analytical techniques are typically implemented in the order shown in the schematic below. It is nonetheless possible to combine some of these techniques or to give preference to one technique in particular, depending on the context.

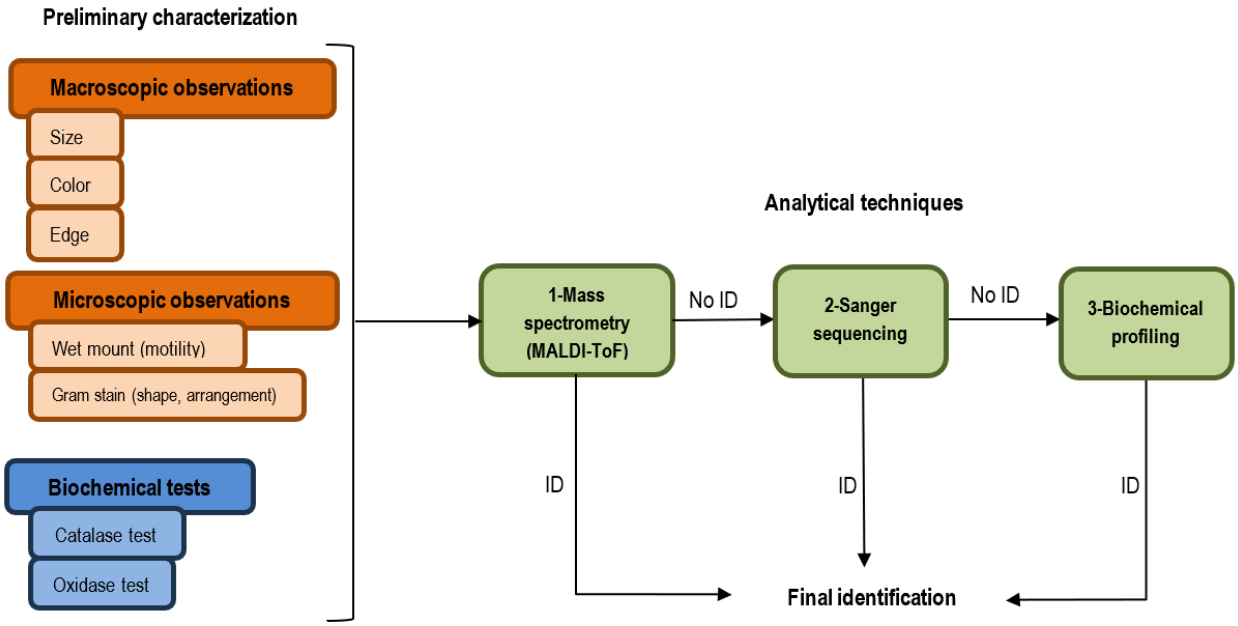

Figure 1. Schematic of bacterial identification process.

ANALYTICAL TECHNIQUE 1 – BACTERIAL IDENTIFICATION BY MALDI-TOF MASS SPECTROMETRY

Principle

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry can be used to identify bacteria by analyzing their protein profiles. Bacterial cells co-crystallized with the matrix are targeted by a laser beam. The molecules of the microorganism are then desorbed and ionized so they can be separated by size through acceleration in an electromagnetic field. The MALDI-TOF instrument measures mass-to-charge ratios of the molecules by determining the time it takes for them to travel the length of the flight tube. The protein profile of the bacteria is then compared to a database of reference spectra, allowing the instrument to return an ID of the microorganism analyzed.

SYSTEM

The bioMérieux VITEK® MALDI-TOF mass spectrometry system is used.

Each VITEK® disposable slide has a unique bar code and comes with three sections of 16 sample spots, for a total of 48 sample spots per slide. Up to four target slides (192 samples) can be run on the instrument at once.

Comments:

A single isolate will typically occupy four sample spots on a slide and will be analyzed in duplicate, with and without addition of formic acid to the target. The formic acid acts as a pretreatment before adding the matrix and seems to improve rates of accurate identification for certain types of bacteria.

Please see manufacturer's instructions for use.

VALIDATION

Note: Different strains of the same species were sometimes tested (x). As per the manufacturer's instructions, the strains were inoculated on TSA media and tested after 18 to 24 hours of incubation at 37°C.

The most recent version of the bioMérieux VITEK® MS knowledge base at the time of the internal validation was V3.2.

Group	Identification results		Comments	
	Correct	Incorrect	Expected ID	ID obtained
Gram-positive cocci				
• *Enterococcus faecalis* (3) • *Enterococcus saccharolyticus* (1) • *Kocuria rhizophila* (1) • *Micrococcus luteus* (4) • *Pediococcus acidilactici* (2) • *Staphylococcus aureus* (3) • *Staphylococcus epidermidis* (3) • *Streptococcus mutans* (4) • *Staphylococcus saprophyticus* (2) • *Streptococcus agalactiae* (1) • *Streptococcus anginosus* (1) • *Streptococcus pneumoniae* [a] (4)	29/29	-	-	-

Gram-positive rods				
• *Bacillus cereus* (3) • *Bacillus circulans* (1) • *Bacillus subtilis* (4) • *Corynebacterium minutissimum* (1) • *Corynebacterium pseudodiphtheriticum* (1) • *Lactobacillus hominis* [b] (1) • *Lactobacillus salivarius* (1) • *Listeria innocua* (2) • *Listeria monocytogenes* (1)	14/15	1/15	*Corynebacterium minutissimum*	*Corynebacterium aurimucosum* [c]
Gram-negative rods				
• *Acinetobacter baumannii* (1) • *Acinetobacter junii* (1) • *Aeromonas hydrophila* (3) • *Alcaligenes faecalis* (2) • *Citrobacter freundii* [a] (3) • *Enterobacter cloacae* (2) • *Escherichia coli* (4) • *Klebsiella aerogenes* (3) • *Klebsiella oxytoca* (2) • *Klebsiella pneumoniae* (2) • *Morganella morganii* (2) • *Proteus mirabilis* (2) • *Proteus vulgaris* (2) • *Providencia stuartii* (1) • *Pseudomonas aeruginosa* (2) • *Pseudomonas alcaligenes* (1) • *Pseudomonas fluorescens* [a] (2) • *Pseudomonas oryzihabitans* (1) • *Pseudomonas putida* (1) • *Pseudomonas stutzeri* (1) • *Serratia marcescens* (3) • *Serratia liquefaciens* (1) • *Shigella sonnei* (1) • *Shigella flexneri* (1) • *Stenotrophomonas maltophilia* (1)	41/45	4/45	*Enterobacter cloacae* (2)	*Enterobacter cloacae/ Enterobacter asburiae* [d]
			Shigella sonnei *Shigella flexneri*	*Escherichia coli* [e]

Other				
• *Aerococcus viridans* (2) • *Arcanobacterium haemolyticum* (1) • *Brevibacterium epidermidis* (1) • *Brevundimonas diminuta* (1) • *Budvicia aquatica* (1) • *Burkholderia cepacia* (2) • *Chryseobacterium indoltheticum* [b] (2) • *Dermabacter hominis* (1) • *Dermacoccus nishinomiyaensis* (1) • *Edwarsiella tarda* (1) • *Kocuria rosea* (2) • *Leclercia adecarboxylata* (1) • *Myroides odoratus* (2) • *Nocardia brasiliensis* (1) • *Oerskovia turbata* (1) • *Oligella urethralis* (1) • *Pedobacter heparinus* [b] (2) • *Rhodococcus hoagii* (1) • *Shingobacterium spiritivorum* (1) • *Sphingomonas trueperi* (1) • *Streptomyces griseus* (1)	25/27	2/27	*Myroides odoratus* (2)	No ID [f]
Total	109/116	7/116		

(a) Incubation extended to 48 hours.
(b) Species not present in the VITEK® MS V3.2. database, hence No ID is an adequate result.
(c) *Corynebacterium minutissimum* is not included in the VITEK® MS V3.2 knowledge base. BioMérieux says that testing species not in the database can yield a No ID result or misidentification.
(d) The species *Enterobacter cloacae* and *Enterobacter asburiae* cannot be distinguished by VITEK® MS, as indicated in the VITEK® MS V3.2 knowledge base. Other systems or ID tests are required to tell them apart.
(e) The species *Shigella sonnei* and *Shigella flexneri* may be identified as *Escherichia coli* because VITEK® MS cannot distinguish between these species, as indicated in the VITEK® MS V3.2 knowledge base.
(f) Though *Myroides* spp. is included in the VITEK® MS V3.2 knowledge base, there is a limitation with respect to the species *Myroides odoratus*, which is not present in this version of the database.

ANALYTICAL TECHNIQUE 2 - BACTERIAL IDENTIFICATION BY SANGER SEQUENCING

Principle

The first step in Sanger sequencing is extraction and purification of the DNA of the microorganism to be identified. A specific DNA segment, the molecular target, is first amplified by PCR. The target segment is then denatured to separate the two strands of the double-stranded DNA. A polymerization reaction is carried out with sequencing primers on both denatured strands using a DNA polymerase and the chain-terminating nucleotides dNTPs and ddNTPS. These chain-terminating nucleotides, marked with a unique fluorescent label, cannot form phosphodiester bonds with the next nucleotide in the chain. When a ddNTP is randomly incorporated in the sequence of a newly synthesized strand, the polymerization terminates. Strands of different lengths are thus obtained marked by a fluorescent label in the terminal position. The sequencer automatically separates the amplified DNA fragments by size using electrophoresis and runs them through a fluorescence detector for identification of the type of nucleotide in the terminal position of each strand. The sequencer generates a chromatogram that can be used to reconstruct the DNA sequence of the initially amplified molecular target. This sequence is used to query databases, where it can be compared with the DNA sequences of referenced microorganisms.

SYSTEM

The target gene region for bacterial identification is the 16S ribosomal RNA (rRNA) gene, that is, the smallest ribosomal subunit found in prokaryotes (Bacteria and Archaea). Sequencing of the 16S rRNA gene is a very well-known procedure used widely for classification and identification of bacteria for the following reasons:

- This gene is present in all bacteria regardless of genus and species.
- This gene includes conserved and hypervariable regions:
    - The conserved regions allow design of virtually universal primers that can be used to identify a wide variety of bacteria.
    - The hypervariable regions make it possible to differentiate between these bacteria, so their genus and sometimes their species can be determined.
- This gene is relatively short (1,500 base pairs (bp)).

Applied Biosystems' integrated 16S Direct workflow system is used for bacterial identification by PCR and Sanger sequencing. This system includes the 16S Direct amplification primers and the BigDye Direct sequencing kit.

- Two amplicons (A and B) that provide virtually full-length coverage of the 16S rRNA gene can be obtained with this kit.
- Four sequencing reactions are set up for each bacteria to be identified.
- The four sequencing reactions can be processed in as little as 30 minutes in the "short" run cycle.

Equipment used: Applied Biosystems™ SeqStudio™ sequence analyzer.

Software used for data analysis: Applied Biosystems™ MicrobeBridge™.

Databases used to analyze results: NCBI 16S rRNA and CDC's MicrobeNet.

Comments:

Please see manufacturer's instructions for use.

VALIDATION

Note: The integrated 16S Direct workflow system was validated using a collection of pre-extracted DNA samples. Validation did not, accordingly, include DNA extraction using the reagent Prepman Ultra mentioned in the manufacturer's protocol.

Criteria for bacterial identification by 16S rRNA gene sequencing

General	
Contig length	≥ 500 bp
Query cover	≥ 90%
E-value	≤ 0
Identity	**Identification level[a]**
If single species ≥ 99% identity	Species
If more than one species ≥ 99% identity	Genus
If only genus ≥ 97%	Genus

ID expected	**Taxonomic level of identification obtained (genus/species) for databases queried**				
	NCBI 16S rRNA		**CDC MicrobeNet**		**Comments[a]**
	genus	**species**	**genus**	**species**	
Gram-positive cocci					
Enterococcus faecalis		✓		✓	Identification to species level possible for some species
Micrococcus luteus	- [b]		✓		High homology at the 16S rRNA gene level in *Micrococcus* spp.
Pediococcus acidilactici		✓		✓	Identification to species level possible for some species
Staphylococcus epidermidis	✓		✓		Limited resolution for *Staphylococcus epidermidis, S. capitis* and *S. caprae*
Staphylococcus saprophyticus	✓		✓		Limited resolution for *Staphylococcus saprophyticus* and *S. xylosus*
Streptococcus agalactiae		✓		✓	Identification to species level possible for Lancefield group species
Streptococcus pneumoniae	✓		✓		*Streptococcus pneumoniae*, *S. pseudopneumoniae* and *S. mitis* cannot be differentiated at the 16S rRNA gene level
Gram-positive rods					
Bacillus cereus	✓		✓		*Bacillus cereus* and *B. anthracis* cannot be differentiated at the 16S rRNA gene level
Bacillus subtilis	✓		✓		Identification to species level impossible
Corynebacterium minutissimum	- [b]		- [b]		Limited identification to species level
Corynebacterium pseudodiphtheriticum		✓		✓	Limited identification to species level
Lactobacillus hominis		✓	✓		Identification to species level possible for some species
Lactobacillus salivarius		✓		✓	Identification to species level possible for some species
Listeria innocua	✓		✓		*Listeria innocua* and *L. monocytogenes* cannot be differentiated at the 16S rRNA gene level
Listeria monocytogenes	✓		✓		*Listeria innocua* and *L. monocytogenes* cannot be differentiated at the 16S rRNA gene level

Gram-negative rods					
Acinetobacter baumannii	✓		✓		Limited identification to species level
Aeromonas hydrophila	✓		✓		n.a.
Alcaligenes faecalis	✓		✓		Limited identification to species level for environmental isolates
Citrobacter freundii	✓		✓		Limited identification to species level
Enterobacter cloacae	✓		✓		Limited identification to species level
Escherichia coli	-		-		*Escherichia coli* and *Shigella sonnei* cannot be differentiated at the 16S rRNA gene level
Klebsiella aerogenes	- [b]		- [b]		Limited identification to species level. *K. aerogenes* is closely related to other *Klebsiella* spp., *Enterobacter* spp. and certain *Raoultella* spp.
Providencia stuartii		✓		✓	Limited identification to species level
Pseudomonas aeruginosa	- [b]		- [b]		Limited identification to species level
Pseudomonas fluorescens	✓		✓		Limited identification to species level
Shigella sonnei	-		-		*Escherichia coli* and *Shigella sonnei* cannot be differentiated at the 16S rRNA gene level
Stenotrophomonas maltophilia	✓			✓	Identification to species level possible for some species
Other					
Burkholderia cepacia	✓		✓		Limited identification to species level; consider reporting as *Burkholderia cepacia* complex
Myroides odoratus	✓		- [b]		Limited identification to species level
Pedobacter heparinus		✓	✓		n.a.
Rhodococcus hoagii	✓			✓	Identification to species level possible for some species
Streptomyces griseus	✓		✓		Limited identification to species level

[a] Indications from the Clinical & Laboratory Standards Institute (CLSI).
[b] According to the CLSI, 16S rRNA gene sequencing should allow genus identification.
n.a.: not applicable to this genus.

ANALYTICAL TECHNIQUE 3 - BACTERIAL IDENTIFICATION BY BIOCHEMICAL PROFILING

Principle

Bacterial identification by biochemical properties is more specifically based on the capacity of an unknown microorganism to use different carbon sources and resist different inhibitory chemicals. A 96-well microplate prefilled with a variety of nutrients and reagents is inoculated with a standardized bacterial suspension of the isolate to be identified. Growth capacity of the microorganism in each well after incubation is indicated by a change in color of the colorimetric indicator. The pattern of colored wells is visually observed and manually recorded in the software. The pattern is then compared to a database of reference patterns, allowing identification of the bacteria analyzed.

SYSTEM

The biochemical analyses are performed on 96-well microplates using the Biolog Gen III MicroLog M system.

Comments:

Please see manufacturer's instructions for use.

REFERENCES

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