COLLEGE of AMERICAN PATHOLOGISTS

Surveys and Anatomic Pathology Education Programs

Bacteriology
D-A 2016

Final Critique

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2016 D-A
FINAL CRITIQUE

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COLLEGE of AMERICAN PATHOLOGISTS

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This activity is approved for continuing education credit in the states of California and Florida.

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Learning Objectives

Upon completing the reading and answering the learning assessment questions, you should be able to:

1. Describe the typical colonial morphology, growth requirements, and clinical significance of isolated organisms.
2. State the key, distinguishing characteristics of the isolated organism.
3. Ensure the organism's identification is consistent with the source and clinical setting.
4. Identify appropriate reporting/interpretation of antimicrobial susceptibilities for an organism, considering the source of the specimen and any relevant clinical information.

This challenge was a simulated blood specimen from a neonate with bacteremia. Participants were asked to report all organisms and perform ungraded susceptibility for the principal pathogen. The challenge contained *Leuconostoc mesenteroides*. A response of *Leuconostoc mesenteroides; Leuconostoc* sp.; Gram-positive cocci, aerobic, not staphylococcus, streptococcus, or enterococcus; Gram-positive cocci, aerobic; Gram-positive coccobacilli, aerobic or was considered satisfactory.

D-01

Identification	Referees (53) No.	Referees (53) %	Participants (1865) No.	Participants (1865) %
Leuconostoc mesenteroides	23	43.4	775	41.5
Leuconostoc sp.	26	49.1	781	41.9
Gram-positive cocci, aerobic, not staphylococcus, streptococcus, or enterococcus	-	-	15	0.8
Gram-positive cocci, aerobic	1	1.9	177	9.5
Gram-positive coccobacilli, aerobic	-	-	15	0.8
Consensus for correct identification of organism	50	94.3	1763	94.5

Table 1. Antimicrobials with CLSI breakpoints that may be appropriate for specimen source.

D-01 *L. mesenteroides*

Antimicrobial	Disk Participants #	Disk Participants S	Disk Participants I	Disk Participants R	MIC Participants #	MIC Participants S	MIC Participants I	MIC Participants R
Ampicillin	See Table 2.				141	109	25	7
Chloramphenicol	See Table 2.				65	60	4	1
Gentamicin	See Table 2.				64	64	-	-
Minocycline	See Table 2.				36	36	-	-
Penicillin	See Table 2.				330	252	66	12
Vancomycin	See Table 2.				105	See Table 2.	-	89

Table 2. Antimicrobials without CLSI breakpoints and/or inappropriate for specimen source

D-01 *Leuconostoc mesenteroides*

Antimicrobial	Disk Participants #	Disk Participants S	Disk Participants I	Disk Participants R	MIC Participants #	MIC Participants S	MIC Participants I	MIC Participants R
Ampicillin	29	23	1	5	See Table 1.			
Azithromycin	7	7	-	-	12	12	-	-
Cefepime	7	3	-	4	19	13	-	6
Cefotaxime	9	2	-	7	41	15	3	23
Ceftriaxone	20	2	1	17	66	17	2	47
Chloramphenicol	16	13	3	-	See Table 1.			
Clindamycin	51	50	-	1	55	54	1	-
Erythromycin	50	48	1	1	57	56	-	1
Fosfomycin +	1	-	-	1	-	-	-	-
Levofloxacin	20	17	1	2	42	40	1	1
Gentamicin	18	18	-	-	See Table 1.			
Minocycline	1	1	-	-	See Table 1.			
Linezolid	16	16	-	-	12	11	-	1
Penicillin	29	23	-	6	See Table 1.			
Tetracycline	28	21	5	2	24	20	1	3
Trimethoprim-Sulfamethoxazole	12	1	-	11	10	7	-	3
Vancomycin	66	3	-	63	105	16	-	See Table 1.

S – Susceptible; I – Intermediate; R – Resistant

\+ Inappropriate use of antimicrobial – for reporting on urine isolates only.

Extended spectrum beta-lactamase, beta-lactamase, carbapenemase, D-zone, and HLAR results are not shown. This testing is considered to be extraneous or not applicable for this organism.

Taxonomy

Leuconostoc species are members of the phylum *Firmicutes*, low-G+C, gram-positive bacteria.[1] They are intrinsically vancomycin-resistant, catalase-negative, gram-positive cocci.[2]

Identification

This isolate grew as small gray, alpha-hemolytic colonies on sheep blood agar and greenish colonies on chocolate agar after 24 hours incubation at 35° in 5% CO_2 (Figure 1). Gram stain of the colonies showed a gram-positive coccus, but can be cocco-bacillary. When a Gram stain is performed from broth, the cocci will be in chains. *Leuconostoc* species are pyrrolidonyl arylamidase (PYR) and leucine amino peptidase (LAP) negative and intrinsically resistant to vancomycin. Vancomycin resistance can be tested by streaking several colonies on a trypticase soy agar with 5% sheep blood plate and placing a 30-µg vancomycin disk in the center of the inoculated area. After overnight incubation at 35° C in a CO_2-enriched atmosphere, any zone of inhibition indicates susceptibility, while resistant isolates exhibit no zone of inhibition.[3] They can also grow in 6.5% sodium chloride. *Leuconostoc* species produce gas from glucose in deMan, Rogosa and Sharpe (MRS) broth and are arginine negative.

Commercially available identification kits or systems are improving in their ability to identify *Leuconostoc* species[4] although there can be difficulty due to phenotypic variation among isolates of the same species, relative metabolic inactivity, and a relatively small number of strains available for inclusion in databases. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI) is a reliable means of identifying *Leuconostoc* species.[5] Sequencing might be necessary for difficult-to-identify isolates.[4]

Clinical Significance

Leuconostoc species are environmental organisms that can be found on vegetable matter and in milk products.[3] *Leuconostoc* was first recognized in clinical specimens in the mid-1980s. They were associated with host defense impairment, invasive procedures, gastrointestinal symptoms, and prior antibiotic treatment.[6] *Leuconostoc* has been isolated from blood, cerebrospinal fluid, peritoneal dialysate fluid, and wounds. There have been case reports of osteomyelitis, ventriculitis, a brain abscess, postsurgical endophthalmitis, and bacteremia in the setting of short gut syndrome. Short gut syndrome leads to microbiota that has a high prevalence of *Lactobacillus* and *Leuconostoc*.[7]

Antimicrobial Resistance and Therapy Considerations

Leuconostoc spp. are somewhat unusual gram-positive cocci, since they are typically susceptible to penicillin and ampicillin but intrinsically resistant to vancomycin. Resistance of *Leuconostoc* spp. to vancomycin and other glycopeptide antimicrobial agents is due to utilization of a peptidoglycan precursor terminating in lactate rather than alanine (vancomycin exerts its effect by binding to the D-alanyl-D-alanine terminus of peptidoglycans).[8] Although this mechanism of resistance is similar to acquired vancomycin resistance in enterococci, the resistance in *Leuconostoc* is inherent. Vancomycin should not be tested, and, if it is reported, it should be reported as resistant.

The current CLSI M45-A3 guidelines covering susceptibility testing of infrequently isolated or fastidious bacteria including *Leuconostoc* species was published in 2015.[9] Broth microdilution MIC testing recommendations (not disk diffusion) and interpretive breakpoints are available for a variety of antimicrobial agents for *Leuconostoc* spp. The test medium is cation-adjusted Mueller-Hinton broth with 2-5% lysed horse blood and incubation is in ambient air. Agents which laboratories should consider for primary testing include penicillin and ampicillin, and isolates from normally sterile sources such as blood

cultures or deep tissue infections should be tested. Thus, the clinical history of a neonate with bloodstream infection from this proficiency challenge should have prompted laboratories to perform susceptibility testing, if consistent with their laboratory practices. Reasons that laboratories may not have tested this isolate include:

- Perception that the isolate did not warrant testing or empiric therapy would be used anyway,
- Lack of available methods to test the isolate,
- Inadequate growth of the isolate for testing, or
- Combination of the above factors.

Specimen source and clinical history are important in guiding appropriate antimicrobial susceptibility testing and reporting.

Susceptible-only breakpoints for penicillin and ampicillin are included in the current M45 guidelines.[10] Although gentamicin breakpoints for *Leuconostoc* were included in previous versions of M45, they are no longer listed. Gentamicin is not administered alone for the treatment of *Leuconostoc* infections, and prior inclusion of breakpoints for gentamicin was thought to be misleading. Therapy of serious infections such as endocarditis due to *Leuconostoc* often involves combined therapy with a penicillin and gentamicin. In these cases, gentamicin results are not useful, and previous experiences of clinicians with the combination regimen would guide the treatment decision. There is a note in CLSI M45 that addresses the combined therapy option, and it is possible that some laboratories may consider including a comment on their laboratory reports to suggest that combined therapy be considered. CLSI breakpoints are available for minocycline (the most active of the tetracyclines against *Leuconostoc*) and chloramphenicol.

Clindamycin is an alternative treatment to penicillin G or ampicillin, as noted by the Sanford Guide to Antimicrobial Therapy.[11] However, no CLSI breakpoints currently exist for clindamycin. In their assessment of MICs ranges of 43 clinical isolates of *Leuconostoc* spp. by broth microdilution, Swenson et al reported relatively low clindamycin MIC50 (MIC which inhibits growth of 50% of isolates) of 0.015 µg/mL and MIC90 of 0.06 µg/mL.[12]

The treatment of choice is penicillin or ampicillin; to date, resistance to these agents using the current breakpoints have not been reported.[12-16] Other antimicrobial agents including the macrolides, linezolid, and cephalosporins have been reported with some success in the treatment of infections due to *Leuconostoc*.[13] However, CLSI interpretive criteria do not currently exist for these agents. Elevated MICs to the cephalosporins as well as the carbapenems have been noted on several occasions. In their survey of 43 *Leuconostoc* spp., Swenson and colleagues reported elevated MICs to a variety of cephalosporins with MIC50s and MIC90s ranging from 8-64 µg/mL.[12] Deye et al reported elevated MICs to carbapenems using broth microdilution testing with lysed horse blood-supplemented Mueller-Hinton broth (imipenem 4 µg/mL; ertapenem >8 µg/mL; meropenem >4 µg/mL) in a *Leuconostoc lactis* isolate from a patient with a ventricular shunt who presented with ventriculitis.[14] Collins and colleagues likewise noted elevated MICs to imipenem in 24 *Leuconostoc* isolates using agar dilution methodology (MIC50 = 4 µg/mL; MIC90 = 8 µg/mL).[15]

A more recent survey by Huang et al of 68 *Leuconostoc* isolates obtained from clinical infections reported elevated MICs to linezolid (range 0.5-8 µg/mL; MIC90 = 4 µg/mL) as measured by broth microdilution.[16] They also compared daptomycin Etest to broth microdilution and reported that Etest did not correlate well with broth microdilution, leading to MICs ≥2-fold lower for 16.2% of the isolates.

Key Points

- *Leuconostoc* species are catalase-negative, PYR and LAP negative gram-positive cocci.
- *Leuconostoc* species have been associated with infections in patients with short gut syndrome.
- *Leuconostoc* species are typically susceptible to penicillin and ampicillin but are intrinsically resistant to vancomycin.
- Penicillin and ampicillin should be considered by laboratories for primary testing of *Leuconostoc* spp.
- Elevated MICs to carbapenems and cephalosporins have been noted.
- CLSI disk diffusion breakpoints for *Leuconostoc* sp. do not exist.

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This challenge was a simulated stool specimen from a male with watery, non-bloody diarrhea accompanied by nausea and vomiting. Participants were asked to report the stool pathogen. The challenge contained *Escherichia coli, Enterococcus faecalis,* and *Plesiomonas shigelloides*. A response of Positive for *Plesiomonas shigelloides*; or Presumptive *Plesiomonas shigelloides*, would refer if needed; AND a response of Negative or Not Tested for all other stool pathogens (*Aeromonas*, *Campylobacter, Escherichia coli*, serogroup O157, *Salmonella, Shigella, Vibrio,* and *Yersinia*) were considered satisfactory.

D-02

Aeromonas	**Referees (50) No.**	**%**	**Participants (1477) No.**	**%**
Negative for *Aeromonas* species	50	100.0	1475	99.9
Campylobacter	**Referees (49) No.**	**%**	**Participants (1488) No.**	**%**
Negative for *Campylobacter* species	49	100.0	1488	100.0
***Escherichia coli,* serogroup O157**	**Referees (47) No.**	**%**	**Participants (1392) No.**	**%**
Negative for *Escherichia coli*, serogroup O157	47	100.0	1389	99.8
Plesiomonas	**Referees (51) No.**	**%**	**Participants (1664) No.**	**%**
Positive for *Plesiomonas shigelloides*	50	98.0	1547	93.0
Presumptive *Plesiomonas shigelloides*, would refer if needed	1	2.0	109	6.6
Salmonella	**Referees (53) No.**	**%**	**Participants (1770) No.**	**%**
Negative for *Salmonella* species	53	100.0	1768	99.9
Shigella	**Referees (53) No.**	**%**	**Participants (1770) No.**	**%**
Negative for *Shigella* species	53	100.0	1765	99.7
Vibrio	**Referees (39) No.**	**%**	**Participants (1239) No.**	**%**
Negative for *Vibrio* species	39	100.0	1232	99.4
Yersinia	**Referees (48) No.**	**%**	**Participants (1387) No.**	**%**
Negative for *Yersinia* species	48	100.0	1386	99.9

Taxonomy

Plesiomonas shigelloides is the only member of the genus *Plesiomonas* and is the only oxidase-positive member of the Enterobacteriaceae family.[1]

Identification

Plesiomonas shigelloides grows well on sheep blood agar and most enteric media. On sheep blood agar, colonies are gray, shiny, smooth, and non-hemolytic. Colonies will be non-lactose and non-sucrose-fermenting on enteric media (Figure 2), so they might be confused with *Shigella* species. *Plesiomonas shigelloides* is oxidase and indole-positive and ferments glucose. It can be distinguished from *Aeromonas* species and other oxidase-positive organisms based on positive inositol but negative mannitol fermentation, positive reactions in Moeller's lysine, arginine, and ornithine and the absence of DNAse or extracellular protease production.

Automated biochemical identification systems can correctly identify *P. shigelloides* as can matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.[2]

Clinical Significance

The primary habitats of *P. shigelloides* are fresh water and marine estuaries in tropical and temperate climates and it is a cause of waterborne infections.[3] It is also found in amphibians, birds, fish, and animals. It is considered an emerging and significant enteric pathogen of water- and foodborne human infections.[1] It is also a major cause of traveler's diarrhea in Japan and China.[3] There are 3 major types of gastroenteritis caused by *P. shigelloides*: a secretory, watery type; an invasive, dysentery-like type; and a subacute or chronic form that can last between 2 weeks and 3 months.[4] Outbreaks have been related to consumption of seafood or untreated water. Infections with *P. shigelloides* have also been implicated in traveler's diarrhea, most commonly in the warm months in tropical countries. Gastrointestinal infections are usually self-limited.

Plesiomonas shigelloides can rarely be the cause of extra-intestinal infections such as meningitis in neonates, bacteremia, sepsis and septic shock with high fatality rates.[1] *Plesiomonas* bacteremia is usually community acquired, rare and polymicrobic.[5] The major risk factors for bacteremia are biliary tract disease and advanced age (>75 years).

Antimicrobial Resistance and Therapy Considerations

Most gastrointestinal *Plesiomonas* infections are self-limiting and do not require antimicrobial treatment. *Plesiomonas* has been moved from the M45 CLSI document covering antimicrobial susceptibility testing of infrequently isolated bacteria and is now included in the M100 CLSI document under Enterobacteriaceae.[6] *Plesiomonas* is typically resistant to ampicillin, carbenicillin, piperacillin, and ticarcillin and is variably resistant to most aminoglycosides and tetracycline. Ceftriaxone, quinolones, amoxicillin-clavulanic acid, carbapenems, and trimethoprim-sulfamethoxazole show good activity against *P. shigelloides*.[1]

Key Points

- *Plesiomonas shigelloides* is the only oxidase-positive member of the Enterobacteriaceae family.
- The most common infections caused by *P. shigelloides* are gastrointestinal and associated with water or seafood.
- Diarrhea caused by *P. shigelloides* is usually self-limited. If treatment is needed, cephalosporins, quinolones, carbapenems, and sulfamethoxazole could be used.

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This challenge was a simulated peritoneal fluid specimen from a child with a ruptured appendix. Participants were asked to report all organisms. The challenge contained *Escherichia coli* and *Bacteroides ovatus.* A response of *Escherichia coli*; *Escherichia* sp.; Gram-negative bacilli, Enterobacteriaceae; or Gram-negative bacilli, aerobic; AND a response of *Bacteroides ovatus*; *Bacteroides fragilis* group; *Bacteroides* sp.; Gram-negative bacilli, anaerobic; or Anaerobe isolated, referred for identification were considered satisfactory.

D-03

Identification	Referees (53) No.	%	Participants (1878) No.	%
Escherichia coli	53	100.0	1862	99.2
Escherichia sp.	-	-	3	0.2
Gram-negative bacilli, Enterobacteriaceae	-	-	-	-
Gram-negative bacilli, aerobic	-	-	3	0.2
Bacteroides ovatus	19	35.9	595	31.7
Bacteroides fragilis group	17	32.1	490	26.1
Bacteroides sp.	13	24.5	324	17.3
Gram-negative bacilli, anaerobic	3	5.7	99	5.3
Anaerobe isolated, referred for identification	-	-	148	7.9
Consensus for correct identification of all organisms	50	94.3	1605	85.4

Members of the *Bacteroides fragilis* group are presumed to be beta-lactamase positive.

Taxonomy and identification

Escherichia coli is a commensal of the human gut, and among the most commonly isolated organisms in the clinical microbiology laboratory. A member of the Enterobacteriaceae family, this facultative gram-negative rod is oxidase-negative, ferments glucose, and reduces nitrate to nitrite. It is indole-positive and non-spore forming. *Escherichia coli* grows well on sheep blood agar and other media, demonstrating lactose fermentation on MacConkey agar. Automated systems and kits successfully identify this organism. Barring specific epidemiologic situations, *E. coli* is not typically further characterized beyond genus and species for clinical purposes.

Taxonomy within the genus *Bacteroides* was extensively revised over recent decades primarily as a result of molecular phylogenetic studies using 16S rRNA gene sequencing. *Bacteroides ovatus* remains a member of the *B. fragilis* group of obligate anaerobic gram-negative rods, along with *B. thetaiotaomicron*, *B. vulgaris*, *B. uniformis, B. eggerthii, Parabacteroides distasonis*, and *B. fragilis*. *Bacteroides ovatus* grows as gray (nonpigmented), circular, convex, and entire colonies on Brucella agar after 24-48 hours at 35°C under anaerobic conditions (Figure 3). Blackening around colonies is observed on Bacteroides bile esculin (BBE) agar, reflecting bile resistance and esculin hydrolysis. Key characteristics among the *B. fragilis* group that may be used for initial testing include resistance to vancomycin, kanamycin, and colistin using special potency disks, and rapid tests for catalase, indole, esculin, and alpha-fucosidase.[1,2] Additional biochemical features of *B. ovatus* used in traditional identification include fermentation of arabinose, rhamnose, sialicin, sucrose, trehalose, xylose, and xylan.[2] Commercially available test kits assess preformed enzyme and carbohydrate fermentation profiles; these require sufficient inoculum for optimal

performance. Gram stain of *B. ovatus* shows short gram-negative rods that may appear somewhat ovoid, and may stain weakly.

Due to the time, effort, and skill required to accurately identify anaerobes with traditional biochemical methods, or with gas chromatography of volatile fatty acid end products of glucose metabolism, many laboratories have implemented alternative and often more rapid approaches to anaerobe identification such as MALDI-TOF mass spectrometry and/or 16S rRNA gene sequencing. *Bacteroides ovatus* is represented in the FDA-cleared Bruker Daltonics MALDI Biotyper CA system database (reference library), currently as "*Bacteroides ovatus* group." [3] The FDA-cleared MALDI system VITEK® MS v2.0 database contains *B. ovatus* (BioMérieux, Inc.), and in a recent multicenter trial, this organism was identified to species level using MALDI analysis in 85% of challenge isolates whose identity had been determined using molecular methods.[4] Earlier studies in 2009[5,6] and 2011[7] emphasized the discriminatory power of MALDI-TOF MS for identification of anaerobes as well as the need for adequate representation of strains in MALDI databases. Improvements along these lines appear to continue to enhance the performance of this technology for anaerobe identification. As this method development and deployment continues in more clinical laboratories, the complementary technology of partial or full 16S rRNA gene sequencing is often used as a comparator gold standard[8] in test evaluations and for clinical purposes. However, 16S rRNA gene sequencing is technically challenging, potentially expensive, and may not be easily available in the majority of clinical laboratories. For clinically important isolates and/or unclear results using other techniques, use of gene sequencing at a reference laboratory may assist in resolving diagnostic uncertainty.

Clinical significance

Peritonitis is among the more severe complications of acute appendicitis. This polymicrobial infection results from a breach in the integrity of the gut epithelial barrier after severe inflammation, with leakage of intraluminal contents into the normally sterile peritoneal cavity. Infection is initially predominated by aerobic/facultatively anaerobic organisms such as *E. coli*, creating local conditions under which anaerobes may proliferate.[9] The underlying etiology of acute appendicitis, particularly the role of individual microbes, remains a focus of study in both adults and children.[10] Mixed aerobic and anaerobic flora are found in cultures of peritoneal fluid, typically *E. coli* with *B. fragilis* group.[9] The most common among the aerobic/facultatively anaerobic organisms recovered from peritoneal fluid was *E. coli* (81%), followed by *Streptococcus anginosus* group (formerly known as *S. milleri* group, 12%), and *Pseudomonas aeruginosa* (12%) in a study of adults and children with peritonitis secondary to perforated appendicitis.[11] Although many anaerobes may be found, *B. fragilis* group was identified frequently in cultures of appendiceal tissue removed from children with suspected acute appendicitis, with *B. fragilis* found overall in 73% and *B. ovatus* in 54% of cultures. This study also reported *E. coli* (88%) and *S. anginosus* group (61%) as the most commonly isolated aerobes.[12] It is essential to isolate and report organisms in the *B. fragilis* group, as these are well known to demonstrate increased virulence as well as antimicrobial resistance.[1]

Antimicrobial resistance and therapy implications

Because many members of the *B. fragilis* group exhibit significant antimicrobial resistance, reporting these organisms should prompt the clinician to assure appropriate antimicrobial coverage. *Bacteroides fragilis* group organisms are presumed to be positive for beta-lactamase and do not need to be routinely tested for this enzyme, and these organisms are presumed to be resistant to penicillin and ampicillin see CLSI M100S-26th edition.[13] Although a growing area of interest, clinical laboratory practices vary regarding routine performance of susceptibility testing for anaerobes.[14,15]

Key points

- *Bacteroides ovatus* is a member of the *B. fragilis* group, along with *B. thetaiotaomicron*, *B. vulgaris*, *B. uniformis, B. eggerthii, Parabacteroides distasonis*, and *B. fragilis*.
- Peritonitis related to a ruptured appendix is expected to grow aerobic/facultative as well as anaerobic organisms from a specimen of peritoneal fluid.
- Identification and reporting of anaerobes enable the clinician to choose appropriate antimicrobial therapy for these organisms.

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This challenge was a bronchoalveolar lavage specimen from an intensive care unit (ICU) patient with pneumonia. Participants were asked to report the principal pathogen only. The challenge contained *Klebsiella oxytoca* and *Neisseria subflava*. A response *Klebsiella oxytoca; Klebsiella* sp.; Gram-negative bacilli, Enterobacteriaceae; or Gram-negative bacilli, aerobic was considered satisfactory.

D-04

Identification	Referees (53) No.	%	Participants (1885) No.	%
Klebsiella oxytoca	53	100.0	1833	97.2
Klebsiella sp.	-	-	32	1.7
Gram-negative bacilli, Enterobacteriaceae	-	-	1	0.1
Gram-negative bacilli, aerobic	-	-	6	0.3
Consensus for correct identification of organism	53	100.0	1872	99.3

Taxonomy

Klebsiella oxytoca belongs to the family of Enterobacteriaceae. Many revisions to the classification of Enterobacteriaceae have been made over the past decades, resulting in occasional confusion among clinical microbiologists and regulatory authorities responsible for the monitoring and control of these organisms.[1] Many of the genera of *Klebsiella pneumoniae* subsp*. ozaenae*, *Klebsiella pneumoniae* subsp. *rhinoscleromatis* are composed of heterogeneous groups of species, which were originally described and classified based on their morphological, biochemical, and phenotypic appearance. In 2003, in a study a 16S rDNA-based phylogenetic tree of the *Klebsiella* genus was constructed and shown to encompass five closely related clusters.[2] This 16S rDNA tree for *Klebsiella* species was in agreement with existing DNA-DNA hybridisation and numerical taxonomy data. In another study in 2008, the genus *Klebsiella* was classified into seven species and subspecies, including *Klebsiella pneumoniae* subsp. *pneumoniae, Klebsiella pneumoniae* subsp. *ozaenae, Klebsiella pneumoniae* subsp. *rhinoscleromatis, Klebsiella oxytoca, Klebsiella planticola, Klebsiella terrigena*, and *Raoultella* (*Klebsiella) ornithinolytica*.[3] Sequence and length polymorphisms of ITS regions have been increasingly used as tools for the identification of bacterial species and/or subspecies. The 16S-23S rRNA gene internal transcribed spacer (ITS) regions of *Klebsiella* spp. are highly conserved within the species or subspecies, however, studies demonstrated that sufficient variations are present to allow differentiation between most of the species. While improvements in DNA sequence-based analyses and whole-genome sequencing analyses have greatly enhanced the understanding of *Enterobacteriaceae*, and likely will have a great influence on future taxonomy changes for these organisms, most of the clinically relevant bacterial species in these groups are still reliably identified by manual and commercial identification systems that are based on biochemical/phenotypic profiles.

Identification

Bacteria within the family of Enterobacteriaceae, including *Klebsiella oxytoca*, are gram-negative, facultative anaerobic rods or coccobacilli. They are typically oxidase-negative and do not form spores. *Klebsiella* species, like many other Enterobacteriaceae are readily isolated from clinical material and grow on agar media routinely used in clinical microbiology laboratories. *Klebsiella pneumoniae* and *Klebsiella oxytoca* are the two most frequently encountered *Klebsiella* species giving rise to infections in humans; however, other *Klebsiella* species can also be found in clinical specimens: *Klebsiella pneumoniae* subsp*. ozaenae, Klebsiella pneumoniae* subsp*. rhinoscleromatis*, *Klebsiella terrigena*, *Klebsiella planticola*, *Raoultella* (*Klebsiella) ornithinolytica*.[1,4] In most clinical microbiology laboratories, strains of *Klebsiella* are currently identified by using automated instruments such as the Microscan, Phoenix, Vitek and API systems which are largely based on classical biochemical tests. While these systems have a reliable and good performance for the two most common *Klebsiella* species, the identification for some of the other

species, however, is at times difficult, because some of the species share similar biochemical profiles.[1,3] However, a study by Hansen et al suggested that it is possible to differentiate *K. pneumoniae* subsp. *pneumoniae* from *K. oxytoca* and other species with 18 biochemical tests.[4] Following the protocols suggested by MacFaddin, *Klebsiella* species could be differentiated by using a basic group of biochemical tests, including the oxidase test; glucose and lactose or sucrose fermentation, gas and H_2S production in triple sugar iron agar; motility and indole production in sulfide indole motility medium; citrate and malonate utilization; arginine, lysine, and ornithine decarboxylation; phenylalanine deamination; urease production; adonitol fermentation; and methyl red and Voges-Proskauer tests.[5] Supplementary tests included growth at 10°C and l-sorbose fermentation and histamine and d-melezitose assimilation.[5] *Klebsiella oxytoca* will grow at 44°C but not at 10°C; in practice, the clinically most important *Klebsiella* species can be distinguished by tests for indole production, ornithine decarboxylase production, the Voges-Proskauer reaction, malonate utilization and o-nitrophenyl-β-D-galactopyranoside (ONPG) production. Specifically *K. oxytoca* and *K. pneumoniae* are distinguished by the indole production, with *K. oxytoca* being indole positive whereas *K. pneumoniae* is negative. Furthermore, *K. oxytoca* has a positive Voges Proskauer reaction, is ONPG positive, but ornithine decarboxylase negative. In a few studies, however, clinical bacterial isolates identified as *K. oxytoca* by commercial identification systems had been subsequently identified to be *Raoultella ornithinolytica* using genotypic systems.[6,7]

More recently, Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry has been shown to be another useful tool for the identification of *Enterobacteriaceae;* in a recent study, 100% of tested *K. oxytoca* and *K. pneumoniae* species were accurately identified.[8] Lastly, *K. oxytoca* has been included in some systems for the detection of organisms directly from positive blood cultures. One study provided a comprehensive evaluation of this methodology for identification of select gram-positive and gram-negative organisms, and demonstrated a resolved sensitivity of the assay for *K. oxytoca* of 98.3%.[9]

Clinical Significance

Klebsiella species are opportunistic human pathogens that can be isolated from various animal and human clinical specimens. Furthermore, *Klebsiella* species are present in the nasopharynx as well as the bowel as part of the transient “normal” flora. However, it should be recognized that feces are the most significant source of patient infections.[1,10] *Klebsiella* species account for a variety of community-acquired and healthcare associated/acquired infections. While *K. pneumoniae* is the most common *Klebsiella* species accounting for disease, *K. oxytoca* is likely a close second in causing disease, and has specifically has been reported as the cause of bacteremia/sepsis, pneumonia, urinary tract, burn wound infections, albeit, it is overall less commonly encountered. Seasonal variation in frequency of infections by *Klebsiella* species likely reflect changes in fecal carriage rates in those patients; typically, infections due to *Klebsiella pneumoniae* and other *Klebsiella* species appear to be more frequent during the warmest months of the year.[1] This observation is likely a reflection of increased presence of these organisms in the environment. Increased rates of antimicrobial resistance in *Klebsiella* species, has accounted for a growing clinical problem when selecting the most appropriate therapy for *Klebsiella* infections. With respect to health-care associated infections in the US, *K. pneumoniae* and *K. oxytoca* together accounted for approximately 8% of these infections, with only *Staphylococcus aureus*, *Enterococcus* species, *E. coli*, coagulase negative staphylococci, and *Candida* species having a slightly higher prevalence.[1]

Antimicrobial Therapy and Antimicrobial Susceptibility Testing

In recent years, an increase in antimicrobial resistance has been observed among many *Enterobacteriaceae*, culminating in the emergence of pan-resistant strains of *Klebsiella pneumoniae*. However, many *K. oxytoca* isolates among other Enterobacteriaceae (eg, *Enterobacter* species, *Serratia*

marcescens) have been reported to possess extended-spectrum β-lactamases (ESBLs) and carbapenemases.

Most strains of *K. oxytoca* produce a chromosomally mediated beta-lactamase (K1) that is in the same group as plasmid-mediated ESBLs. Like the plasmid-mediated ESBLs, K1 hydrolyzes extended-spectrum cephalosporins and aztreonam and is inhibited by clavulanic acid.[1] Like *K. pneumoniae, K. oxytoca* can contain IRT beta-lactamases, plasmid-mediated extended spectrum beta-lactamases and plasmid-mediated ampC type beta-lactamases. A recent study, described resistance rates among organisms identified from burn wound infections, and found *Klebsiella* species (including *K. oxytoca*) to be the second most common organisms identified in such infections, with 54% of these isolates being multi-drug resistant.[11] Interestingly, the study on nasal colonization by *Klebsiella* species referenced above did describe very low rates of antimicrobial resistance among *Klebsiella* species colonizing the anterior nares.[10] Antimicrobial susceptibility profiles of *K. oxytoca* isolates are quite similar to those found in many isolates of *K. pneumoniae* and treatment guidelines are virtually identical.

Key Points

- *Klebsiella oxytoca* is a common cause of human infections causing pneumonia, meningitis, bloodstream infections, and urinary tract infections.
- *Klebsiella oxytoca* is oxidase-negative, indole-positive, with a positive VP reaction, and grows well on routine laboratory agar media, including sheep blood agar.
- Antimicrobial resistance in *Klebsiella oxytoca* has recently increased, and the organisms have been shown to express ESBLs and carbapenemases.

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This challenge was a simulated CSF specimen from a neonate with meningitis. Participants were asked to report the principal pathogen only, perform graded susceptibility and Gram stain. The challenge contained *Citrobacter koseri*. A response of *Citrobacter koseri*; *Citrobacter* sp.; Gram-negative bacilli, Enterobacteriaceae; Gram-negative bacilli, aerobic was considered satisfactory.

D-05

Identification	Referees (53) No.	Referees (53) %	Participants (1853) No.	Participants (1853) %
Citrobacter koseri	52	98.1	1785	96.3
Citrobacter sp.	1	1.9	57	3.1
Gram-negative bacilli, Enterobacteriaceae	-	-	-	-
Gram-negative bacilli, aerobic	-	-	3	0.2
Consensus for correct identification of organism	53	100.0	1845	99.6

For the Gram stain challenge, a response of gram-negative bacilli was considered satisfactory.

D-05

Gram Stain Reaction	Referees (53) No.	Referees (53) %	Participants (1861) No.	Participants (1861) %
Gram-negative	53	100.0	1859	99.9

Morphology	Referees (53) No.	Referees (53) %	Participants (1858) No.	Participants (1858) %
Bacilli	53	100.0	1854	99.8

Extended spectrum beta-lactamase, beta-lactamase, carbapenemase, D-zone, and HLAR results are not shown. This testing is considered to be extraneous or not applicable for this organism.

Table 1. Antimicrobials with CLSI/FDA breakpoints that may be appropriate for specimen source.

		Disk Participants						MIC Participants					
	Antimicrobial	#	S	I	R	% Acceptable	Acceptable Response/ Non-graded Reason Code♦	#	S	I	R	% Acceptable	Acceptable Response/ Non-graded Reason Code♦
D-05 *Citrobacter koseri*	Amikacin	54	53	-	1	98.2	S	592	590	2	-	99.7	S
	Ampicillin	69	-	1	68	98.6	R	661	1	1	659	99.7	R
	Ampicillin-Sulbactam	32	30	-	2	93.8	S	394	324	-	70	82.2	S
	Aztreonam	13	13	-	-	100.0	S	371	369	1	1	99.5	S
	Cefepime	50	50	-	-	100.0	S	1001	1000	1	-	99.9	S
	Cefotaxime	43	42	-	1	97.7	S	376	376	-	-	100.0	S
	Ceftazidime	40	40	-	-	100.0	S	703	702	1	-	99.9	S
	Ceftriaxone	83	83	-	-	100.0	S	1380	1376	1	3	99.7	S
	Cefuroxime-parenteral	4	4	-	-	-	20	22	22	-	-	100.0	S
	Doripenem	4	4	-	-	-	20	40	40	-	-	100.0	S
	Ertapenem	31	31	-	-	100.0	S	595	595	-	-	100.0	S
	Gentamicin	99	99	-	-	100.0	S	1556	1556	-	-	100.0	S
	Imipenem	53	53	-	-	100.0	S	660	659	1	-	99.9	S
	Meropenem	53	53	-	-	100.0	S	755	754	-	1	99.9	S
	Piperacillin	2	2	-	-	-	20	55	5	1	49	89.1	R
	Piperacillin-Tazobactam	61	61	-	-	100.0	S	1061	1060	-	1	99.9	S
	Ticarcillin-Clavulanate	3	3	-	-	-	20	57	57	-	-	100.0	S
	Tobramycin	26	26	-	-	100.0	S	949	948	1	-	99.9	S
	Trimethoprim-Sulfamethoxazole	74	74	-	-	100.0	S	1057	1056	-	1	99.9	S

♦ S – Susceptible; I – Intermediate; R – Resistant; 20 – No appropriate target (less than 10 participants)

Table 2. Antimicrobials without CLSI breakpoints and/or inappropriate for the organism or specimen source

D-05 *Citrobacter koseri*	Antimicrobial	Disk Participants #	Disk Participants S	Disk Participants I	Disk Participants R	MIC Participants #	MIC Participants S	MIC Participants I	MIC Participants R
	Amoxicillin-Clavulanate (Oral) [c]	31	29	1	1	217	202	1	14
	Cefazolin (1st gen cephalosporin) [c]	26	23	3	-	228	223	1	4
	Cefepime meningitis (S. pneumoniae only) [b]	-	-	-	-	1	1	-	-
	Cefotaxime meningitis (S. pneumoniae only) [b]	-	-	-	-	1	1	-	-
	Cefotetan (2nd gen cephalosporin) [c]	1	1	-	-	12	12	-	-
	Cefoxitin (2nd gen cephalosporin) [c]	7	7	-	-	109	109	-	-
	Ceftriaxone meningitis (S. pneumoniae only) [b]	-	-	-	-	1	1	-	-
	Cefuroxime (not appropriate for oral use) [c]	20	20	-	-	194	192	1	1
	Ciprofloxacin (fluoroquinolones) [c]	35	35	-	-	293	293	-	-
	Fosfomycin [a]	-	-	-	-	1	1	-	-
	Levofloxacin (fluoroquinolones) [c]	12	11	-	1	225	225	-	-
	Nitrofurantoin [a]	-	-	-	-	7	7	-	-
	Norfloxacin [a]	1	1	-	-	2	2	-	-
	Tetracycline (tetracyclines) [c]	5	4	-	1	38	37	1	-
	Tigecycline (tetracyclines) [c]	1	1	-	-	45	45	-	-

S – Susceptible; I – Intermediate; R – Resistant

a. Code 25 – inappropriate use of antimicrobial – for reporting on urine isolates only.

b. Code 25 – inappropriate use of antimicrobial – for reporting on *S. pneumoniae* only.

c. Code 45 – Antimicrobial agent is likely ineffective or inappropriate for this organism or site of infection. See CLSI Warning box below for further explanation in parenthesis.

The following reporting instructions are printed in CLSI M100-S26:[1]

"Warning": The following antimicrobial agents should not be routinely reported for bacteria isolated from CSF that are included in this document. These antimicrobial agents are not the drugs of choice and may not be effective for treating CSF infections caused by these organisms (ie, the bacteria included in Tables 2A to 2J):

agents administered by oral route only
1st- and 2nd-generation cephalosporins (except cefuroxime parenteral)
and cephamycins
clindamycin
macrolides
tetracyclines
fluoroquinolones

Reference:

1. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing.* Twenty-sixth Informational Supplement. M100-S26. Wayne, PA: CLSI, 2015: page 43.

Identification and Taxonomy

Citrobacter spp. organisms are part of the *Enterobacteriaceae* family and are facultative, enteric gram-negative bacilli that are oxidase negative, ferment glucose, reduce nitrate to nitrite, and are non-spore forming. These organisms are indole positive and typically grow as relatively large dull gray colonies on sheep blood agar and may or may not be lactose positive on MacConkey agar (Figure 4). These organisms are easily identified by most bacteriology systems into individual species or into the subgroups of *C. braakii-C. freundii-C. sedlakii, C. werkmanii-C. youngae,* or *C. koseri-C. amalonaticus.* This particular organism was lactose-positive and identified on the Vitek 2 with a good confidence score. Identification by MALDI-TOF has also been very successful in identifying these organisms.[1,2] Historically *C. koseri* has been known as *C. diversus* and *Levinea malonatic.*[3] It is of interest that a new genera, *Pseudocitrobacter* with two species (*P. faecalis* and *P. anthropi*) initially phenotypically identified as *Citrobacter* species, has recently been characterized from several patients in Pakistan.[4]

Clinical Significance

Citrobacter spp. organisms may cause a wide variety of infections including urinary tract infections, wound infections, respiratory tract infections, intra-abdominal infections, bacteremia, endocarditis, meningitis (primarily infants), and sepsis. These organisms are ubiquitous in our environments as well as in the intestinal tracts of humans and animals. There are 12 species of *Citrobacter* of which 3 are more commonly involved in nosocomial infections: *C. freundii, C. braakii,* and *C. koseri. Citrobacter koseri* is almost exclusively involved with meningitis primarily involving children less than 2 months old. This organism is also associated with sequelae of brain abscess and neurological defects. Nosocomial spread from health care worker to patient, and less commonly by the mother are the routes of spread of the organism to the infant. This case was a neonate presenting with meningitis where this organism is known to play a pathogenic role.

Antimicrobial Resistance and Therapy Considerations

Citrobacter koseri is intrinsically resistant to ampicillin, piperacillin, and ticarcillin. Intrinsic antimicrobial resistance is defined as inherent or innate (eg, not acquired by resistance plasmids or gene mutation), which is reflected in wild-type antimicrobial patterns of all or almost all representative isolates of a species. If ampicillin, piperacillin or ticarcillin are reported for *C. koseri*, the results should be reported as resistant. Refer to Appendix B in the M100S CLSI *Performance Standards for Antimicrobial Susceptibility Testing* for a list of intrinsic resistance profiles for a variety of organisms.[5] These tables can be useful when confirming the susceptibility results on a specific isolate and also when developing testing and reporting protocols. A small percentage (1% to 3%) of isolates included in the CLSI's intrinsic resistance table may test susceptible due to method variation, mutation, or low levels of resistance expression. When this occurs, a laboratory should confirm the organism identification and consider repeating the susceptibility test by the same or an alternative method. Once results are confirmed, most would edit a "susceptible" result to "resistant" for agents to which the species is intrinsically resistant. However, when an unexpected "susceptible" result occurs, there is always the chance that the test was underinoculated or performed incorrectly and results for other agents may be falsely susceptible. All these factors must be considered when determining the best strategy for additional testing and final reporting.

In general, *C. koseri* isolates demonstrate >95% susceptibility to many agents, including piperacillin-tazobactam, cephalosporins, carbapenems, aminoglycosides, tetracyclines, and fluoroquinolones. *Citrobacter koseri* typically is more susceptible than *C. freundii* to various antimicrobial agents due in

large part to the chromosomal inducible AmpC β-lactamases and various extended-spectrum β-lactamases (ESBLs) carried by *C. freundii* and some *C. koseri*. The *C. koseri* isolate used in this Survey was susceptible to most antimicrobial agents, with the exception of those to which the species is intrinsically resistant. Isolates of *C. koseri* in recent years have demonstrated resistance to a broader range of antimicrobial agents as a result of the organism's ability to acquire antimicrobial resistance determinants.[6] Resistance to third-generation cephalosporins due to ESBLs, particularly CTX-M-15 has been reported.[7] Carbapenemases such as KPCs, VIMs and NDM metallo-β-lactamases have recently been identified in *C. koseri* but are rare.[8-10] In 2015, a report emerged from a nationwide antimicrobial resistance survey in the Balkans (Croatia) of the first *Citrobacter* spp., not *C. freundii* to contain the $bla_{\text{NDM-1}}$ gene.[10] This single *C. koseri* isolate from a patient with a urinary tract infection in 2011 expressed multiple β-lactamase genes including $bla_{\text{NDM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1,}}$ $bla_{\text{SHV-12}}$, and various fluoroquinolone resistance genes. The isolate tested resistant to third-generation cephalosporins, cefepime, ertapenem and fluoroquinolones but intermediate to meropenem and imipenem. Other cephalosporinases such as CMY AmpC β-lactamases have also rarely been identified in *C. koseri* in combination with other β-lactamases.[9]

This challenge was a CSF isolate. Most laboratories followed the CLSI reporting guidelines for isolates from CSF cultures. The antimicrobial agents listed below should not be routinely reported for bacteria isolated from CSF. They are not the drugs of choice and may not be effective for treating infections from this source. Laboratories that reported susceptibility results for these antibiotics for this challenge of a CSF isolate should review their testing and/or reporting practices and address them.

- Agents administered by oral route only
- 1st- and 2nd- generation cephalosporins (except cefuroxime parenteral)
- Cephamycins
- Clindamycin
- Macrolides
- Tetracyclines
- Fluoroquinolones

Key Points

- *Citrobacter koseri* is associated with neonatal meningitis.
- *Citrobacter koseri* used to be called *C. diversus*.
- *Citrobacter koseri* is intrinsically resistant to ampicillin.
- Certain antimicrobial agents should never be reported on organisms obtained from the CSF, due to their lack of efficacy in treating infections in the CSF.

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Bacterial Antigen Detection

D-06

Group B *Streptococcus*	Referees (35) No.	%	Participants (447) No.	%
Negative for Group B *Streptococcus* Ag	34	97.1	444	99.3

Haemophilus influenzae type b	Referees (34) No.	%	Participants (433) No.	%
Negative for *Haemophilus influenzae* Ag type b	34	100.0	431	99.5

Neisseria meningitidis	Referees (34) No.	%	Participants (432) No.	%
Positive for *N. meningitidis* serotype B/E. coli KI	24	70.6	257	59.5
Positive for *N. meningitidis* serotype A/Y/C/W135	6	17.6	93	21.5
Positive for *N. meningitidis* serotype A/Y	-	-	2	0.5
Positive for *N. meningitidis* serotype C/W135	1	2.9	2	0.5
Positive for *N. meningitidis* Ag/serotyping not performed	1	2.9	20	4.6

Streptococcus pneumoniae	Referees (38) No.	%	Participants (594) No.	%
Negative for *Streptococcus pneumoniae* Ag	38	100.0	593	99.8

D-06

Manufacturer	Response (a) Negative	Positive
Group B *Streptococcus*		
BD Directigen	267	2
Remel Wellcogen	135	-
Other (b)	16	1
***Haemophilus influenzae* type b**		
BD Directigen	267	-
Remel Wellcogen	122	1
Other (b)	15	1
Neisseria meningitidis		
BD Directigen	20	245
Remel Wellcogen	26	97
Other (b)	9	8
Streptococcus pneumoniae		
BD Directigen	263	1
Binax NOW	151	-
Remel Wellcogen	120	-
Other (b)	17	-

a. Data combines referee and participant results.
b. Includes Other methods not listed and peer groups with less than 10 laboratories reporting.

Bacterial Antigen Detection

D-07

C. difficile Antigen/Toxin	Referees No.	Referees (53) %	Participants No.	Participants (1667) %
Toxigenic *C. difficile* negative	53	100.0	1597	95.8
GDH antigen negative, no further testing performed	-	-	64	3.8

D-07

C. difficile Strain	Participant No.	Participant (301) %
BI/NAP1/027 Presumptive Negative	301	100.0

D-07

Manufacturer *C. difficile* Antigen/Toxin	Response (a) Negative	Positive	GDH Ag negative
BD Max	35	-	-
BioMerieux VIDAS/miniVIDAS	14	-	-
Cepheid Xpert	519	1	-
Great Basin Portrait Analyzer	39	-	-
Meridian illumigene	254	3	1
Meridian ImmunoCard Toxins A&B	90	1	1
Meridian Premier Toxin A+B	33	-	-
Remel Xpect Toxin A/B	18	-	-
TechLab/Alere QUIK CHEK	7	-	5
TechLab/Alere QUIK CHEK Complete	324	-	39
TechLab/Alere Tox A/B QUIK CHEK	22	-	1
TechLab/Alere TOX AB II	13	-	-
Other (b)	55	1	10

a. Data combines referee and participant results.
b. Includes Other methods not listed and peer groups with less than 10 laboratories reporting.

The CAP wishes to thank Richard Britton Thomson Jr. PhD, for providing these photographs. Unless permission is received from Dr. Thomson, these photographs may not be used for any purpose except in connection with this Survey.

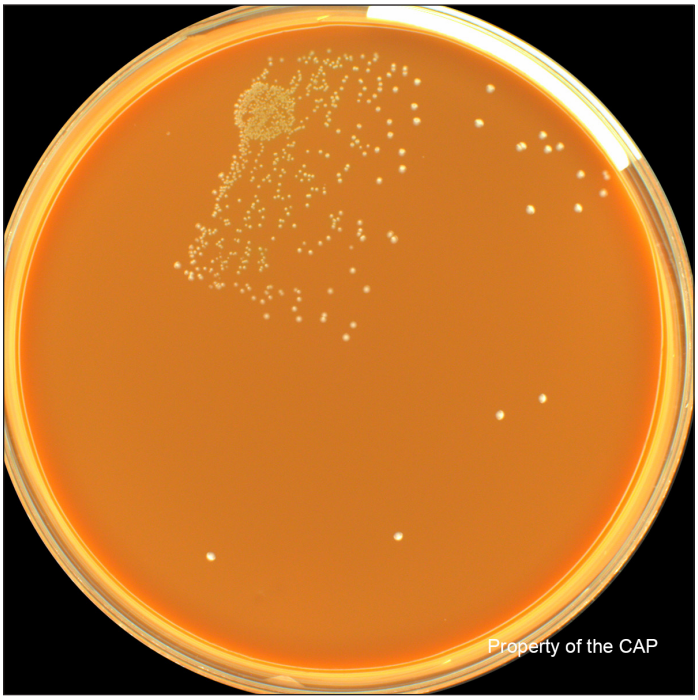

Figure 1. Colonies of *Leuconostoc mesenteroides* on chocolate agar after 24 hours at 35°C in 5% CO_2.

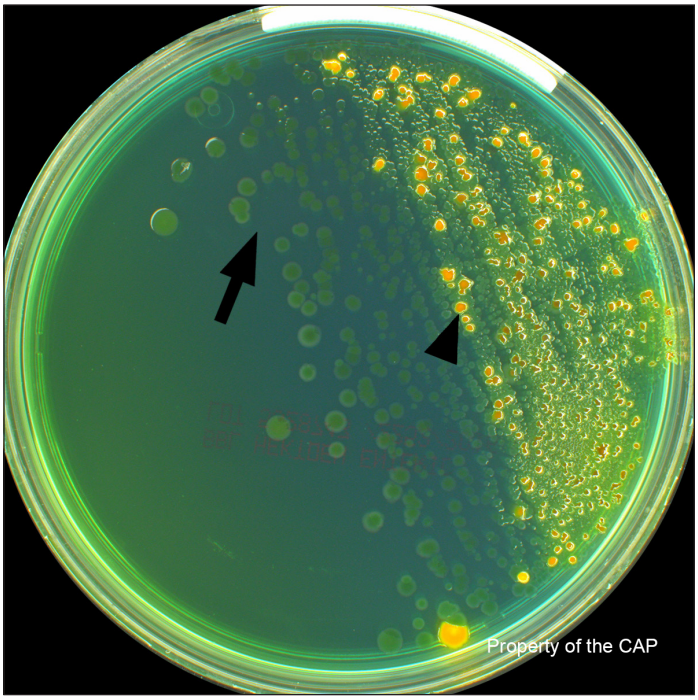

Figure 2. Hektoen enteric agar with non-lactose fermenting colonies of *Plesiomonas shigelloides* (arrow) and lactose fermenting colonies of *E. coli* (arrowhead).

Figure 3. *Bacteroides ovatus* on anaerobic CNA media.

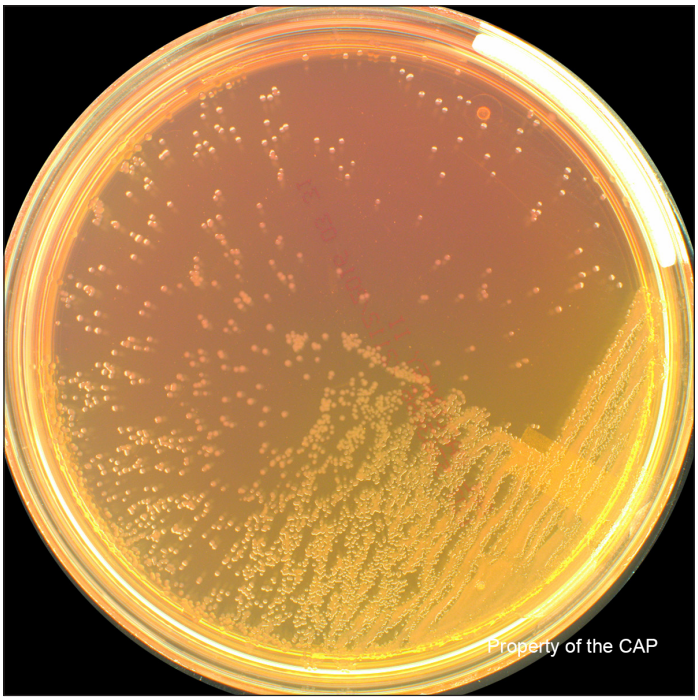

Figure 4. *Citrobacter koseri* on MacConkey agar.

NOTES

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