ASCP CASE REPORTS

MICROBIOLOGY **JUNE 2016** **MB 16-3**

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LEARNING OBJECTIVES

On completion of this exercise, the participant should be able to

- recognize risk factors for extragenital *Mycoplasma* infections;
- describe the challenges in identification of pathogenic mycoplasmas;
- apply current microbiological testing systems in the diagnosis of *Mycoplasma* species; and
- describe which antibiotic classes are active against *Mycoplasma* species.

HISTORY

A 25-year-old gravida 3 para 2 woman who was 10 days postpartum presented to the emergency department with worsening headaches. The patient had a medical history of migraine headaches and depression. Her most recent pregnancy was complicated by preterm labor with premature rupture of membranes 7 days before delivery. Before admission, it was thought that the patient was experiencing a postepidural spinal headache, but computed tomography of the head showed a thin, right-sided, chronic-appearing subdural hematoma with minimal mass effect and no midline shift. This finding was later confirmed by magnetic resonance imaging. The patient was admitted for further investigation and monitoring. On admission, the patient was afebrile, but had an elevated white blood cell count of 14,900/μL (reference range, 4800-10,800; to convert to $\times 10^9$/L, multiply by 0.001) and an absolute neutrophil count of 13,400 /μL (reference range, 1500-6600; to convert to $\times 10^9$/L, multiply by 0.001).

During observation, the patient developed progressively worsening left-sided seizures and became intermittently febrile, with fevers as high as 39.4°C. Imaging studies of the chest, abdomen, pelvis, and blood vessels of the head were all unremarkable. Prophylactic vancomycin, ceftriaxone, and metronidazole were started and cerebrospinal fluid, blood, sputum, and urine cultures were performed, but all results were negative. Follow-up computed tomography of the head (**Image 1)** demonstrated that the right-sided subdural hematoma had a moderate degree of peripheral enhancement and a very mild degree of midline shift; this finding was suggestive of an infectious process.

A diagnostic burr hole, a small, surgically drilled opening in the skull created to insert a catheter to allow for draining of the subdural hematoma, was performed to rule out a subdural empyema.[1] During the procedure, evidence of an old hematoma and mucopurulent material were noted. The mucopurulent material was collected and cultured for aerobic and anaerobic bacteria. The aerobic cultures of the empyema were negative; however, tiny clear colonies were observed on the anaerobic sheep blood agar plate after 5 days of incubation (**Image 2**). Gram and acridine orange stains of the colonies showed hazy-appearing "clouds," but no obvious microorganisms could be discerned (**Image 3**). Sequencing of the first 500 base-pairs of the 16S rRNA gene of the isolate definitively identified the isolate as *Mycoplasma hominis*. Following empyema drainage and bacterial identification, the patient was prescribed levofloxacin and her symptoms resolved.

A CASE OF *MYCOPLASMA HOMINIS* BRAIN ABSCESS IN A 25-YEAR-OLD POSTPARTUM WOMAN

The colloquialism "mycoplasma" can refer to any one of many genera and species of bacteria belonging to the class Mollicutes. This class is divided into 4 orders, 5 families, 8 genera, and more than 150 validly named species.[2] These microorganisms are a highly divergent clade, and are most closely related to low-G+C%, gram-positive bacteria, including *Erysipelothrix* species, *Bacillus* species, and the clostridia.[3] Of the mycoplasmas, only *Mycoplasma* species, *Ureaplasma* species, and Candidatus *Mycoplasma haematoparvum*[4] (order Mycoplasmatales, family Mycoplasmataceae) are frequently encountered as human pathogens. These bacteria possess the smallest genomes of any prokaryotes that are capable of axenic growth; however, as a result of reductive evolution, their biosynthetic capabilities are limited.[3] Consequently, these microorganisms are reliant on hosts, including arthropods, plants, and vertebrates, for all nutrients and macromolecules. In humans, mycoplasmas are generally found in association with the mucosal linings of the body, including the oropharynx, upper respiratory tract, and genitourinary tract, where they exist primarily as harmless commensals; although some species, discussed below, are opportunistic or professional pathogens.

A striking feature that distinguishes Mollicutes from virtually all other bacteria is the complete absence of a cell wall. To date, no genes encoding peptidoglycan precursors have been identified in any of the mycoplasmas whose genomes have been annotated. The lack of a cell wall imparts an inability to be Gram stained; as a result, these organisms stain pink-red, although they are truly not gram negative. The absence of a wall also renders them intrinsically resistant to many frequently used antimicrobial agents, including β-lactams, glycopeptides, and fosfomycin. Mycoplasmas are also smaller than most bacteria; the average single cell of wild-type strains is generally less than 2 μm long and less than 1 μm wide. As a consequence of their wall-less structure, the morphology of most mycoplasmas varies considerably. However, a small number of species exhibit strikingly consistent morphologies, including coccoid, spindle-shaped, rod-shaped, flask-shaped, and helical forms.

Mycoplasmas are facultative anaerobes and some require specific substrates for growth (eg, urea or arginine). In addition, they require a source of sterols for cell membrane biogenesis, a feature not seen in other bacteria. Despite their fastidious nutritional requirements, several culture media have been formulated for their axenic cultivation.

Clinical Significance

Although most human commensal mycoplasmas are harmless, some are known to be opportunistic pathogens. Common opportunists include *Mycoplasma hominis*, *Mycoplasma fermentans*, and *Ureaplasma* species Professional pathogens are those not known to be harmless commensals of most hosts; examples include *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. *Mycoplasma pneumoniae* is often transmitted through respiratory secretions and by contaminated fomites. It is implicated in 10% to 30% of community-acquired pneumonias, specifically so-called walking pneumonia, and is a major cause tracheobronchitis.[5] In addition, *M pneumoniae* has also been isolated from extrapulmonary sites, including cerebrospinal, pericardial, and synovial fluids.[6] Genitourinary mycoplasmas, including *M genitalium*, are generally transmitted by direct contact, such as through sexual intercourse or from mother to child at birth or in utero.[5,6]

Mycoplasma hominis harmlessly colonizes at least 13% of men and 31% of women.[5] It has been implicated in infections of the genitourinary tract and can be found in high concentrations in pelvic inflammatory disease and bacterial vaginosis, but its role as the causative agent in these conditions is the subject of debate.[7] It is also speculated that *M hominis* can play a role in endometritis, chorioamnionitis, and premature rupture of membranes.[5] Less commonly, extragenital infections, including pneumonia and meningitis, have been reported in neonates exposed to the bacterium during passage through the birth canal. Cases of bacteremia and postpartum central nervous system infections have been reported in previously colonized mothers. Zheng et al[8] reported a case with similar constellation of symptoms, diagnostic workup, and treatment in a 22-year-old postpartum woman who had an otherwise uncomplicated vaginal delivery.[8] *Mycoplasma hominis* has also been reported in association with osteomyelitis,[9] necrotizing pleuropneumonia,[10] endocarditis,[11] wound infections,[12] and brain abscess.[8],[13,] Risk factors for extragenital mycoplasma infections include immunosuppression, hypogammaglobulinemia, cancer, trauma, and procedures or surgeries involving the genitourinary tract, but it is important to note that extragenital infection has been reported in previously healthy adults.[6,14] Extragenital, nonpulmonary infections caused by *Mycoplasma* species may occur more frequently than currently thought. Because these organisms are generally underappreciated for their potential to cause such infections and means for their detection and definitive identification are not widely available to most clinical laboratories, they are rarely included on a differential diagnosis. Instead, typical bacterial and fungal pathogens are tested for and, perhaps by chance, the diagnosis of a mycoplasmosis can be made if attempts to identify all other etiologies have been exhausted. However, in recent years, infections caused by mycoplasmas have been increasingly

diagnosed, mainly as a result of improved diagnostics, including the use of mycoplasma-specific nucleic acid amplification methods.[6]

Culture and Molecular Testing

Specimens for mycoplasma culture can originate from a number of body sites, including sterile body fluids, urine, blood, wounds, and respiratory samples. Care should be taken when transporting specimens, as mycoplasmal viability is affected by temperature and pH extremes as well as desiccation. Transport media for mycoplasmas include 2 SP medium and tryptic soy broth containing 0.5% bovine albumin.[5] Universal transport media suitable for maintaining mycoplasmal viability that are more commonly used include M4, M5, M6, universal transport medium (UTM), and the BD Universal Viral Transport System (BD worldwide).[6] To successfully isolate mycoplasmas from blood cultures, anticoagulant-free blood should be inoculated into liquid mycoplasmal growth medium.[6] Commercial blood culture media are not recommended because mycoplasmas are inhibited by the anticoagulant sodium polyanethol sulfonate, which is found in common formulations of these media.[6]

Media used for the propagation of mycoplasmas include SP-4, Hayflick, Friis, Shepard 10B, and Frey media, among others. Many species require the addition of a cholesterol source, most commonly fetal bovine serum or another animal serum, to media. Other substances, including growth factors such as yeast extract, amino acids, glucose, supplemental arginine, and other nutrients, are needed to support their growth as well.[5,6] *Ureaplasma* species require urea for growth, so it is an essential component of media meant for recovery of these organisms. Cell wall–targeting antibiotics such as penicillin, and sometimes antifungal agents, are added to suppress the growth of contaminating microorganisms. Typically, a pH indicator such as phenol red is added; changes in its color serve as a visual indicator of mycoplasmal growth, as mycoplasmas rarely produce turbidity or other macroscopically visible signs of growth, especially in broths.[5] For example, growth of *M pneumoniae* creates an acidic pH by metabolizing glucose to lactic acid, whereas *M hominis* creates an alkaline pH shift as a result of arginine hydrolysis.[5]

Mycoplasmas are optimally grown within a temperature range that reflects the normal physiologic temperature of their hosts; for human and other mammalian mycoplasmas, this range is generally 35°C to 37°C. Broths should be incubated under ambient atmospheric conditions, whereas agar plates should be incubated in an

ambient atmosphere supplemented with 5% to 10% carbon dioxide or in an anaerobic environment containing 95% nitrogen and 5% carbon dioxide.[6] Cultures for genital mycoplasmas should be incubated for at least 7 days; those for *M pneumoniae* are generally held for 6 weeks before a culture is determined to be negative.[6]

On solid media, many *Mycoplasma* species produce umbonate colonies that are often reported as resembling fried eggs (**Image 4**). The central mass of the colony consists of a cone-shaped column of bacterial cells that is embedded in the agar, surrounded by an apron of cells growing on the surface of the medium. Some species, including *M pneumoniae* and *Ureaplasma* species, often produce colonies that are distinct in appearance and often resemble cauliflower or brown-pigmented granules, respectively. Regardless of the species, analysis with low-power magnification is often needed to distinguish colonies from debris and to observe colony details.

To isolate *M pneumoniae*, primary isolation media commonly used include SP-4 broth and agar (pH 7.5) containing glucose or methylene blue–glucose diphasic medium.[5,6] A shift to an acidic pH or colony growth on the agar media after 8 to 15 days of incubation should be subcultured to mycoplasma glucose agar or SP-4 agar. Preliminary identification of *M pneumoniae* can be made if slow-growing, cauliflower- or dome-shaped colonies that hemadsorb guinea pig or sheep erythrocytes are isolated from respiratory tract specimens. The latter property exploits the cytoadherence capability of *M pneumoniae* and is rapidly and easily detectable, permitting high-volume screening of cultures. Briefly, agar cultures of mycoplasmas are overlaid with a saline suspension of erythrocytes, incubated for a short period of time, rinsed, and observed using low-power magnification. Colonies of hemadsorbing mycoplasmas (eg, *M pneumoniae*) are coated with adherent erythrocytes. Confirmatory testing, including phenotypic, serologic, and molecular (polymerase chain reaction [PCR]) methods, is needed for definitive identification.[5]

For specific isolation of *M hominis* and *Ureaplasma* species, Shepard 10B broth (pH 6.0), along with A8 agar, can be used.[6] *Mycoplasma hominis* has also been known to grow on routine bacteriologic culture media such as standard sheep blood, anaerobic blood, and chocolate agars, and on these media, colonies typically appear as pinpoint and clear droplets of moisture. Often, though, these colonies are overlooked, as they often appear similar to inoculum debris or condensation on the agar surface. No discernable cells are noted on Gram stain, as mentioned above. *Ureaplasma urealyticum* colonies typically grow on A8 agar after 1 to 3 days of incubation and are granular and gold to light brown. This colonial appearance on A8 agar is unique to *U urealyticum*,

which is why further definitive testing is not usually required.[5] On A8 agar, *M hominis* typically grows after 1 to 3 days of incubation and often displays the characteristic fried-egg morphology. Definitive testing for *M hominis* can then be done by growth inhibition using specific antisera or nucleic acid testing.[5]

Culture methods are the criterion standard for identification of *Mycoplasma* species but are, for the most part, becoming obsolete. Culture tends to be very time-consuming, costly, and requires specialized technical expertise that is at a premium in today's clinical laboratories. For these and other reasons, most laboratories, including large reference laboratories, have abandoned this approach. Rapid sample-to-answer molecular tools are quickly replacing culture as a means of detecting pathogenic mycoplasmas directly from patient specimens and are becoming more commonplace in the diagnostic microbiology laboratory, especially in the form of real-time PCR assays. Available formats include single-analyte tests and multiplex assays such as syndromic panels that can detect numerous microorganisms in the sample specimen aliquot. Molecular testing methods can typically be performed within a single day of specimen receipt, and most and do not require a viable organism as they rely on detection of specific genetic sequences that often remain detectable for some time following cell death, as long as storage conditions are favorable.[6] Many commercially available and "home-brew" molecular tests for *M pneumoniae* have been developed, and the targets of these tests include the P1 gene and others.[15,16] *Mycoplasma pneumoniae* is also a target on the FilmArray Respiratory Panel (BioFire Diagnostics), which permits an approximately 1-hour sample-to-answer result. For *M hominis* and *U urealyticum*, similar assays have been developed that target species-specific gene sequences, which include *gap, fst*Y, and *yid*C, along with urease genes and their subunits.[6,17]

Recently, numerous reports have demonstrated that matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is able to accurately speciate a variety of human and veterinary *Mycoplasma* species, In many studies, protein extracts derived from broth cultures were analyzed.[6,18]

Treatment

The absence of a cell wall makes treatment of mycoplasmas more difficult than other bacteria, as many commonly used drugs target components of the cell wall synthesis and maintenance machinery. Agents such as β-lactams (eg, penicillins, cephalosporins, monobactams, and carbapenems), glycopeptides, lipoglycopeptides, and fosfomycin are completely ineffective for treatment of mycoplasmal infections. Mycoplasmas are also

intrinsically resistant to sulfonamides, trimethoprim, and rifampin, and resistance to macrolides is emerging in some strains.[6] Tetracyclines were the standard drugs of choice for the treatment of *M hominis* and *U urealyticum* infections, but emerging resistance to these agents has been described.[19] Fluoroquinolones, including levofloxacin, are broadly effective for treatment of mycoplasmoses, but resistance has been reported in urogenital mycoplasmas (*M hominis*, *M genitalium*, and *Ureaplasma* species).[5]

Summary

Although rarely a pathogen of extragenital sites, including the brain, *Mycoplasma hominis* should be suspected if evidence of other pathogens cannot be demonstrated, and pinpoint and clear colonies grow after extended incubation on blood-containing media. It is not identifiable by most conventional bacterial identification methods, but this and many other species can be identified by a number of methods, including nucleic acid sequencing and mass spectrometry. Owing to the rarity of this organism as an agent of brain abscesses, inclusion of these organisms in the differential diagnosis can facilitate implementation of curative antibiotic treatment if culture-based methods fail to demonstrate other pathogens.

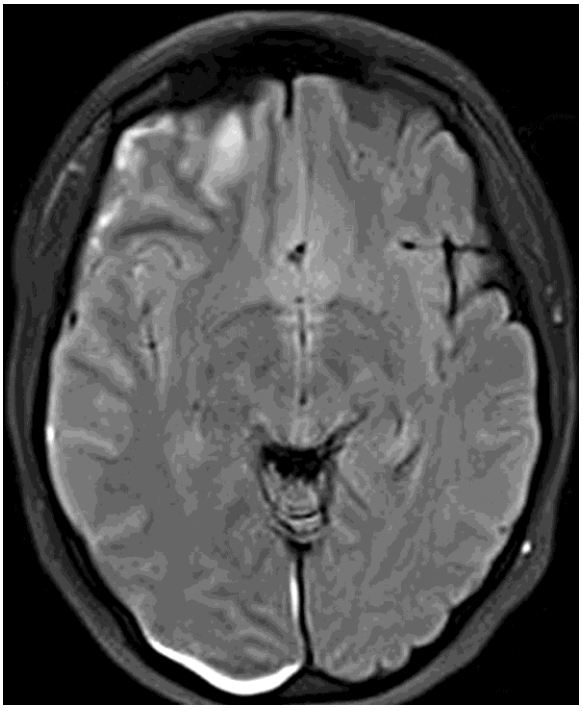

Image 1. The patient's follow-up CT scan of the brain, axial plane view, showing a mild left midline shift and development of frontal infarct with stable right subdural hematoma.

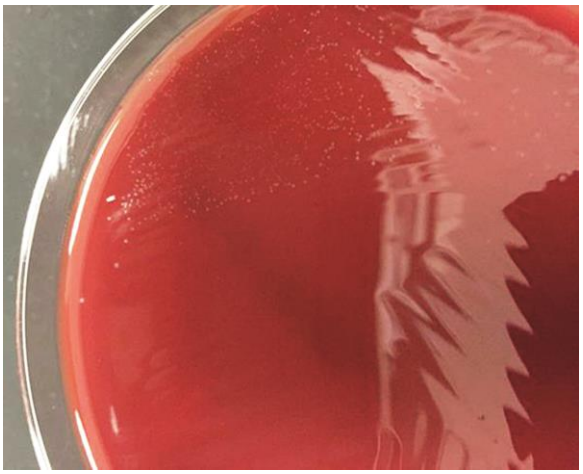

Image 2. The patient's *Mycoplasma hominis* isolate growing on CDC anaerobic blood agar after 5 days of incubation at 35°C in an atmosphere enriched with 5% CO_2. Colonies are punctate, smooth, and entire. The characteristic umbonate "fried-egg" morphology is not readily apparent on blood-containing bacteriologic media; however, *M hominis* is readily recovered from clinical specimens on blood-containing media, including standard sheep's blood agar, anaerobic blood agar, and chocolate agar.

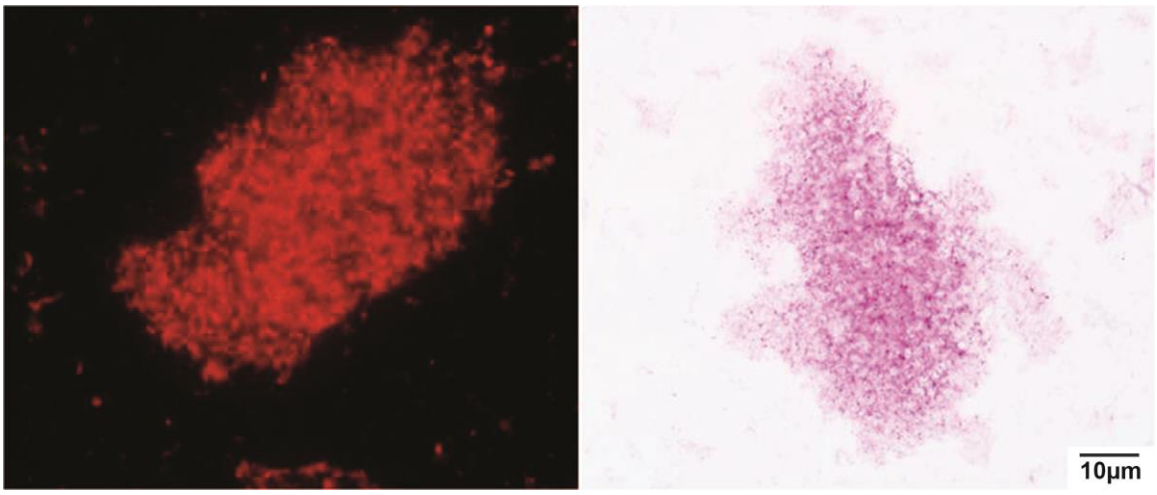

Image 3. Acridine orange (AO) stain (left) and Gram stain (right) of the *Mycoplasma hominis* isolate. Mycoplasmas appear as pink-staining masses of amorphous material when colonies, broth cultures, or body fluids containing these organisms are Gram stained. DNA fluorochrome stains, including the AO stain, permit better visualization of individual organisms, but the resolving power of most standard light microscopes is insufficient to gather detailed morphologic information.

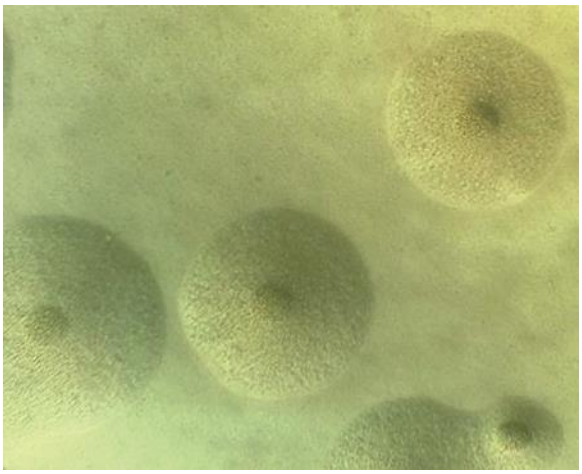

Image 4. Typical umbonate colonies of *Mycoplasma hominis* grown on SP-4 agar containing arginine. Photo credit: Dr. Meghan May, University of New England College of Osteopathic Medicine, in Biddeford, Maine.

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CME QUESTIONS

1. If a clinical sample is received by the microbiology laboratory for cultivation-based detection of *Mycoplasma pneumoniae*, which of the following is the ideal combination of culture media and identification tests?
   A. Sheep blood agar and hemadsorption testing of isolates
   B. Anaerobic sheep blood sheep agar and indirect immunofluorescence staining of isolates
   C. SP-4 agar containing glucose and hemadsorption testing of isolates
   D. SP-4 agar containing glucose and oxidase and indole testing

2. A 15-day-old neonate with a fever of 38.1°C and focal seizures had a cerebrospinal fluid (CSF) culture performed and no microorganisms were detected on a Gram stained CSF smear. The patient was empirically treated with intravenous ampicillin and cefotaxime; however, over the next few days the patient's symptoms gradually worsened. After 4 days, the CSF culture grew what was eventually identified as *Mycoplasma hominis*. Which of the following explains why the patient's condition worsened despite treatment with 2 different antibiotics?
   A. *Mycoplasma hominis* is known to elaborate numerous β-lactamases that hydrolyze both ampicillin and cefotaxime.
   B. Ampicillin and cefotaxime are not able to penetrate the central nervous system.
   C. Standard doses of these drugs are insufficient to kill *M hominis.*
   D. *Mycoplasma hominis* has no cell wall, which confers resistance to both ampicillin and cefotaxime.

3. Which of the following characteristics distinguishes most mollicutes, including *Mycoplasma* species and *Ureaplasma* species, from most other bacteria?
   A. They are acid fast.
   B. They require sterols, such as cholesterol, for cell membrane biogenesis.
   C. They require high concentrations of iron in growth media.
   D. They are obligate intracellular organisms.

4. A medical laboratory scientist receives a throat-swab specimen from a patient with a suspected *M pneumoniae* upper respiratory tract infection. The specimen is subsequently inoculated into SP-4 broth, which is incubated at 37°C and observed periodically for signs of growth. Which of the following combinations of visual indicators should the medical laboratory scientist expect to see if *M pneumoniae* grows in the culture, and how many days following inoculation would these indicators appear?
   A. Acidic shift in broth pH with little to no turbidity after 12 days of incubation
   B. Acidic shift in broth pH with gross turbidity after 2 days of incubation
   C. Basic shift in broth pH with little to no turbidity after 12 days of incubation
   D. Basic shift in broth pH with gross turbidity after 2 days of incubation

5. Some mycoplasmas, including *M hominis*, can be isolated from clinical specimens using standard bacteriological media such as sheep blood agar. However, recovery of this and other *Mycoplasma* species is generally not possible using commercial blood culture media, which is intended for isolation of a diverse array of microorganisms. Which of the following explains why mycoplasmas are not recoverable from commercial blood culture media?
   A. The resin in resin-containing blood culture media inactivates mycoplasmas.
   B. Standard blood culture media contain antimycoplasmal antibiotics.
   C. *Mycoplasma* species are inhibited by the anticoagulant sodium polyanethol sulfonate found in many formulations of blood culture media.
   D. Commercial blood culture media destroy the cholesterol in the blood, which is required for mycoplasmal growth.

6. Which of the following risk factors has been reported to predispose individuals for extragenital infections with *Mycoplasma* species?
   A. Medical procedures involving the urogenital tract, immunosuppression, and trauma
   B. Previous sexually-transmitted infection, such as gonorrhea
   C. Recent antibiotic use
   D. There are no known risk factors; all extragenital *Mycoplasma* infections are spontaneous