Staphylococcus pseudintermedius: Look What the Dog Dragged In

AAVMC / APTR One Health Case Studies Initiative

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Student Case Materials

Case presentation:

A 36 year old man fractured his left humerus in a bicycling accident. Several weeks later, he presented to his physician with drainage from a sinus tract at the site of the fracture. A culture was performed on the exudate and Staphylococcus pseudintermedius *was recovered from the specimen.*

Overview:

Although *S. pseudintermedius* can be a member of the oral, nasal and skin flora of healthy dogs, it is also the leading cause of skin and soft tissue infections in dogs. The true incidence of *S. pseudintermedius* infection in humans is unknown, but is likely underestimated. This is attributed to the fact that the traditional methods used in human clinical microbiology laboratories would be likely to misidentify these isolates as *Staphylococcus aureus*. With the introduction of Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a diagnostic tool for identification of microbes in clinical laboratories, it is becoming clear that *S. pseudintermedius* is indeed a cause of human infections. This is clinically significant as the methodologies to predict beta-lactam susceptibility are different for *S. aureus* and *S. pseudintermedius*.

Learning Objectives:

1. Explain laboratory methods used to differentiate *Staphylococcus aureus* from other bacteria.
2. Describe the importance of *S. pseudintermedius* in veterinary and human infections.
3. Recall the mechanism for methicillin resistance in staphylococcus.
4. Outline the principle of MALDI-TOF MS for microbial identification.
5. For the practitioner (human medical or veterinary), identify appropriate communication points to convey to the patient, client/owner, or other health professional regarding this infection.

Preparatory Materials:

1) Becker K, Skov R, von Eiff C. (2015) *Staphylococcus*, *Micrococcus*, and Other Catalase-Positive Cocci. *In* Jorgensen J, Pfaller M, Carroll K, Funke G, Landry M, Richter S, Warnock D (ed), *Manual of Clinical Microbiology, 11th Edition*. ASM Press, Washington, DC. p 354-382.

2) Que Y and Moreillon P. (2010) *Staphylococcus aureus* (Including Staphylococcal Toxic Shock). *In* Mandell GL, Bennett JE, Dolin R (ed), *Principles and Practice of Infectious Diseases, 7th Edition.* Churchill Livingstone Elsevier, Philadelphia, PA. p 2543-2578.

A) Songer JG, Post KW (2005) The Genus *Staphylococcus. Veterinary Microbiology: Bacterial and Fungal Agents of Animal Diseases*. Elsevier-Saunders, St. Louis, MO. p 35-41.

B) Weese, JS (2012) Staphylococcal Infections. *In* Greene CE (ed), *Infectious Diseases of the Dog and Cat, 4th Edition.* Elsevier Saunders, St. Louis, MO. p 340-348.

Additional recommended references can be found within the case study. These references are not required but are supplemental reference materials.

Key Questions for Class Discussion:

1. How are staphylococci identified in clinical laboratories?
2. Which species of staphylococcus are coagulase positive?
3. What laboratory tests could be used to differentiate *Staphylococcus aureus* from *Staphylococcus pseudintermedius*?
4. How is mass spectrometry being used to identify microbes in clinical settings?
5. How is methicillin resistance detected in *S. aureus* and *S. pseudintermedius*?
6. Is the recovery of *S. pseudintermedius* from this patient an unusual finding? Would the answer be the same if the patient were a dog?

Part 1: Clinical Microbiology Overview

Questions for Part 1:

1. How is the Gram stain performed?
2. How is the Gram stain interpreted?
3. What type of culture media are commonly used to recover staphylococci in clinical specimens?
4. What is the principle of MALDI-TOF MS for microbe identification?
5. How are the results of antimicrobial susceptibility tests reported?

Specimen Processing and Growth Media

Clinical microbiology laboratories receive a variety of specimen types for culture, such as tissue, respiratory secretions, wound swabs, and feces. Most types of specimens are plated directly onto a variety of solid media (agar) when they are received in the laboratory. The media selected depends on the specimen source (sputum, urine, stool, wound drainage, etc.) and the organisms that are expected. Specimens that are typically polymicrobial (i.e. many different types of bacteria and/or normal flora are in the specimen, such as stool) are plated onto media that will enhance detection of pathogens while minimizing overgrowth of normal flora. For example, stool specimens are plated onto 5 or 6 different media; each of these can help isolate selected pathogens (e.g., *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, etc.). Specimens from sterile body sites, such as cerebral spinal fluid, are plated to enriched media and incubated in an environment that supports the growth of fastidious organisms. Sheep blood agar plates are used most commonly and support the growth of most (but not all)

bacteria. In addition to being an enriched media, blood agar is a differential media; colonies growing on this agar type can be examined for their hemolytic pattern (beta-, alpha- or non-hemolytic), which can guide identification of the organism.

Gram Stain

A Gram stain is one of the most important tests done by the clinical microbiology laboratory. The Gram stain consists of a sequence of four reagents designed to stain bacteria and related organisms. The interpretation of the Gram stain includes the color of an organism, shape or morphology of individual organisms (rods, cocci, etc.), and grouping of organisms (in clusters, chains, etc.), all of which provide clues to an organism's identity.

A sample of a body fluid, blood culture broth, or bacterial colony growing on solid media is placed on a glass slide and heat or methanol fixed before staining. The slide is first stained with a basic dye (crystal violet) that is taken up by almost all bacteria, then treated with an iodine-containing compound (a mordant or fixative). Next, the slide is decolorized with acetone/ethanol, which destains only Gram-negative bacteria while Gram-positive organisms retain the purple complex of crystal violet and iodine. Finally, the slide is counterstained (usually with a pink stain called safranin), which allows one to visualize the Gram-negative organisms. When a Gram stain is performed on material from a clinical specimen, it can impact empiric therapy and may also drive the extent of workup in the laboratory. The stain will be examined for the absence or presence of inflammatory cells, and specific bacterial morphotypes and staining patterns are noted. A Gram stain performed directly from a clinical specimen can provide clues regarding the patient's illness and the absence or presence of cells such as epithelial cells may also provide valuable information regarding the quality of the specimen.

Overview of Gram Stain Procedure:

a. Clinical specimens or cultured organisms are placed onto clean glass slides, air dried, and then "fixed" to the slide, either using heat or by flooding the slide with methanol.
b. Crystal violet: The slide is flooded with crystal violet (purple stain) for about one minute, and then rinsed with water to remove excess stain.
c. Gram's iodine: The slide is flooded with iodine (mordant) for about one minute. During this time, crystal violet-iodine complexes are formed inside the bacterial cells. The slide is rinsed with water to remove excess stain.
d. Acetone-alcohol decolorizer: is added to the slide for a few seconds and then the slide is immediately washed with tap water. The decolorizer removes crystal violet/iodine complexes from Gram-negative organisms but these complexes are retained in Gram-positive organisms. This is a result of the difference in the cellular properties of Gram-positive and Gram-negative bacteria.
e. Safranin: The slide is flooded with safranin for about a minute as a counterstain: Gram negative organisms are stained red for visualization. Slides are blotted and air

dried.

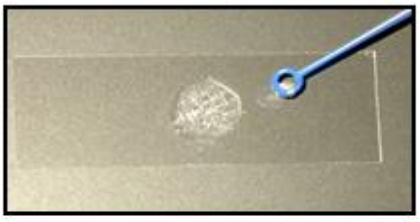
f. Microscopic examination: Gram-stained slides are examined using oil immersion under 1000X magnification.

Interpretation: The color of an organism, shape or morphology of individual organisms (rods, cocci, etc.), and grouping of organisms (in clusters, chains, etc.), all of which provide clues to an organism's identity. Gram-positive organisms appear purple and Gram-negative organisms appear pink.

Bacterial Identification

After the Gram stain morphology is known, organisms are further identified by the use of biochemical tests. Some are very simple bench top tests that allow the technologist to navigate major branch points in identification. For example, among Gram-positive cocci, the catalase test distinguishes generally between staphylococci (catalase-positive) and streptococci (catalase-negative). Staph-like organisms are then classified by the coagulase test (*S. aureus* is coagulase-positive, while most other staphylococci are coagulase-negative). Many Gram-positives (e.g., *S. aureus* and beta-hemolytic streptococci) are subjected to simple tests in which a colony is mixed with a suspension of latex beads coated with antibody to surface determinants of the organism. Agglutination of the beads upon mixing confirms the identification.

Catalase Test



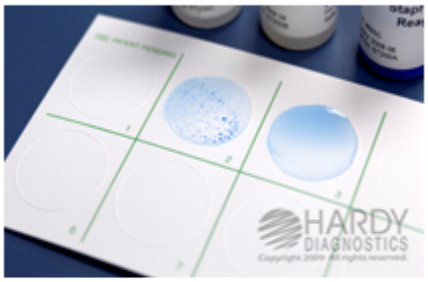
- Catalase is an enzyme that cleaves hydrogen peroxide (H_2O_2) into oxygen and water

$H_2O_2 \rightarrow H_20$ and O_2 (gas bubbles)

Positive	Negative	Variable
Staphylococcus	*Aerococcus*	*Rothia*
Micrococcus	*Streptococcus, Enterococcus*	

Staphylococcal Latex Agglutination Assay

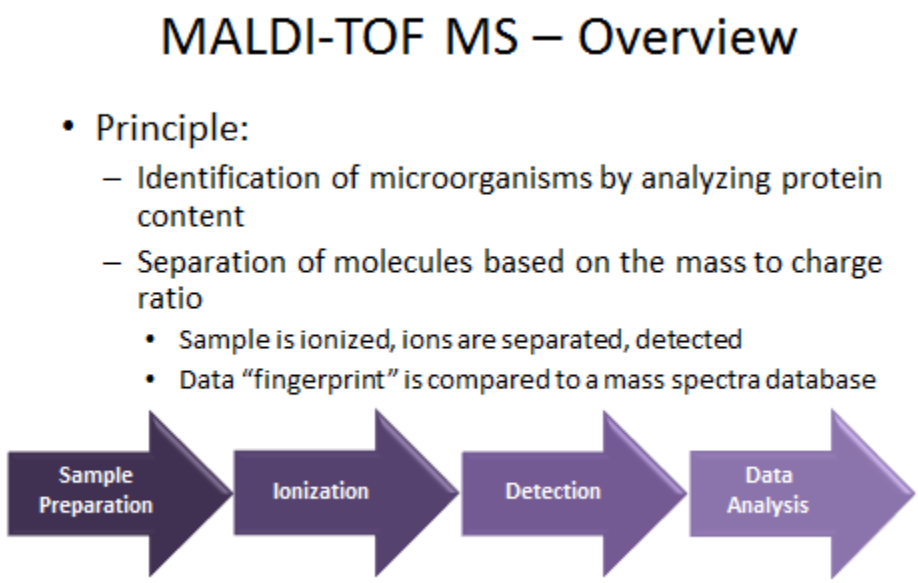
- Latex particles coated with fibrinogen and IgG
- **Fibrinogen:**
  - Detection of **cell-wall bound coagulase ("clumping factor")**
  - Does NOT differentiate coagulase positive from coagulase negative staphylococci
- **IgG:**
  - Protein A detection (on surface of *S. aureus*)
- Positive test---***S. aureus***



Clumping Factor	
Positive	**Negative**
S. aureus	Other staphylococci
S. lugdunensis	
S. intermedius	
S. schleiferi	

MALDI-TOF MS: Although biochemical testing has been the mainstay of microbial identification for more than a century, a new technology is emerging that is poised to supplant biochemical testing for identification of organisms in the clinical laboratory. This technology is matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS). Using this technique, only a single colony of microorganism is required. A portion of the colony is placed onto a stainless steel "target" plate using a toothpick, and once dry, overlaid with a "matrix" solution that co-crystallizes with the bacterial proteins. Once this mixture is dry, the target plate is placed into the MALDI-TOF MS instrument, and the sample is shot with a laser, ionizing the organism/matrix crystals. The ions then move through a vacuum (flight tube) and arrive at a detector at a rate proportional to their mass to charge ratio. This process generates a protein "fingerprint" of the organism that is compared to a database to assign an identity to the isolate. This process is very rapid (occurs in minutes) and has a very low reagent cost. Currently, this method can be reliably used for aerobic and anaerobic Gram-positive and Gram-negative bacteria, yeast and mycobacteria (1-6). The method is under continued development for filamentous fungi.

Overview of MALDI-TOF MS

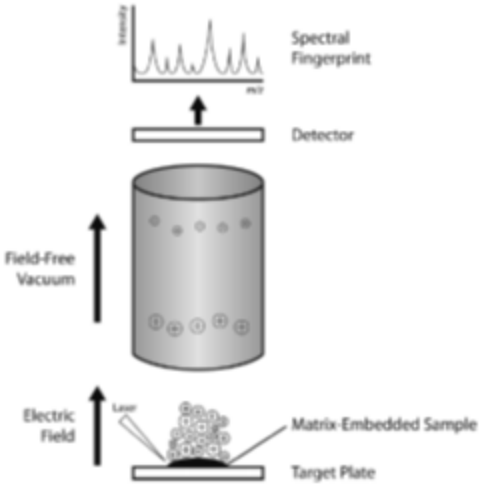


Sample Preparation for MALDI-TOF MS

Application of the organism to the MALDI slide

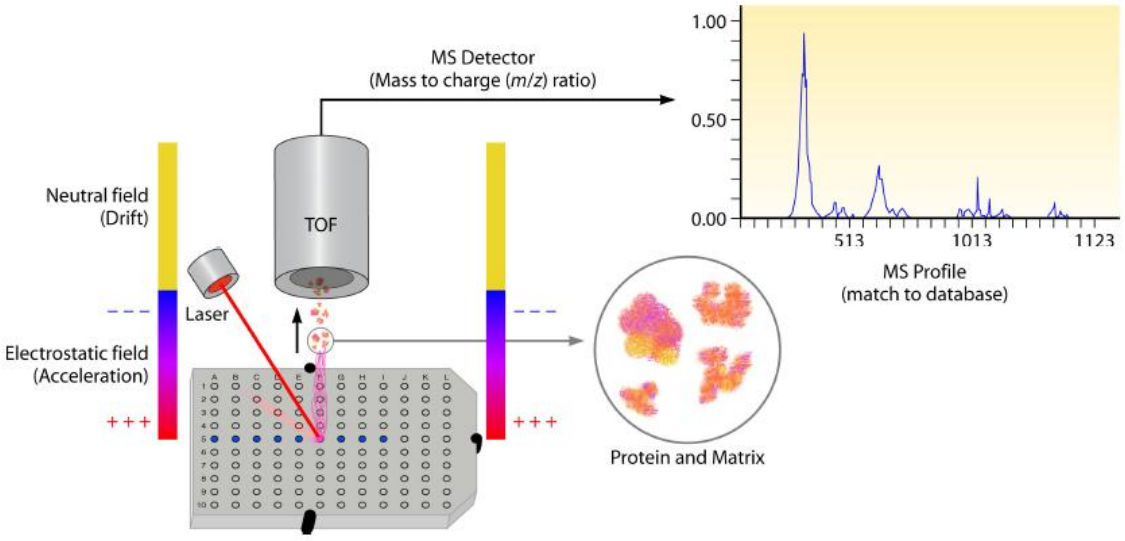
Overlay with matrix
Load slide into MS

MALDI-TOF MS for Microbiology



- A molecular fingerprint is generated
- Primarily ribosomal proteins detected (relative abundance)
- Low reagent cost
- Identification is performed in minutes
- Potential to indentify many organism types

Dingle and Butler-Wu. Clin Lab Med 2013. 33: 589-609.



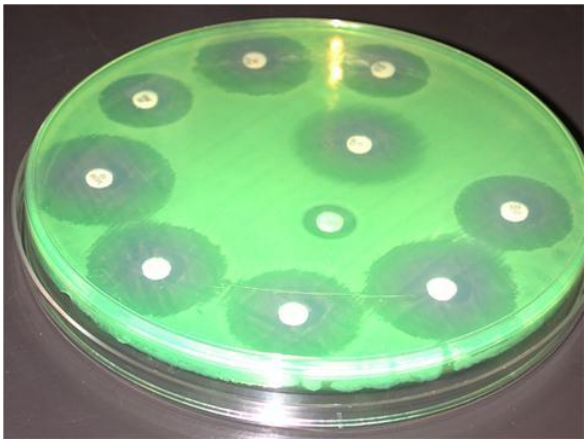
Antibiotic Susceptibility Testing

Once a pathogenic organism is identified, antimicrobial susceptibility testing may be performed on the isolate. The objective of this testing is to predict the outcome of treatment with the agent tested, and guide the clinician in selecting the most appropriate agent. There is a growing emphasis in utilizing the most narrow-spectrum, least expensive, least toxic agent.

In addition to providing treatment guidance for a particular patient, susceptibility testing also serves a larger, global purpose, such as monitoring the acquisition of resistance in a species over time, identification of new resistance phenotypes, and collection of data for antibiogram preparation. An antibiogram is a report on the rates of resistance for isolates recovered from a particular laboratory or institution. Each clinical laboratory is required to create an annual antibiogram report.

There are several ways to determine the susceptibility of an isolate to various antibacterial agents. These methods may be based on genotype, or phenotype. The traditional method is **disk diffusion**, in which a suspension of the organism is plated in a "lawn" onto a specific type of agar plate (usually Mueller-Hinton agar, a nutritionally replete type of media) and a number of filter-paper disks, each impregnated with a given antibiotic at a specific concentration, are then placed onto the agar. The antibiotic diffuses from each disk into the agar, setting up concentration gradient of antibiotic in the surrounding agar. If the organism is susceptible to a particular antibiotic, it does not grow near the disk, resulting in a clear zone around the disk. The technician measures the diameter of this "zone of inhibition" around each disk and compares that to standard tables established by CLSI (the Clinical and Laboratory Standards Institute). On the basis of these measurements, the organism is then classified as Susceptible, Intermediate, or Resistant to each of the tested antibiotics. Interpretive criteria do not exist for all organism-antimicrobial combinations.

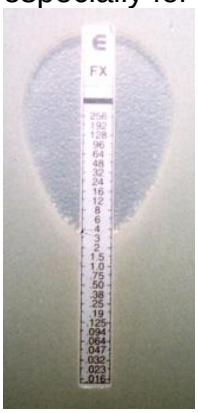
Example of a disk diffusion susceptibility test for an isolate of *Pseudomonas aeruginosa*. Note the presence of a green pigment, which is characteristic of *P. aeruginosa*.



An alternate (but more labor-intensive) method is broth microdilution, in which one attempts to grow the organism in a series of two-fold concentration dilutions of an antibiotic. The lowest concentration that prevents growth (the minimum inhibitory concentration, or MIC) is again compared to standards established by the Clinical and Laboratory Standards Institute (CLSI), and used to classify the organism as S, I, or R to the antibiotic. This testing method is especially helpful for organisms for which no disk-diffusion criteria exist, or certain fastidious organisms that do not grow on Mueller-Hinton agar. To correlate an MIC with interpretive breakpoints, one must consider the levels of drug achievable in various body compartments/fluids as compared to the MIC. An interpretation of Susceptible (S) implies that an infection due to the strain may be appropriately treated with the proper dosage of drug. Intermediate isolates (I) are strains with MICs that can be achieved *in vivo* but that response rates might be lower than for S isolates. The intermediate category also provides a buffer zone to prevent major discrepancies in interpretation. Resistant (R) suggests that strains will not be inhibited by achievable concentrations of the drug or that specific resistance mechanisms will prevent the activity of the drug.

In many laboratories, automated systems are now used to perform modified broth microdilution antibiotic susceptibility testing for some organisms on cards or panels placed into the instrument. Some of these automated systems can perform identification and antibiotic susceptibility testing in as little as 8-12 hours.

Another alternate susceptibility testing method is a hybrid of disk diffusion and broth microdilution is a gradient diffusion method called Etest. This is a commercially available testing method that has a gradient of an antimicrobial agent applied to one side of a plastic strip. This strip is applied to an agar plate, and the antibiotic diffuses into the agar, as with disk diffusion. However, following incubation, an MIC is read directly from a scale on the top of the Etest strip at the point where the ellipse of inhibition of organism growth intercepts the strip. Etest strips are much more expensive than the disks, but their main strength is simplicity for determining a specific MIC value, especially for infrequently tested drugs or organisms.



Example of an Etest. This isolate has an MIC of 4 ug/mL.

The clinician usually does not receive detailed reports about zone sizes or MICs, but rather a simplified summary report offering only S, I, or R for each antibiotic tested. The intent is that these reports can be used as guidelines to select an appropriate antibiotic for treatment of the patient's infection.

Part 2: *Staphylococcus aureus* and *Staphylococcus pseudintermedius*

Questions for Part 2:

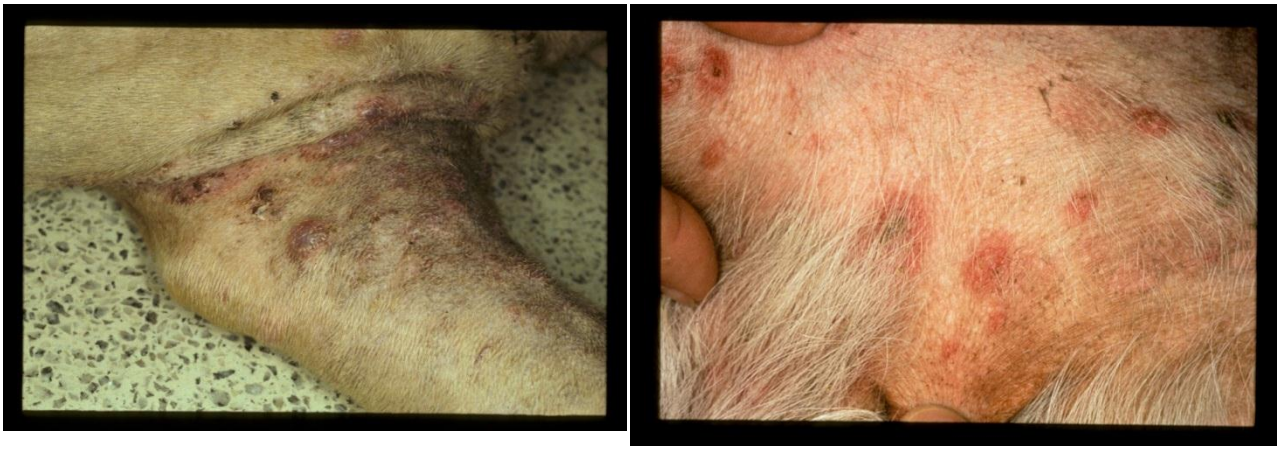
1. Provide examples of Gram positive bacteria:
    a. Gram positive cocci in clusters
    b. Gram positive cocci in chains
2. Which organisms are part of the *Staphylococcus intermedius* complex?
3. What biochemical assay(s) produce the same results of *Staphylococcus aureus* and *Staphylococcus pseudintermedius*? What biochemical assay(s) can differentiate these two organisms?

Staphylococcus aureus is an opportunistic pathogen of both humans and animals. In humans, clinical presentations can range from toxin-mediated food poisoning (7) to endocarditis (8) to skin / soft tissue infections [SSTI] (9). Population surveys estimate that 30-35% of people in the United States are asymptomatically colonized with *Staphylococcus aureus* (10). This means that it may be found on body surfaces, such as the nares, axilla or groin, but does not cause any disease state.

In animals, *Staphylococcus aureus* also causes a wide variety of clinical syndromes, including orthopedic implant infections, mastitis, pyoderma and also asymptomatic carriage (11). *Staphylococcus aureus* carriage in dogs was shown to occur with a frequency of less than 10% (12).

Staphylococcus intermedius group is a complex of organisms including *Staphylococcus intermedius*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini*. The most commonly isolated member of the complex is *S. pseudintermedius*, which can be normal flora, found on the skin, nose and mucosal surfaces of dogs. It has been found to colonize other animals, such as pigeons, minks, horses, raccoons and goats. It is also the leading cause of skin and soft tissue infections, also known as pyoderma.

A dog with pyoderma.



Historically, *S. pseudintermedius* was thought to be a very uncommon human pathogen, associated only with animal bite wounds. The first report of a human infection that was not associated with a dog bite appeared in the literature in 1994 (7), but other than that, very little literature associating *S. pseudintermedius* and human infection can be found until recently.

We now recognize that the incidence of human *S. pseudintermedius* infection has been under-reported as an artifact of laboratory methods. *S. pseudintermedius* and *S. aureus* share important phenotypic features: both are Gram-positive cocci in clusters, catalase positive, coagulase positive, and typically form beta-hemolytic colonies on sheep's blood agar.

Staphylococcus aureus growing on sheep's blood agar, demonstrating beta-hemolysis.

In addition, selective medium commonly used for identification of *S. aureus*, such as mannitol salt agar and DNase agar, show common reactivity profiles for *S. aureus* and *S. pseudintermedius*. Thus, isolates recovered from human infection were likely commonly misclassified as *S. aureus*.

DNase Agar

- Hydrolysis of DNA releases phosphate groups
- Free phosphate groups increase pH of the media
  - Toluidine blue becomes pink
- *S. aureus* is positive
  - So is *S. intermedius!*

Mannitol Salt Agar

- Contains:
  - 7.5% NaCl
  - Mannitol
- Fermentation of mannitol raises pH, turning phenol red indicator to yellow
- *S. aureus* can utilize mannitol
  - So can *S. intermedius!*

A biochemical assay that can be useful for differentiation of *S. aureus* from *S. pseudintermedius* is the Pyrrolidonyl Arylamidase (PYR) Test. *S. aureus* is PYR negative and *S. pseudintermedius* is PYR positive. However, this test may be reserved only for screening select specimen types, such as isolates recovered from blood cultures.

PYR Test

- Detects the presence of pyrrolidonyl arylamidase
  - If present, cleaves PYR causing a color change

Positive	Negative
S. lugdunensis	*S. aureus*
S. intermedius	*S. epidermidis*
S. haemolyticus	*S. saprophyticus*

Summary of Biochemical Reactions for *S. aureus* and *S. intermedius* group

	Coagulase	Clumping Factor	PYR	DNase Activity	Mannitol Fermentation	Ornithine Decarboxylase	Novobiocin Resistance
S. aureus	+	+	-	+	+	-	-
S. intermedius	+	+	+	+	v/+	-	-

MALDI-TOF MS is an accurate method to differentiate *S. aureus* and *S. pseudintermedius*. As MALDI-TOF MS is becoming a common tool used in clinical microbiology laboratories, it is now understood that *S. pseudintermedius* is a more common cause of human infection than previously thought. In one laboratory in the United Kingdom, *S. pseudintermedius* had "never" been recovered, but when the laboratory actively sought to differentiate *S. aureus* from *S. pseudintermedius*, 32 isolates were identified during a 32 month period (13). During the same time period,

10,777 isolates of S. aureus were recovered from 39,380 specimens (13). At Barnes Jewish Hospital in St. Louis, MO, *S. pseudintermedius* was recovered from 23 patients in 2014 and 31 patients in 2015, whereas *S. aureus* was recovered from approximately 2,000 patients in each of those years (Burnham, unpublished personal observation). *S. pseudintermedius* was recovered from wound, blood, tissue, bone, joint, and respiratory specimens.

Part 3: Antimicrobial Resistance

Questions for Part 3:

1. What are some potential mechanisms for beta-lactam resistance in bacteria?
2. What gene confers methicillin resistance in staphylococci?
3. What gene confers penicillin resistance in staphylococci?
4. Disk diffusion with which antimicrobial can be used to predict methicillin resistance in *Staphylococcus aureus*? *Staphylococcus pseudintermedius*?
5. Why is accurate identification of S. *pseudintermedius* in human clinical specimens important?

Just as antimicrobial resistance is increasing in *S. aureus*, it is also increasing in *S. pseudintermedius*. Most isolates of *S. pseudintermedius* are resistant to penicillin via beta-lactamase production conferred by bla_Z. Methicillin resistance is becoming more common in *S. pseudintermedius*; this is conferred by the gene *mecA*, as it is in *S. aureus* (14). Methicillin resistant *S. pseudintermedius* strains are commonly resistant to other antimicrobial agents, such as doxycycline and trimethoprim-sulfamethoxazole. Resistance to these two agents is very uncommon in *S. aureus*.

Methicillin Resistance In *Staphylococcus*

- Encoded by *mecA*, carried on SCC*mec*
- *mecA* encodes PBP2a, altered binding site for beta lactam antibiotics, can't get to active site
- *mecA* positive isolates are resistant to all beta-lactam antibiotics
  - Penicillins (all)
  - Cephalosporins
    - Some exceptions: **Ceftaroline**
  - Carbapenems

In methicillin-resistant *S. aureus* (MRSA) a cefoxitin disk is used as a surrogate marker for detection of methicillin resistance.

Detection of MRSA by Disk Diffusion

- Cefoxitin (FOX) has been long known to be the most sensitive and specific indicator drug for MRSA
- FOX disk diffusion zone size:
  - ≤21: Resistant (MRSA)
  - ≥22: Susceptible (MSSA)

(MSSA)

Cefoxitin Susceptible

Cefoxitin Resistant

(MRSA)

Increasing Oxacillin Resistance in Isolates from Dogs

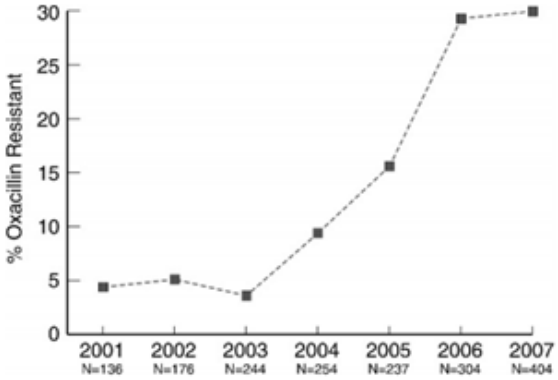
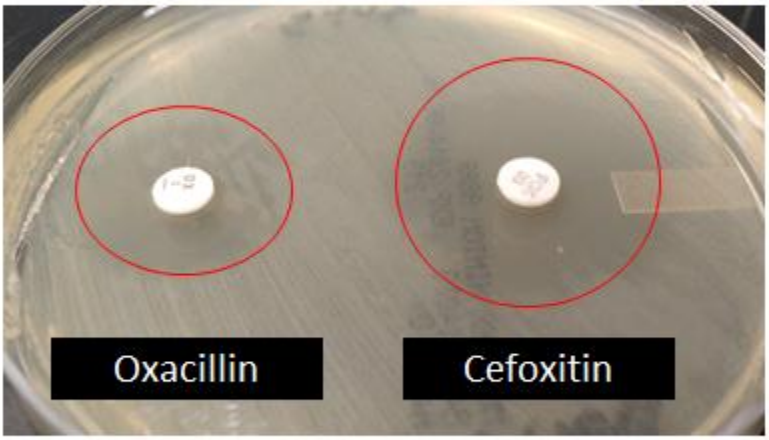


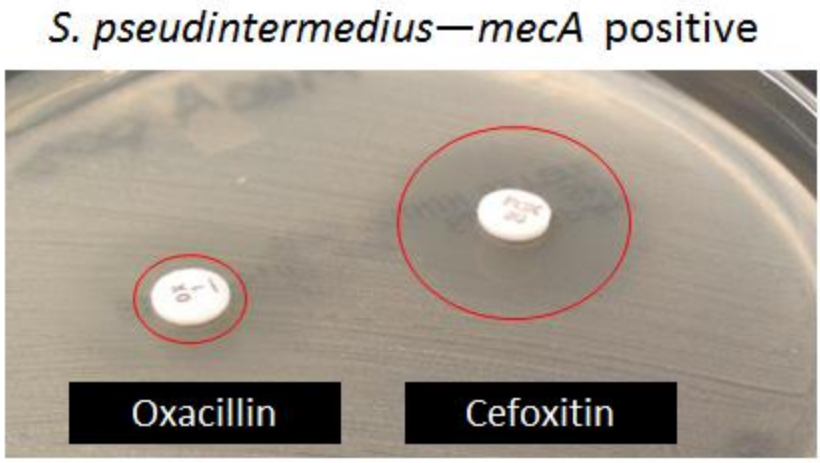
Figure 2. Frequencies of oxacillin resistance among *Staphylococcus pseudintermedius* isolates from dogs from 2001 to 2007. Disk diffusion method; resistant: ≤17 mm, susceptible: ≥18 mm.

Bemis et al. 2009. J Vet Diagn Invest. 21: 53-58. Knoxville, TN

However, in *S. pseudintermedius*, cefoxitin is not an accurate method for predicting mecA status, but rather an oxacillin disk should be used as the surrogate marker (14-16).

S. pseudintermedius—mecA negative





Thus, the ability to accurately differentiate these two species is more than a matter of novelty or trivia, but rather important to provide accurate antimicrobial susceptibility data to drive treatment decisions. As more laboratories adopt MALDI-TOF for bacterial identification, we will have a better idea of the incidence of *S. pseudintermedius* infection in humans.

Part 4: Clinical Impact

Question for Part 4:

1. For the practitioner (human medical or veterinary), what are the appropriate communication points to convey to the patient, client/owner, or other health professional regarding this infection?

In humans and in animals, *S. pseudintermedius* can both colonize and cause disease. This means that the microbe may live on body surfaces without any harm, or it can cause a variety of types of infection. The true incidence of *S. pseudintermedius* in humans is not known. Infection with methicillin-resistant *S. pseudintermedius* is emerging as a major health problem in small animal veterinary medicine. These infections are usually treated with antibiotics. However, as antimicrobial use continues to increase, this is an increasing challenge. Unfortunately, there are currently no studies to provide data on the best ways to prevent transmission and decrease the risk

of infection. In addition, data on the benefit of decolonization (i.e. using an antimicrobial therapy to eradicate colonization with the organism) are not well studied at this time.

A number of guidelines currently exist to aid in the prevention of infection and transmission of methicillin resistant *S. aureus* in human households (17-21). Some of the key recommendations from these guidelines, and studies on this topic include:

- Personal hygiene is the primary recommendation for controlling MRSA within the household.
    - Open / draining wounds should be covered / bandaged.
    - Regular bathing and hand-washing, particularly after touching infected skin or draining wounds.
    - Avoid sharing personal items (razors, towels, linens) with infected persons.
- Environmental hygiene can be considered when basic personal hygiene measures have not been effective, but the overall effectiveness of environmental measures has not been proven. In addition, the most effective methods or regimens for environmental hygiene are not known at this time.
- Decolonization of humans should only be considered in selective cases when personal hygiene measures have failed and ongoing transmission is suspected. Some examples of decolonization measures include intranasal mupirocin, bleach baths, and/or chlorhexidine bathing.
- Routine decolonization of pets is not recommended.
    - Anatomical site of *Staphylococcus aureus* carriage is not known for pets.
    - Concerns with antimicrobial resistance propagation with decolonization exposure.

It is possible that in the future, with additional study, that these guidelines may be helpful to prevent *S. pseudintermedius* infection as well.

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