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Title: Antibiotic resistance in Coagulase Negative Staphylococci isolated from Cope's Gray Treefrog (*Hyla chrysoscelis*)

Abstract approved: Scott S. Cypher

Two hundred and twenty two Coagulase Negative Staphylococci (CoNS) were isolated from the feces of Cope's gray treefrogs (*Hyla chrysoscelis*). Seven species were identified with *S. sciuri* and *S. xylosus* being the most prevalent. Antibiotic susceptibility of all isolates was determined using the disk diffusion method. Our results indicated 99% of the isolates were resistant to penicillin G and 59% were resistant to oxacillin, a clinical substitute for methicillin. Ten randomly chosen isolates were analyzed for the presence of the *mecA* gene, which codes for methicillin resistance. The gene was detected in 4 of the 10 isolates. These data suggest the gray treefrog may be harboring inordinate amounts of methicillin resistant CoNS and the mechanism of resistance in some isolates may be *mecA* independent. The environmental impact of these microbes is unknown, especially due to the recommended use of frogs in elementary and secondary classrooms.

Antibiotic Resistance in Coagulase-Negative Staphylococci isolated from Cope's Gray Treefrog (*Hyla chrysoscelis*)

A Thesis

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Approved for the Graduate Council

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PREFACE

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Introduction

The use of live animals, including reptiles and amphibians, as educational tools in classrooms is very common. However, benefits gained by the use of these animals is somewhat tempered by safety and health concerns. In 1997, the Kansas Department of Health and Environment (KDHE) published a document detailing proper handling and maintenance of animals in classrooms (14). The amphibian and reptile segment of this publication focused on the risk of infection by *Salmonella,* which is shed by these animals. Children are especially susceptible to infection since their immune systems are not as well developed as adults. Additionally, they have a propensity for putting their hands in their eyes and mouths, further compounding the problem.

The risks of contracting a *Salmonella* infection from handling animals have been well documented, however, there have been no studies concerning the risk of infection from *Staphylococcus*. The pathogenicity of members comprising the genus *Staphylococcus* is well documented. Staphylococci are gram-positive cocci that grow in clusters. They are non-spore forming but are able to survive for long periods of time on inanimate objects (13, 18). Though they may be considered normal flora of the skin, mucous membranes, and gastrointestinal tract, injury or immune suppression can lead to pathogenic colonization by these organisms (31). Diseases may include skin and soft tissue infection, endocarditis, pulmonary infections, and genitourinary tract infections (16, 17, 18).

One of the major medical innovations of the 20th century was the discovery of antibiotics, beginning with penicillin in the 1920s. Since that time, hundreds of antimicrobial compounds have been developed from both natural and synthetic sources.

Antibiotic use saves many lives, however, it also provides the selective pressure to produce drug resistant strains of bacteria (10). Acquired antimicrobial resistance can be the result of a mutation in an organism's genome or the acquisition of additional genetic information. These events alter the bacteria's physiology or structure rendering drug treatment ineffective. Because of the random nature of genetic alteration, some strains within a species may be resistant while others may not. Comprehensive laboratory testing must be done to discover resistant strains in bacteria of all genera (34).

In the early 1940s penicillin G began to be used for the treatment of Staphylococcal infections. In 1961, the first methicillin-resistant staphylococci were reported in Great Britain, shortly after introduction of the antibiotic. The same problem was reported in the United States in the mid-1970s (35). The rate of resistance has continued to increase and resistant organisms are no longer only isolated from hospital populations (1). To combat this problem, new antibiotics are continually being developed. With each new drug, however, new resistant strains are eventually selected for. Of particular concern is the development of methicillin resistant *S. aureus* (MRSA) due to the acquisition of the *mecA* gene (29). These organisms are not only resistant to methicillin, a drug commonly used to treat *Staphylococcus* infections, but are also resistant to all other β-lactams (9).

The penicillins, or β-lactams, inhibit several bacterial enzymes necessary for the formation of peptidoglycan, especially penicillin-binding proteins (PBPs) (37). There are several mechanisms of resistance to this group of antibiotics, with the most common being the production of β-lactamases by the bacteria. Other mechanisms include the alteration of the cell's PBPs, or acquisition of novel β-lactamases insensitive PBPs.

Staphylococcus species may harbor a plasmid containing the β-lactamase (*bla*) operon and/or the *mecA* gene (5, 29). The *mecA* gene codes for a β-lactam resistant PBP, which will take over cell wall production if the cell is exposed to this family of antibiotics. In rare cases *Staphylococcus* may constitutively contain modified PBPs, which have a low affinity for oxacillin and are therefore resistant to the drug. Presence of the *mecA* gene, therefore, indicates but is not necessary for oxacillin resistance in Staphylococci (5, 34).

Coagulase-negative staphylococci (CoNS), are distinguished from coagulase-positive staphylococci (e.g. *S. aureus*) by their inability to clot rabbit plasma (16). Although infections by *S. aureus* receive the most attention, CoNS are a frequent cause of nosocomial, or hospital acquired, infections (36). In fact, CoNS are the microorganisms most frequently isolated from catheters (36). CoNS were generally considered to be non-virulent normal flora, however, within the past ten years it has been shown that they can and will cause infection (16). Even more alarming is the rate of antibiotic resistance observed among isolates. For example, in a study of CoNS isolated from an intensive care unit of a pediatric hospital, 97% were methicillin resistant (33). Additional studies have shown similarly alarming rates (8, 12,13, 19, 21) and that antibiotic resistant CoNS can be found in a variety of domestic animal populations (1, 4, 22, 23, 24).

In this study, the Staphylococcal flora from the gray treefrog (*Hyla chrysoscelis*) was examined for levels of antibiotic resistance. This organism was chosen as a study organism for several reasons. It is a common amphibian in Kansas and is routinely used in classrooms as a hands-on teaching tool. The risk of *Salmonellosis* is well known, however, there have been no studies examining the presence of *Staphylococcus*. Two hundred and twenty two CoNS were isolated from the feces of Cope's Gray Treefrog, *H.*

chrysoscelis, the species of each isolate determined, and antimicrobial resistance patterns examined for five different antibiotics. Alarming rates of both penicillin G and oxacillin (a clinical substitute for methicillin) resistance were found. These results illustrate the need to educate those who will be in contact with these animals.

Methods and Materials

Collection and maintenance of gray treefrogs

Twelve gray treefrogs were collected in the spring of 1999 under a valid Kansas Scientific Collecting Permit (#SC-113-2000) issued to Greg Sievert (Department of Biological Sciences, Emporia State University, Emporia, KS) at two different sites in Lyon County, KS. The frogs were housed in individual plastic containers maintained at 35°C with a heat lamp. Wet paper towels were placed in the containers to provide moisture and the frogs were fed crickets *ad libitum*.

Isolation of *Staphylococcus* species

Feces of the captive gray treefrogs were collected within 1 h of defecation. Each frog's fecal material was placed in a sterile 15 ml tube containing 1 ml saline and homogenized by vortexing. One hundred microliter aliquots of this solution were plated on mannitol salt agar (MSA) plates and incubated at 37°C for 24 h. Mannitol fermenting colonies were randomly picked and streaked for isolation on MSA and subsequently two tryptic soy agar (TSA) plates to ensure purity. A single isolated colony was presumptively identified as *Staphylococcus* if a catalase test was positive and Gram staining yielded gram-positive cocci. To conclusively determine *Staphylococcus*, isolates were incubated in the presence of of lysostaphin. Briefly, a TSA plate was streaked for isolation with the colony in question. Lysostaphin (10 µl of a 10 µg/ml solution) was placed on the plate prior to incubation at 37°C overnight. A zone of inhibition where the lysostaphin was placed indicated cell lysis and confirmed the genus *Staphylococcus*.

Species identification of *Staphylococcus*

Various biochemical tests were performed according to standard protocols to determine the species of each isolate (16). Each confirmed staphylococci was tested for the ability to grow anaerobically, reactions in the coagulase, urease and oxidase tests, and the ability to produce acid from various sugars. Table 1 illustrates in detail how each procedure was performed and table 2 shows the control species used for each test.

Antibiotic disk susceptibility testing

Each isolate was tested for susceptibility to six different antibiotics according to guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) (3, 27). The antibiotics oxacillin, penicillin, clindamycin, erythromycin and sulfamethoxazole-trimethoprim were chosen based on staphylococcal testing standards established by the NCCLS (28). Briefly, each isolate was grown overnight in 3 ml of Mueller Hinton broth containing 2% NaCl (MH-NaCl) at 37°C. A loop full of each culture was used to inoculate a fresh 3 ml tube of MH-NaCl followed by incubation at 37°C with shaking at 250 rpm to a turbidity of a 0.5 McFarland standard. A sterile cotton swab was dipped into the turbid broth and used to inoculate the entire surface of a MH-NaCl agar plate in 3 different directions (Fig. 1). The antibiotic discs listed above were placed on each plate using a Becton Dickinson disc stamper (Franklin Lakes, NJ). After incubation for 24 h at 37°C, zones of inhibition were measured in millimeters to determine the susceptibility of each isolate to the five antibiotics tested according to criteria established by NCCLS (27). Figure 2 illustrates a typical antibiotic sensitivity plate showing variable zones of inhibition.

Table 1. Diagnostic procedures

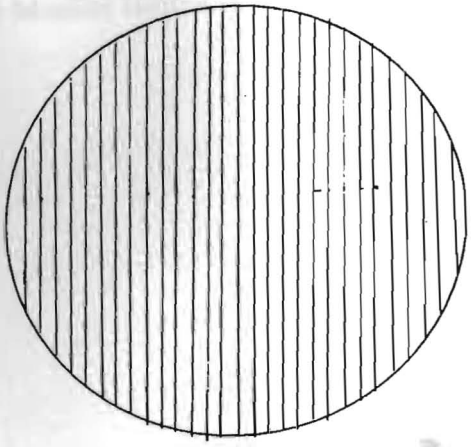
Test	Reaction Conditions
Coagulase	A loop full of bacteria from an overnight broth culture was used to inoculate 0.5 ml of rabbit blood plasma. After incubation at 37°C for 24 h, tubes were checked for coagulation, which indicated a positive reaction.
Oxidase	Three drops of oxidase reagent were placed on filter paper. A sterile toothpick was used to place each isolate from a plate onto the wet paper. A color change after 10 min indicated a positive reaction.
Sugar Fermentation	Three ml of 1% sugar solutions in phenol red broth were placed in 15 x 150 mm tubes and inoculated with a single colony from a plate culture. Tubes were incubated at 37°C and observed for a yellow color change, which indicated acid production and a positive result.
Anaerobic Growth	TSA plates were streaked with each isolate from plate cultures and placed in an anaerobe jar. Plates were incubated at 37°C for 24 h. Growth indicated the organism could grow anaerobically.
Urease	Each isolate was used to inoculate a 3 ml tube of urea broth from plate cultures. Tubes were incubated at 37°C for 24 h. A red color change indicated ammonia formation and a positive result.
Catalase	Each isolate was placed on a glass microscope slide from plate cultures. One drop of hydrogen peroxide was placed on the bacteria. Bubbling indicated the production of catalase and a positive reaction.

Table 2. Positive and negative controls used in diagnostic procedures

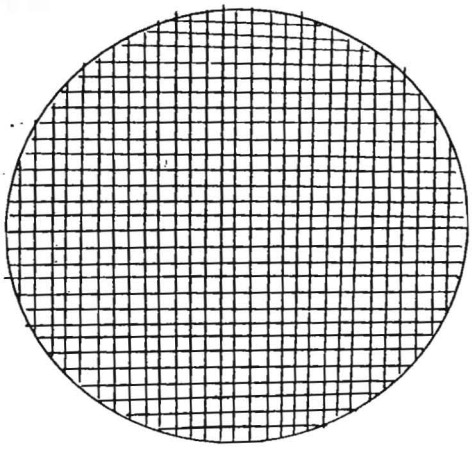
Test	Control Organism	Result [a]
Coagulase	*Staphylococcus aureus*	+
Oxidase	*Micrococcus luteus*	+
	Staphylococcus aureus	-
Sugar Fermentation		
Raffinose	*Klebsiella pneumoniae*	+
	Staphylococcus aureus	-
Xylose	*Escherichia coli*	+
	Staphylococcus aureus	-
Mannose	*Staphylococcus aureus*	+
	Proteus mirabilis	-
Trehalose	*Staphylococcus aureus*	+
	Staphylococcus epidermidis	-
Anaerobic Growth	*Escherichia coli*	+
	Micrococcus luteus	-
Urease	*Klebsiella pneumoniae*	+
	Escherichia coli	-
Catalase	*Staphylococcus aureus*	+
	Streptococcus mutans	-

[a] A (+) indicates the control organism was positive for the test, whereas a (-) indicates a negative result

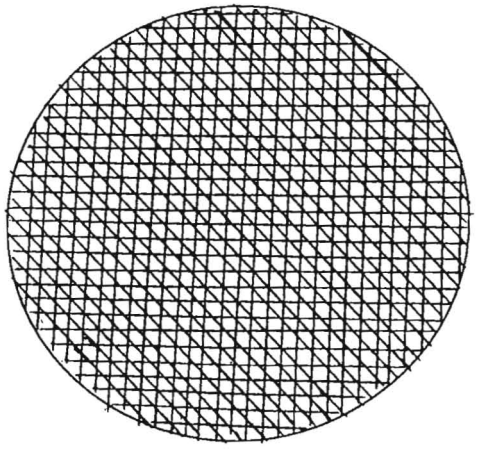
Figure 1. Three directional inoculation for antibiotic susceptibility testing



Step one, vertical streaking



Step two, horizontal streaking



Step three, diagonal streaking

Figure 2. Antibiotic disks on Mueller Hinton with 2% NaCl agar


SXT
OX
CC
2
AM
30
SXT

Determination of minimum inhibitory concentration of oxacillin

Ten of the isolates determined to be resistant to oxacillin via the disk method were randomly chosen to undergo minimum inhibitory concentration (MIC) testing according to NCCLS guidelines (26). Briefly, isolates grown overnight in MH-NaCl at 35°C with shaking at 250 rpm were used to inoculate 3 ml of MH-NaCl to a McFarland turbidity standard of 0.5. Twenty microliter of this suspension was used to inoculate each of 5 tubes containing 2ml MH-NaCl containing variable amounts of oxacillin (0.25-1 μg/ml). Inoculated tubes were incubated at 35°C with shaking at 250 rpm for 24 h. The tube with the lowest concentration of antibiotic containing no visible bacterial growth was determined to be the MIC.

Chromosomal DNA isolation

Chromosomal DNA was isolated from the 10 isolates for which MIC determinations were performed using an established procedure (30). Briefly, isolates were inoculated individually from plates into 6 ml of TSB contained in 20 ml test tubes and incubated for approximately 2h at 35°C with shaking at 250 rpm. Cultures were transferred to 15 ml conical tubes and cells isolated by centrifugation at 4000 rpm for 5 min in a Sorvall GLC-1 tabletop centrifuge (Newton, CT). The supernatant was discarded and cells resuspended in 2 ml of tris-ethylenediaminetriacetate (TE) buffer. One hundred microliter of lysostaphin (10mg/ml) was added and the suspension incubated at 37°C for 30 min. Complete cell lysis was accomplished by adding 100 μl of 20% sodium dodecyl sulfate (SDS) with subsequent incubation at 60°C for 15 min. Twenty microliter of RNase (10mg/ml) was added to degrade RNA during incubation at 37°C for 20 min followed by addition of 15 μl of proteinase K (10 mg/ml) and incubation

at 60°C for 1 h to degrade protein. Subsequently, a phenol extraction was performed to remove any remaining contaminants by adding 2 ml of a saturated phenol (pH 8) solution and mixing. After centrifugation at 4,000 rpm for 5 min, the top (aqueous) layer was removed and placed in a clean 15 ml conical tube. A second phenol extraction was performed as described. To further clean the DNA preparation, 2 ml of chloroform/isoamyl alcohol (24:1) was added after the second phenol extraction. After centrifugation at 4,000 rpm for 5 min, the aqueous layer was removed and placed in a clean 15 ml conical tube. Ten milliliter of ice cold 95% ethanol was added and the solution mixed by inversion to precipitate DNA. The DNA was removed with a glass Pasture pipette, placed in a clean 15 ml conical tube to air dry, resuspended in 2-4 ml of TE, and stored at 4°C.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify the *mecA* gene from DNA templates. The primers used were 5' AAA ATC GAT GGT AAA GGT TGG C 3' and 5' AGT TCT GCA GTA CCG GAT TTG C 3' as previously described (7). Typical reactions consisted of 500 ng DNA, 200 μM deoxynucleotide triphosphates (dNTPs), 1.5 μM $MgCl_2$, 1 μl *Taq* polymerase, and 50 nM of each primer in buffer containing 50 mM TrisCl, 50 mM KCl and 0.01% Triton-X100 in a final volume of 100 μl. Reactions were carried out on a MJ Research Minicycler model PT 150 (Watertown, Mass). Thirty cycles were used and consisted of a 94°C denaturation step for 1 min, a 55°C annealing step for 1 min, and a 2 min extension step at 72°C. Additionally, an initial denaturation step of 5 min at 94°C and a final extension step at 72°C for 5 min were used.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize PCR products. The gel was prepared by dissolving 0.38 g of agarose in 30 ml of 1X TAE, which was prepared from a 50X stock (242 g tris, 57.1 ml acetic acid and 4 ml 0.5 M EDTA/1L). Ethidium bromide (1 µl of a 10 mg/ml solution) was added to aid visualization of the DNA. The electrophoretic chamber was a Minicell EC370M (Fisher, St. Louis, MO) powered by a Bio-RAD model 250/2.5 power supply (Bio-RAD; Hercules, CA). Agarose gels were visualized using a UV Intensity Transilluminator (Fisher) and documented with Panasonic CCD Ultra Lum camera and scion image software (Ultra Lum; Paramount, CA).

Cloning and sequencing of 16s ribosomal RNA gene

To further confirm the *Staphylococcus* identification, the 16s ribosomal RNA gene of one of the isolates was sequenced. Briefly, PCR products were gel purified using a Qiagen QIAEXII Gel Extraction kit (Valencia, CA) according to the manufacturers recommendations. Purified products were incubated with end conversion mix (Novagen, Madison, WI) at 22°C for 15 min to prepare it for ligation into pT7Blue-3 (Novagen, Madison, WI). After inactivation at 75°C for 5 min, 10 µl of the conversion reaction was mixed with 1 µl pT7Blue-3 (50 ng) and 1 µl T4 DNA ligase at 22°C for 2 h. The ligation mix was used to transform competent *E. coli* DH5α cells prepared according to standard procedures (30). Briefly, the ligation mixture was added to 100 µl of competent cells, incubated on ice for 15 min, heat shocked for 90 sec at 42°C, and placed on ice for 2 min. Nine hundred microliter of TSB was added to the transformed cells and 100 µl of the resulting suspension was spread plated on Luria-Bertani (LB) agar plates containing 5-

Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal) (20 μg/ml) and ampicillin (100 μg/ml). Following incubation at 37°C for aproximately 24 h, white colonies were used to inoculate 3 ml tubes containing TSB with 100 μg/ml ampicillin. Plasmids were isolated using a Qiagen QIAprep Spin Miniprep kit according to manufacturers guidelines and visualized by agarose gel electrophoresis. Recombinant plasmids were sent to the DNA sequencing facility at Kansas State University for DNA sequencing. Obtained sequence data were compared to known *Staphylococcus* 16s rRNA genes using the Basic Local Alignment Search Tool (BLAST).

Figure 3. Identification flow chart for coagulase negative *Stapylococci*.

Mannitol Fermentation

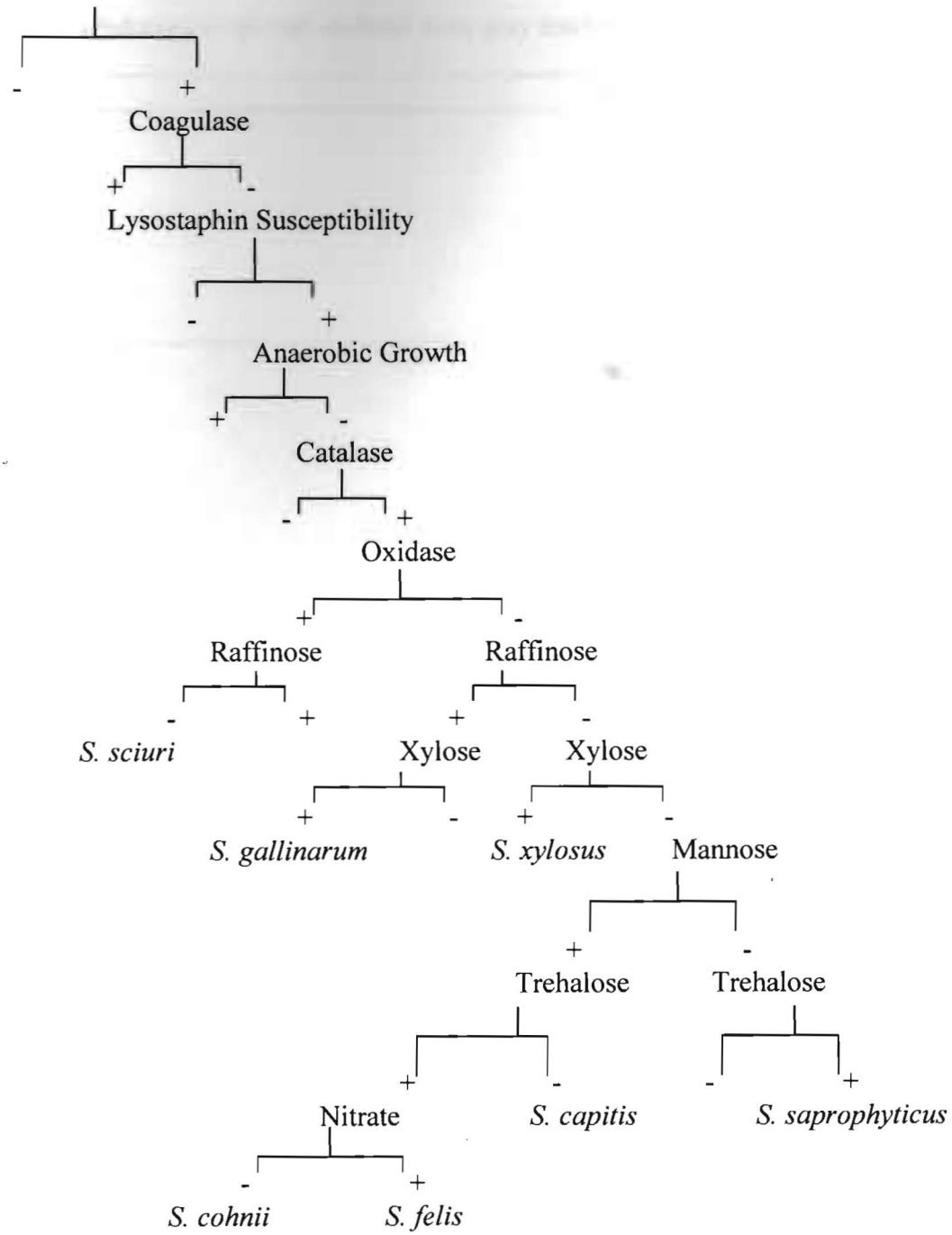


TABLE 3. *Staphylococcus* species isolated from gray treefrog feces

Species	Number isolated
S. sciuri	112
S. xylosus	95
S. felis	5
S. cohnii	4
S. gallinarum	1
S. saprophyticus	1
S. capitis	1

Figure 4. 16s ribosomal RNA sequence from F10C8, *Staphylococcus xylosus* isolate

GAT TTT TGC GGG CGC CAC TCG GGC CCT TCA CGT GTG GTC TAG AGC
TAG CCT AGG CTC GAG AAG CTT GTC GAC GAA TTC AGA TGC TCA GAT
TGA ACG CTG GCG GCG TGC CTA ATA CAT GCA AGT CGA GCG AAC AGA
TGA GAA GCT TGC TTC TCT GAT GTT AGC GGC GGA CGG GTG AGT AAC
ACN GTG GGT AAC CTA CCT ATA AGA CTG GGA TAA CTC CGG GAA ACC
GGG GCT AAT ACC GGA TAA TAT TTT GAA CCG CAT GGT TCA ATA GTG
AAA GAC GGT TTC GGC TGT CAC TTA TAG ATG GAC CCG CGC CGT ATT
AGC TAG TTG GTA AGG TAA CGG CTT ACC AAG GCG ACG ATA CGT AGC
CGA CCT GAG AGG GTG ATC GGC CAC ACT GGA ACT GAG ACA CGG TCC
AGA CTC CTA CGG GAG GCA GCA GTA GGG AAT CTT CCG CAA TGG GCG
AAA GCC TGA CGG AGC AAC GCC GCG TGA GTG ATG AAG GTC TTC GGA
TCG TAA AAC TCT GTT GTT AGG GAA GAA CAA ATT TGT TAG TAA CTG
AAC AAG TCT TGA CGG TAC CTA ACC AGA AAG CCA CGG CTA ACT ACG
TGC CAG CAG CCG CGG TAA TAC GTA GGT GGC AAG CGT TAT CCG GAA
TTA TTG GGC GTA AAG CGC GCG TAG GCG GTT TCT TAA GTC TGA TGT
GAA AGC CCA CGG CTC AAC CGT GGA GGG TCA TTG GAA ACT GGG AAA
CTT GAG TGC AGA AGA GGA GAG TGG AAT TCC ATG TGT AG

Table 4. Antibiotic Disc Test Results[a]

Number	Species	OX	VA[b]	P	CC	E	SXT
F1C1	*sciuri*	17	17	20	16	28	28
F1C2	*sciuri*	20	20	24	20	30	32
F1C3	*sciuri*	16	15	15	18	23	24
F1C6	*sciuri*	14	15	23	17	21	22
F1C7	*sciuri*	14	15	18	18	24	25
F1C8	*sciuri*	17	15	23	17	21	24
F1C9	*sciuri*	21	19	24	22	28	31
F1C10	*sciuri*	15	15	23	17	22	25
F1C11	*sciuri*	13	16	25	19	22	22
F1C12	*sciuri*	15	15	22	18	22	24
F1C13	*sciuri*	14	16	22	20	24	27
F1C14	*sciuri*	15	16	20	18	23	25
F1C15	*sciuri*	15	15	20	20	22	24
F1C16	*sciuri*	16	15	20	20	23	28
F1C17	*sciuri*	14	15	19	18	23	25
F1C18	*sciuri*	20	15	18	25	23	29
F1C19	*sciuri*	17	16	18	24	25	26
F1C20	*sciuri*	15	16	26	19	24	25
F2C1	*sciuri*	20	17	20	17	14	28
F2C2	*sciuri*	18	16	16	17	0	30
F2C3	*sciuri*	17	18	25	20	26	28
F2C4	*sciuri*	18	18	18	20	27	28
F2C5	*sciuri*	20	19	25	22	29	30
F2C6	*sciuri*	14	16	23	19	26	27
F2C7	*sciuri*	16	17	24	20	25	27
F2C8	*sciuri*	15	16	22	19	25	26
F2C9	*sciuri*	15	17	23	19	25	26
F2C10	*sciuri*	17	17	24	20	23	25
F2C11	*sciuri*	17	17	22	21	25	29
F2C12	*sciuri*	16	19	28	20	27	26
F2C14	*sciuri*	17	16	26	18	24	24
F2C15	*sciuri*	17	17	25	21	26	27
F2C17	*sciuri*	18	17	17	19	25	29
F2C18	*sciuri*	14	16	23	19	25	28
F2C19	*sciuri*	18	18	20	19	27	26
F3C1	*sciuri*	17	14	20	18	23	25
F3C2	*xylosus*	19	15	18	19	23	27
F3C3	*sciuri*	17	16	23	18	25	29
F3C4A	*xylosus*	20	17	22	20	27	30
F3C4B	*xylosus*	20	16	19	20	22	25
F3C5	*xylosus*	21	18	23	22	27	30
F3C6	*sciuri*	15	16	26	19	24	25
F3C7A	*xylosus*	21	19	26	28	30	31

Table 4. continued

F3C7B	*xylosus*	21	18	24	25	28	33
F3C8	*xylosus*	18	15	19	20	25	26
F3C9	*xylosus*	21	17	21	24	29	30
F3C10	*felis*	19	15	23	19	22	25
F3C11	*xylosus*	20	17	20	21	26	29
F3C12	*xylosus*	20	15	19	20	25	26
F3C13	*sciuri*	20	17	21	21	25	27
F3C14	*sciuri*	17	17	22	22	27	30
F3C15	*sciuri*	20	20	27	23	22	33
F3C16	*sciuri*	15	15	19	18	25	25
F3C17	*sciuri*	17	17	24	22	28	30
F3C18	*sciuri*	17	15	21	19	25	27
F3C19	*sciuri*	19	20	25	22	33	35
F3C20	*sciuri*	22	2	30	25	32	30
F3C21A	*sciuri*	30	25	35	33	33	40
F3C21B	*sciuri*	25	19	27	32	32	35
F5C1	*sciuri*	15	15	19	15	24	23
F5C2	*sciuri*	16	16	25	18	27	25
F5C3	*sciuri*	14	15	18	16	24	22
F5C4	*sciuri*	14	15	20	17	24	24
F5C5	*sciuri*	15	16	23	19	24	28
F5C6	*sciuri*	15	17	23	20	26	28
F5C7	*sciuri*	14	15	21	19	24	26
F5C8	*sciuri*	14	15	20	17	24	25
F5C9	*sciuri*	15	16	22	19	24	26
F5C10	*sciuri*	17	17	23	18	23	25
F5C11	*sciuri*	15	16	21	18	26	25
F5C12	*sciuri*	16	17	24	20	25	28
F5C13	*sciuri*	13	16	21	18	24	25
F5C14	*sciuri*	14	16	22	18	24	27
F5C15	*sciuri*	14	16	22	18	25	27
F5C16	*xylosus*	20	20	24	27	27	35
F5C18	*sciuri*	15	16	24	18	25	25
F5C19	*sciuri*	16	15	20	17	25	25
F5C20	*sciuri*	18	16	27	21	25	26
F6C5	*sciuri*	16	15	23	18	24	25
F6C6	*sciuri*	18	18	25	20	24	26
F6C7	*xylosus*	16	15	24	18	24	26
F6C9	*sciuri*	16	16	21	19	22	25
F6C10	*xylosus*	17	16	18	22	24	25
F6C11	*sciuri*	14	16	24	18	24	25
F6C12	*sciuri*	15	16	20	19	23	25
F6C13	*xylosus*	20	18	19	25	27	28
F6C14	*sciuri*	13	16	17	17	22	25

Table 4. continued

F6C15	*xylosus*	17	16	18	21	24	25
F6C16	*sciuri*	16	16	21	18	23	26
F6C17	*sciuri*	14	16	19	17	24	27
F6C18	*gallinarum*	23	18	20	16	25	28
F6C19B	*xylosus*	15	14	18	17	23	22
F7C1	*saprophyticus*	20	17	20	25	25	29
F7C2	*xylosus*	19	16	21	20	26	26
F7C3	*sciuri*	13	16	25	18	25	26
F7C4	*sciuri*	17	15	19	20	25	26
F7C5	*sciuri*	11	15	22	18	24	26
F7C6	*sciuri*	16	15	27	?	14	14
F7C7	*xylosus*	22	18	22	20	28	30
F7C8	*xylosus*	17	13	16	20	23	24
F7C9	*xylosus*	18	16	21	22	28	29
F7C10	*sciuri*	15	16	20	17	25	23
F7C11	*sciuri*	18	17	29	24	30	30
F7C12	*xylosus*	19	16	18	21	25	25
F7C13	*sciuri*	15	14	20	17	23	21
F7C14A	*felis*	16	15	22	22	25	27
F7C14B	*xylosus*	18	16	16	22	22	24
F7C15	*xylosus*	20	15	20	22	25	26
F7C17	*xylosus*	19	16	21	25	27	30
F7C18	*xylosus*	18	15	20	16	15	24
F7C19	*xylosus*	19	19	24	20	12	29
F8C1	*sciuri*	16	15	20	18	26	26
F8C3	*sciuri*	16	15	19	18	24	25
F8C4	*sciuri*	20	20	24	22	30	31
F8C5	*scirui*	16	16	20	17	26	28
F8C6	*capitis*	22	19	25	23	14	30
F8C8	*sciuri*	15	15	20	16	26	27
F8C9	*sciuri*	16	16	24	19	25	29
F8C11	*xylosus*	20	17	19	21	27	27
F8C15	*gallinarum*	21	17	23	20	27	30
F8C16	*xylosus*	22	17	21	22	28	28
F8C17	*sciuri*	16	15	19	17	25	26
F8C18	*felis*	17	17	21	20	28	28
F8C19	*sciuri*	16	15	20	17	14	25
F8C20	*sciuri*	17	15	23	17	22	24
F9C1	*xylosus*	20	17	19	19	30	29
F9C2	*xylosus*	19	16	16	20	25	27
F9C3	*sciuri*	15	14	23	18	22	24
F9C5A	*xylosus*	20	15	16	17	26	28
F9C5B	*xylosus*	20	15	16	17	23	26
F9C6	*gallinarum*	20	16	19	26	29	29

Table 4. continued

F9C7	*xylosus*	18	15	18	21	25	25
F9C8	*sciuri*	14	15	22	17	24	23
F9C9	*sciuri*	11	14	23	17	23	26
F9C10	*sciuri*	14	13	18	15	21	23
F9C11	*sciuri*	16	15	20	18	23	27
F9C12	*sciuri*	16	14	22	18	22	25
F9C13	*sciuri*	14	15	22	17	25	25
F9C15	*sciuri*	16	15	23	17	22	25
F9C16	*sciuri*	17	16	25	18	25	27
F9C17	*sciuri*	15	14	22	17	24	26
F9C18	*xylosus*	19	14	17	17	26	26
F9C19	*sciuri*	17	16	21	19	25	26
F9C20	*sciuri*	16	14	18	17	23	22
F10C2	*cohnii*	20	18	20	18	15	25
F10C3	*xylosus*	16	14	17	21	22	24
F10C5	*xylosus*	21	18	22	20	26	27
F10C7	*xylosus*	18	15	19	20	24	25
F10C8B	*xylosus*	17	14	18	20	23	24
F10C9A	*xylosus*	17	15	20	23	25	26
F10C9B	*xylosus*	18	10	20	21	25	26
F10C10	*xylosus*	20	17	20	20	27	18
F10C11	*xylosus*	22	17	20	20	17	30
F10C12	*xylosus*	19	16	20	22	25	25
F10C13	*xylosus*	17	15	19	21	25	27
F10C14	*xylosus*	16	13	17	21	23	24
F10C15	*xylosus*	20	17	22	25	28	30
F10C16	*xylosus*	19	16	20	23	25	25
F10C17A	*xylosus*	18	15	18	22	25	25
F10C17B	*xylosus*	17	15	20	22	25	25
F10C18	*xylosus*	16	14	19	22	25	26
F10C19	*xylosus*	17	15	18	20	23	23
F10C20	*xylosus*	18	15	19	22	24	25
Dry 2	*xylosus*	20	16	20	20	29	29
Dry 3	*felis*	21	18	23	20	30	32
Dry 5	*xylosus*	22	16	20	20	28	28
Dry 6	*xylosus*	20	11	19	19	27	26
Dry 7	*xylosus*	20	17	21	20	29	30
Dry 9A	*xylosus*	22	18	22	22	30	30
Dry 9B	*xylosus*	21	17	21	20	30	30
Dry 10	*xylosus*	20	17	21	20	29	30
Dry 11	*xylosus*	18	11	23	25	27	29
Dry 13	*sciuri*	13	15	19	18	25	27
Dry 14	*xylosus*	17	15	20	23	26	27
Dry 15	*xylosus*	20	16	19	21	27	27

Table 4. continued

Dry 16	*sciuri*	16	15	25	19	25	26
Dry 17	*sciuri*	17	15	24	19	25	26
Dry 18	*xylosus*	16	15	18	22	24	24
Dry 19A	*sciuri*	16	16	20	19	24	28
Dry 19B	*sciuri*	15	15	20	19	24	26
Fresh 11	*sciuri*	15	14	21	19	24	25
Fresh 13	*sciuri*	14	15	20	18	24	24
Fresh 14	*xylosus*	19	16	19	20	27	26
Fresh 15	*xylosus*	20	16	20	27	25	30
Fresh 16	*sciuri*	16	16	22	18	15	29
Fresh 17	*sciuri*	15	16	22	18	20	26
Fresh 18	*sciuri*	16	15	21	17	15	25
Fresh 19	*sciuri*	16	16	22	18	15	28
Fresh 20	*xylosus*	16	17	14	10	28	30
Fresh21A	*felis*	18	17	25	24	28	28
Fresh21B	*xylosus*	18	15	20	18	10	27
Fresh22A	*xylosus*	18	16	20	23	26	26
Fresh22B	*xylosus*	19	16	21	24	27	28
Fresh 23	*xylosus*	17	16	19	21	12	29
Fresh 24	*xylosus*	17	11	19	20	28	27
Fresh 25	*xylosus*	19	15	21	23	28	28
Fresh 26	*xylosus*	17	15	19	21	25	24
Fresh 27	*cohnii*	18	17	21	21	28	30
Fresh 28	*xylosus*	17	15	19	21	25	25
Fresh 29	*xylosus*	13	16	21	18	26	27
Fresh30A	*xylosus*	19	17	21	22	27	27
Fresh 31	*xylosus*	18	15	20	23	26	26
Fresh 32	*xylosus*	18	17	21	23	26	27
Fresh 33	*xylosus*	15	16	20	20	27	26
Fresh34A	*xylosus*	19	16	20	23	26	27
Fresh34B	*xylosus*	14	19	20	23	30	30
Fresh 36	*xylosus*	15	17	20	21	28	28
Fresh 37	*xylosus*	13	17	19	19	28	26
Fresh 38A	*xylosus*	14	16	20	25	28	27
Fresh 38B	*xylosus*	15	17	20	27	29	28
Fresh 39	*xylosus*	17	17	21	23	27	27
Fresh 40A	*xylosus*	18	16	20	27	27	26
Fresh 40B	*xylosus*	19	18	23	20	27	30
Fresh 41	*xylosus*	16	16	21	26	28	28
Fresh 42A	*xylosus*	15	17	20	30	27	27
Fresh 42B	*xylosus*	17	17	22	27	28	27
Fresh 44	*xylosus*	14	15	18	21	26	24
Fresh 45	*xylosus*	15	15	18	22	25	26
Fresh 46	*xylosus*	15	15	20	22	26	26

Table 4. continued

Fresh 47	*xylosus*	15	15	18	22	25	25
Fresh 48	*xylosus*	18	17	18	10	26	26
Fresh 49	*xylosus*	16	17	21	25	28	28

[a] numbers represent the diameter of the zone of inhibition in millimeters. OX, oxacillin; P, penicillinG; CC clindamycin; E, erythromycin; SXT, sulfamethaoxazole-trimethoprim

[b]Vancomycin resistance data is included in this table but was not used in the final analysis of these results

TABLE 5. Percentage of *Staphylococcus* isolates resistant to individual antibiotics

Antibiotic	Number of resistant isolates[a]	Percent of isolates with resistance
Oxacillin	132	59
Penicillin	221	99
Clindamycin	2	0.9
Erythromycin	3	1
SXT[b]	0	0

[a] As determined by the disk diffusion method according to NCCLS guidelines (27).
[b] Sulfamethoxazole-trimethoprim

TABLE 6. Antibiotic resistance patterns of *Staphylococcus* by species

Species	Number	Antibiotics[a]				
		OX	P	CC	E	SXT
S. sciuri	93	R	R	S	S	S
S. sciuri	15	S	R	S	S	S
S. sciuri	3	S	S	S	S	S
S. sciuri	1	S	R	S	R	S
S. xylosus	60	S	R	S	S	S
S. xylosus	35	R	R	S	S	S
S. xylosus	1	S	R	S	R	S
S. xylosus	1	R	R	R	S	S
S. felis	3	S	R	S	S	S
S. felis	3	R	R	S	S	S
S. gallinarum	3	S	R	S	S	S
S. saprophyticus	1	S	R	S	S	S
S. capitis	1	S	R	S	S	S
S. cohnii	1	S	R	S	S	S

[a] OX, oxacillin; P, penicillin G; CC clindamycin; E, erythromycin; SXT, sulfamethoxazole-trimethoprim; R, resistant; S, sensitive

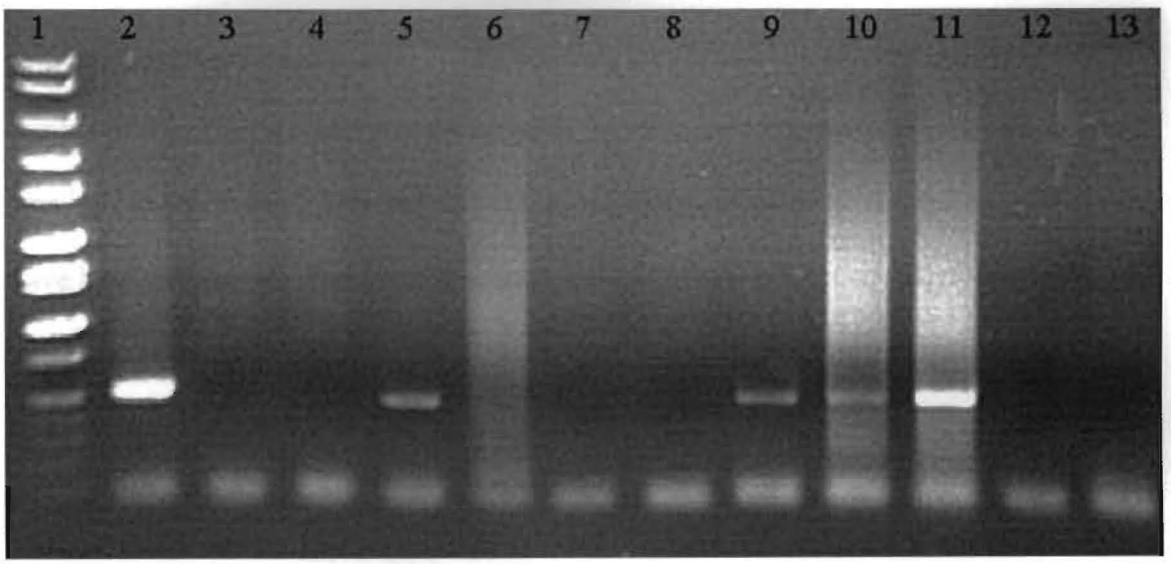
TABLE 7. Minimum inhibitory concentration of oxacillin for resistant staphylococci

Isolate number	Species	MIC (μg/ml)
F1C6	*S. sciuri*	0.5
Dry 13	*S. sciuri*	0.5
Fresh 29	*S. xylosus*	0.75
Fresh 44	*S. xylosus*	0.5
F2C18	*S. sciuri*	0.5
F9C10	*S. sciuri*	0.5
F9C9	*S. sciuri*	0.5
F6C19B	*S. xylosus*	0.25
F7C13	*S. sciuri*	0.5
F9C3	*S. sciuri*	0.5

Polymerase chain reaction (PCR)

PCR was performed on DNA isolated from 10 of the oxacillin resistant isolates using primers targeting the *mecA* gene. Amplification products were analyzed visually by agarose gel electrophoresis (Fig. 5). A 534 base pair amplicon, indicative of the *mecA* gene fragment, was amplified in 4 of the 10 samples in addition to the methicillin resistant *Staphylococcus aureus* (MRSA) positive control.

Figure 5. Agarose gel of the 534 bp *mecA* amplicons from 10 oxacillin resistant *Staphylococcus* isolates. Reaction conditions are described in the Methods and Materials. Lane 1, molecular weight markers; lane 2, MRSA positive control; lane 3, *S. aureus* negative control; lane 4, F9C9; lane 5, F9C3; lane 6, F7C13; lane 7, F2C18; lane 8, F9C10; lane 9, F1C6; lane 10, Fresh 29; lane11, F6C19B; lane 12, Fresh 44. Lanes 4-10 are *S. sciuri* and lanes 11-13 are *S. xylosus*.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Discussion

Antibiotic resistance is a becoming a topic of great concern. Increasing use of prescription antibiotics as well as antibacterials in household products, livestock feed and our water supply place pressure on bacterial populations to develop resistance. Penicillin was introduced in the 1920s and since then, hundreds of natural and synthetic antibiotics have been developed. Oxacillin is an antibiotic in the penicillin, or β-lactam, family. By 1961, oxacillin resistant staphylococci were being reported (5, 35). Resistance to β-lactams in staphylococci is primarily conferred by the *mecA* gene, a chromosomal gene of unknown origin that encodes an enzyme for building peptidoglycan (29). Additionally, some strains may carry a plasmid containing a β-lactamase operon resulting in the overproduction of this enzyme (29).

Antibiotic resistant *Staphylococcus* have been isolated from many different domestic animal populations (1, 23, 24). In one study, approximately 39% of the 44 staphylococci isolated from the external ear of dogs were resistant to penicillin G (24), which was in agreement with previous studies (21, 22). Resistance to oxacillin, however, was low in this previous investigation. A study of 104 CoNS isolated from cats resulted in a 65% penicillin G resistance rate as well as a 22% oxacillin resistance rate (23). Higher rates of resistance in these populations should not be surprising as they are domestic and probably have a large amount of contact with antimicrobial substances. Alarmingly, an even higher rate of resistance was found in the present study from a wild population of gray treefrogs.

Many studies have been done on β-lactam resistance in *S. aureus*, however, CoNS are now being recognized as a significant concern. They are a leading cause of

nosocomial infections, especially among immunocompromised people, newborns and those with internal prosthetic devices (38). In a 1998 study, 64% of the organisms isolated from catheters were CoNS (36). An even more startling statistic is that approximately 75% of hospital strains are resistant to penicillins (38).

The decision to study staphylococci from amphibians was made for several reasons. The use of these animals in elementary and secondary classrooms as hands-on educational tools is very common. They are useful tools for illustrating differences in animal anatomy and physiology as well as teaching children responsibility through proper care and maintenance of a pet. Recently, a campaign has been launched to educate Kansas teachers about the possible health risks of handling animals (14). The amphibian and reptile information focuses mainly on the possibility of acquiring *Salmonella* infections.

There have not, however, been any studies examining the presence of *Staphylococcus* species in amphibians and reptiles. It has been known for many years that staphylococci are a clinically significant pathogen and therefore a study of their presence in these animals and antibiotic resistance profile seemed prudent. *Staphylococcus aureus* has been considered the major pathogen in this genus, however, it has been shown that CoNS may also produce cytotoxic proteins (39). Additionally the rapid increase in β-lactam resistance among all *Staphylococcus* species illustrates the ability of these organisms to transfer the *mecA* gene between species. Resistance is not limited to this genus and has been observed in other, serious pathogens such as pneumococci (34).

MSA plates provided a convenient way to initially isolate *Staphylococcus* species from the fecal material of gray treefrogs. The 222 CoNS identified were comprised of 7 species. Of the isolates, *S. sciuri* was the most predominant at 50% followed by *S. xylosus* at 43% (Table 1). Both of these organisms are considered part of the normal flora of rodents (15, 16) and *S. xylosus* can act as an opportunistic pathogen (17). Infection of the soft tissue, endocarditis, pulmonary infections and genitourinary tract infections can result from colonization by CoNS. These findings emphasize the importance of handling amphibians in a safe and appropriate manner.

Further emphasizing the necessity for caution were the antibiotic resistance profiles determined using the disk diffusion method. Ninety nine percent of the 222 CoNS isolated were resistant to penicillin G and 59% were resistant to oxacillin (Table 2). As shown in Table 3, many of the isolates also exhibited multiple antibiotic resistances. A previous study showed that the use of Mueller-Hinton media containing 2% NaCl for the testing of oxacillin susceptibility in staphylococci significantly increased the accuracy of the results (11). This method is now recommended by the NCCLS and was, therefore, used in this study. Since oxacillin is a very clinically important antibiotic, 10 of the oxacillin resistant isolates were randomly chosen and screened for the *mecA* gene. Gene specific PCR primers revealed the presence of the gene in 4 of the 10 isolates (Fig. 3). These results may suggest divergence at the primer binding site or a *mecA* independent resistance mechanism. The disk diffusion method would indicate resistance in isolates that could be overproducing β-lactamases or have intrinsically modified, low affinity PBPs (29). These mechanisms, however, would not be indicated by a *mecA* primed PCR. No attempt was made to test these hypotheses. Additionally,

the outer edges of the zones of inhibition in some isolates were diffuse. This observation may indicate that certain strains are inhibited by an antibiotic via a strain specific made of action. However, no data support this conclusion and future studies may address this issue

The levels of antibiotic resistance found in this study are high. Of particular concern, however, is the fact that the frogs used in this study were captured in their natural environments. These areas were adjacent to land that could be classified as agricultural and therefore it is possible that these frogs were exposed to antibiotic runoff. One area, however, was only used intermittently for farming purposes and the other was next to a national wildlife refuge. It would be impossible to say that these frogs never encountered antibiotics. However, the likelihood of prolonged exposure is very small.

The physiology of the frog may be a more likely explanation. It has been documented that antibiotic compounds can be found in the skins of amphibians (2, 20, 32). It is possible that one of these peptides mimics the antibiotics that were tested. If this is the case, the normal flora of gray treefrogs would have had to develop resistance to these compounds. Additionally, it has been established that methicillin resistance is more pronounced in bacteria grown at 30°C vs. 37°C (25). Amphibians are cold-blooded and, therefore, lower body temperatures may be playing a role in the development and maintenance of resistance in the gray treefrogs' bacterial flora.

Although the CoNS isolated are not as pathogenic as *S. aureus*, they can still be considered opportunistic pathogens. These infections could be made even more serious with the addition of antibiotic resistance. Additionally, there is the possible danger of resistance transfer. Indeed, it has been shown that plasmids from *Staphylococcus* can be

transferred to *Listeria* (6). The increase of antibiotic resistance among all genera of bacteria is of grave concern both scientifically and medically. Much care needs to be taken in the handling of all organisms and in the study of the extent of this problem.

The present study was limited by the lack of previous research on this topic. Previous studies have been focused on antibiotic resistance in *S. aureus*. The inclusion of other species of bacteria in this study gave it a broader scope than previous studies, however it limited possibilities for comparison of results. Additionally, the study could have been made stronger by the collection of gray treefrogs from more than two locations. Future studies could include a comparison of staphylococci isolated from gray treefrogs captured in a wider variety of environments. A comparison of the flora from other species of amphibians found in these locations would be interesting as well.

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Antibiotic Resistance in Coagulase-Negative Staphylococci Isolated from Cope's Gray Treefrog

Title of Thesis

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April 30, 2001

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