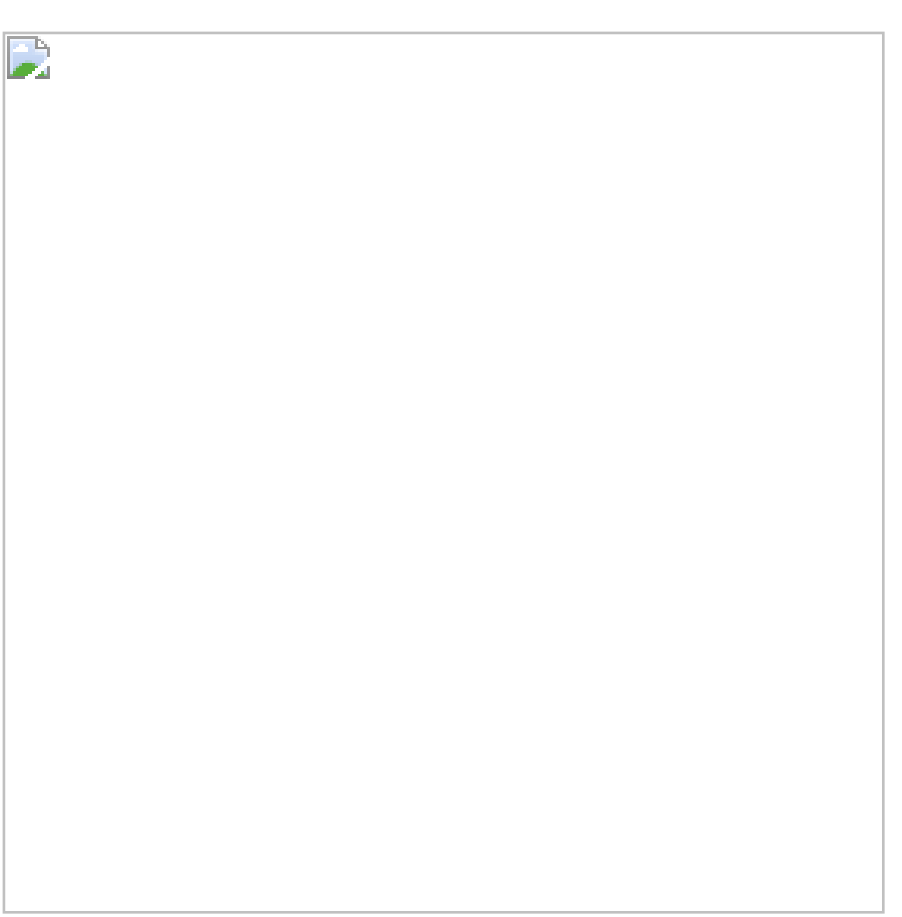

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Authors: Pinard, Chantale L., Brightman, Alan H., Yeary, Teresa J., Everson, Troy D., Cox, Linda K., et al.

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Normal Conjunctival Flora in the North American Opossum (*Didelphis virginiana*) and Raccoon (*Procyon lotor*)

Chantale L. Pinard,[1,3] Alan H. Brightman,[1] Teresa J. Yeary,[2] Troy D. Everson,[1] Linda K. Cox,[2] M. M. Chengappa,[2] and Harriet J. Davidson[1] [1] Department of Clinical Sciences, Mosier Hall, Kansas State University, Manhattan, Kansas 66506, USA; [2] Department of Pathology and Diagnostic Medicine, Kansas State University, Manhattan, Kansas 66506, USA; [3] Corresponding author (email: cpinard@vet.ksu.edu)

ABSTRACT: We documented the normal conjunctival bacterial flora from 17 opossums (*Didelphis virginiana*) and 10 raccoons (*Procyon lotor*) trapped in Manhattan, Kansas (USA) from November 1999 to January 2000. Both raccoons and opossums were free of apparent ocular disease. The inferior conjunctival sacs of each animal were swabbed for aerobic bacterial and *Mycoplasma* culture and polymerase chain reaction (PCR) for *Mycoplasma* and *Chlamydia* detection. All conjunctival samples were positive for one or more species of aerobic bacteria. The most common isolate from opossums was *Staphylococcus* spp. Other isolates included *Streptococcus* spp., *Bacillus* spp., *Corynebacterium* spp., and *Enterococcus faecalis.* The most common isolate in raccoons was *Bacillus* spp. Other isolates included *Streptococcus* spp., *Staphylococcus* spp., non-hemolytic *Escherichia coli,* and *Enterococcus faecalis. Mycoplasma* culture was negative in samples from opossums and raccoons. Evidence of *Mycoplasma* and *Chlamydia* presence was detected by PCR.

Key words: Chlamydia, conjunctiva, *Mycoplasma,* normal flora, opossum, survey, raccoon.

Opossums (*Didelphis virginiana*) and raccoons (*Procyon lotor*) are common in rural and urban communities throughout much of North America. They are occasional household pets and are used in research (Asoh and Goyal, 1978; Christensen and Percy, 1984). Therefore, these animals may be presented as patients with ocular disease. Although some ocular features have been described in both species (Oswaldo-Cruz et al., 1979; Rohen et al., 1988; McMenamin and Krause, 1993), the normal conjunctival flora in these animals has not been reported. Normal conjunctival flora have been studied in dogs, cats, horses, cattle, sheep, llamas, rabbits, birds (Moore and Nasisse, 1998) and deer (Dubay et al., 2000). Gram-positive bacteria were reported as the most common isolates. *Mycoplasma* was identified in normal canine (Rosendal, 1973), feline (Campbell et al., 1973), bovine (Barber et al., 1986) and ovine eyes (Dagnell, 1994), but not in equine (Whitley and Moore, 1984), llama (Gionfriddo et al., 1991), bison (*Bison bison*) (Davidson et al., 1999) and avian eyes (Wolf et al., 1983). *Chlamydia* was detected in normal porcine eyes (Davidson et al., 1994). The purpose of this study was to describe the normal conjunctival flora of the opossum and raccoon eye.

Opossums and raccoons were trapped in the suburbs of Manhattan, Kansas (USA) as part of a study of external parasites and evaluation of sedatives in these species. The study took place from November 1999 to January 2000. Traps were set at night and checked in the morning. Seventeen opossums (eight males/nine females) and 10 raccoons (seven males/three females) were sedated with intramuscular injections of medetomidine hydrochloride (0.1–0.132 mg/kg in opossums; 0.75 mg/kg in raccoons; Animal Health, Exton, Pennsylvania, USA) and ketamine hydrochloride (10 mg/kg in opossums; 2.5 mg/kg in raccoons; Phoenix Scientific, Inc., St. Joseph, Missouri, USA). All eyes were examined with a direct ophthalmoscope (Welch Allyn, Arden, North Carolina, USA). Age was determined by examination of the dentition.

A total of three eye swabs were taken from each animal for bacterial isolation and identification. Two samples for aerobic bacterial and mycoplasma testing were taken from the left inferior conjunctival sac using sterile pre-moistened dacron swabs (Mini-Tip Culturette, Becton-Dick-

inson Microbiology Systems, Franklin, New Jersey, USA). Testing for *Chlamydia* and *Mycoplasma* spp. by polymerase chain reaction (PCR) was performed on DNA extracted from swabs taken from the right inferior conjunctival sac that were immersed in Bovarnick's transport buffer.

Bacterial swabs were plated for culture within 1–3 hr of collection. The first left eye swab taken was used for culture on blood and MacConkey plates and in Schdaedler's enrichment media and incubated at 37 C in 5% CO_2 for 24 hr. The enrichment cultures were streaked onto blood agar after 24 hr. Colonies isolated from all plates after 24–48 hr incubation were identified by biochemical reactions and the Gram reaction following standard protocols (Quinn et al., 1994; Carter et al., 1995). To distinguish *Staphylococcus epidermidis* from other non-hemolytic *Staphylococcus* spp., oxidase-negative, catalase-positive, and Gram-positive cocci were tested for mannitol, maltose, and trehalose fermentation. *Staphylococcus aureus* and *Staph. intermedius* were differentiated by hemolysis and fermentation of mannitol, maltose, and trehalose. To differentiate between α-hemolytic *Streptococcus* spp. and α-hemolytic *Enterococcus* spp., acid production in trehalose, sorbitol, mannitol, salicin, lactose, raffinose, inulin, and esculin broth was documented for all catalase-negative, Gram-positive cocci. In addition, alpha-hemolytic *Streptococcus* spp. were tested for bile-esculin hydrolysis and growth in 6% NaCl. *Corynebacterium* spp. was identified by Gram stain, triple sugar iron, urea, casein, hydrolysis, and catalase reaction. Further identification of this bacterium was done with nitrate, glucose, maltose, lactose, and sucrose medium reactions.

The second swab was used to culture *Mycoplasma* spp. by inoculating modified Friis' agar plates (Lauerman, 1994), then placing the swab into Friis' broth, a PPLO-based medium. Additional Friis' agar plates were streaked from the broth culture on days 3, 7, 10, and 14. At 10 days postinoculation the plates were examined for *Mycoplasma*-like colonies.

Bacterial DNA from the right eye swab was extracted using the QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, California, USA) according to the manufacturer's instructions for bacteria from eye swabs. The *Mycoplasma* genus-specific PCR protocol was followed exactly as described (Lauerman, 1998a, b) using primers developed in Japan (Harasawa et al., 1986): JGM*F*: 5′-ACA CCA TGG GAG CTG GTA AT-3′ and JGM*R*: 5′-CCT CAT CGA CTT TCA GAC CCA AGG CAT-3′. The thermal cycles were: 40 cycles of 94 C for 30 sec, 55 C for 30 sec, 72 C for 60 sec ending with incubation at 72 C for 5 min. A nested PCR scheme using two pairs of oligonucleotide primers which flank variable domains 3 and 4 of the *Chlamydia omp*1 gene were used that uniformly amplify all species of *Chlamydia* (Kaltenboeck, 1998). Samples were subjected to PCR in two separate reactions designated PRIM3, the first reaction, and SEC3, the secondary, internal, nested reaction. PRIM3 amplification with primers 191CHOMP: 5′-GCI YTI TGG GAR TGY GGI TGY GCI AC-3′ and CHOMP37: 5′-TAG AA ICK GAA TTG IGC RTT IAY GTG IGC IGC-3′ began with a 10 min denaturation at 96 C followed by 50 3-step cycles of denaturation at 96 C for 1 sec, annealing at 46 C for 1 min, and chain elongation at 72 C for 1 min. Amplification of the secondary genus-specific *omp*1 gene region was performed using SEC3 primers, 201CHOMP: 5′-GGI GCW GMI TTC CAA TAY GCI CAR TC-3′ and CHOMP336: 5′-CAA GMT TTT CTG GAY TTM AWY TTG TT-3′ and proceeded for 35 cycles with denaturation at 96 C for 1 sec, annealing at 46 C for 1 min, and chain elongation at 72 C for 1 min. Standard precautions were followed throughout to avoid amplicon cross contamination of the PCR reactions. Amplifications were performed in a PTC-100® Programmable Thermal Controller (MJ Research, Inc., Watertown, Massachusetts, USA). Aliquots of the PCR prod-

ucts were analyzed following electrophoresis in 1.5% (w/v) agarose gels and ethidium bromide staining.

The captured animals were yearlings, with the exception of four adults (three opossums, one raccoon). All animals appeared to be in good body condition and health except for one opossum and one raccoon that had several bite wounds on their bodies. No obvious eye diseases were observed upon examination with the direct ophthalmoscope.

Aerobic bacteria were cultured from all opossums. Eleven samples required growth on enrichment media. A Gram-negative inert rod and a Gram-positive catalase-negative coccus could not be identified with standard methods. These unknown bacteria were found on two unrelated animals. *Staphylococcus* spp. was the most common bacteria isolated in opossums (82%). Not all the *Staphylococcus* could be identified; however, *Staph. intermedius, Staph. aureus,* and *Staph. epidermidis* were identified. The second most common bacteria were *Streptococcus* spp. (29%) and *Bacillus* spp. (29%). *Corynebacterium* spp. (12%) and *Enterococcus faecalis* (6%) were also isolated. Sixty-five percent of opossums had more than one bacterium grown from the conjunctival swabs. Polymerase chain reaction for *Chlamydia* was positive for 18% of opossum samples. Results of PCR for *Mycoplasma* were similar (12%). *Mycoplasma* cultures were negative in both species.

Aerobic bacteria were isolated from all ten raccoon samples submitted. Four samples required growth on enrichment media. *Bacillus* spp. was the most common bacteria isolated (60%). *Streptococcus* spp. and *Staphylococcus* spp. were isolated from 30% of raccoons. Not all the *Staphylococcus* spp. could be identified; however, *Staph. intermedius* and *Staph. aureus* were found. Non-hemolytic *Escherichia coli* (20%) and *Enterococcus faecalis* (20%) also were isolated. One colony of *Pseudomonas aeruginosa* was cultured from an individual raccoon with skin lacerations that also had colonies of *Bacillus* spp. and *Staph. aureus* present in its conjunctival flora. Eighty percent of the samples submitted from the raccoons had more than one bacterium. Polymerase chain reactions for *Chlamydia* were positive for 30% of raccoons. Similar results were obtained on PCR for *Mycoplasma* (30%).

The normal conjunctival flora of the opossum and raccoon is similar to the dog and cat (Gerding and Kokamo, 1990; Espinola and Lilenbaum, 1996). In our study, *Mycoplasma* was identified by PCR but could not be confirmed by culture. The low percentage of *Mycoplasma* recovered was similar to that found in cats (Campbell et al., 1973). Campbell et al. (1973) cultured *Mycoplasma* from 5% of 240 samples. Isolation of *Chlamydia* in cats, however, is most often associated with conjunctivitis (Ramsey, 2000). The pig is the only species reported to have *Chlamydia* recovered from normal appearing eyes (Davidson et al., 1994). Therefore, it is interesting to find the DNA of this organism in normal appearing opossum and raccoon eyes.

Mycoplasma and chlamydia nucleic acids were detected by PCR. Ideally, confirmation of mycoplasmal and/or chlamydial infection by culture would have substantiated our PCR results, but many *Mycoplasma* and *Chlamydia* spp. are fastidious, requiring special media for propagation, and neither genus has been described as being cultured from opossum or raccoon eyes. The PCR can be very sensitive and specific if proper technique and protocols are followed. High sensitivity and specificity has been reported for the detection of chlamydial DNA in human adults with chlamydial conjunctivitis (Kowalski et al., 1995). Both mycoplasma and chlamydia have conserved genus-specific sequences that allow these organisms to be reliably detected by PCR (Kaltenboeck et al., 1992; Lauerman, 1998a, b) and established methods were employed in this study. Since PCR identifies DNA, latent infections or dead organisms can be de-

tected when cultural methods yield negative results. False positive results may occur if contamination occurs during testing, however, standard precautions were followed in this study to avoid amplicon cross contamination of the PCR reactions.

Sampling technique, geography, season, and ambient temperature at collection time may influence the prevalence of certain bacteria (Gerding and Kokamo, 1990). In this study, the aforementioned factors were the same throughout the study. The ambient temperature at collection was approximately 5 C. The winter climate may have influenced presence of certain organisms and therefore the flora identified might have been different in summer. The small sample size in this study could also be another factor influencing the culture results and percentages reported.

The normal bacterial conjunctival flora in the opossum and raccoon are similar to those reported in other species. The PCR finding of *Mycoplasma* and *Chlamydia* warrants further study.

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