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Effectiveness of Nasal Flush Treatments in Mycoplasma PCR- or DNA Sequencing–Positive Tortoises

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Abstract

Mycoplasmas can cause severe chronic nasal sinusitis in tortoises and box turtles, both in captive and wild populations. The aim of this study was to compare positive and negative *Mycoplasma* sp. test results before and after a nasal flush protocol combined with systemic enrofloxacin and to compare positive and negative results between DNA sequencing and PCR in tortoises with nasal discharge. Fifteen mycoplasma-positive tortoises were included in the study. All were treated with the nasal flush protocol. At the end of the nasal flush protocol, 26.7% of the treated tortoises remained mycoplasma positive. One month after the end of the nasal flush protocol, 20% remained positive. The PCR and DNA sequencing tests had good agreement based on a weighted kappa of 0.64. Based on these results, we conclude that the outlined nasal flush protocol is overall successful at acutely resolving nasal discharge and leading to a PCR- or DNA sequencing–negative tortoise 1 month after treatment.

Keywords: Chelonian, DNA sequencing, mycoplasmosis, nasal flush, PCR, upper respiratory tract disease

Introduction

Mycoplasma (*Mycoplasmopsis*) *agassizii* causes upper respiratory tract disease (URTD) in desert (*Gopherus agassizii*) and gopher (*Gopherus polyphemus*) tortoises (Brown *et al*., 1994, 1995). The most common clinical sign is a serous, mucoid, or purulent nasal discharge. Similar clinical presentations with *M. agassizii*–documented infections have been reported in many species of wild and captive tortoises as well as in box turtles (*Terrapene carolina*, *Terrapene ornata ornata*) and even red-eared sliders (*Trachemys scripta elegans*) in many countries worldwide. All tortoises should be considered potentially susceptible (Jacobson *et al*., 2014). Mycoplasmosis in tortoises is believed to result in initial high mortality, with low mortality and high morbidity to follow (Jacobson *et al*., 2014).

Mycoplasma agassizii attaches to the nasal sinus and choanal epithelium of tortoises and causes an overexuberant, chronic host immune response that fails to clear infection. The nasal mucosa suffers from loss of ciliated epithelium, mucosal hyperplasia, infiltration of lymphocytes and histiocytes, and submucosal lymphoid hyperplasia, resulting in nasal discharge and epithelial ulceration, dysplasia, and necrosis (Jacobson *et al*., 1991; Brown *et al*., 1999).

The most common clinical sign of mycoplasmosis in tortoises is nasal discharge, but ocular discharge, conjunctivitis, and palpebral edema may also be seen. Normal olfactory mucosa becomes replaced with leukocytes, proliferating mucosal epithelial cells, and basal cells (Jacobson *et al*., 1991). It is reported that mycoplasmosis decreases olfaction, potentially suppressing appetite because tortoises use olfaction to find and select food, and may also interfere with predator detection and reproduction because tortoises also use olfaction to track mates and mark territory (Germano *et al*., 2014).

Over time, mycoplasmosis has become one of the most important chronic infectious diseases and most reported infectious disease of wild and captive North American and European tortoises (Soares *et al*., 2004; Jacobson *et al*., 2014; McGuire *et al*., 2014; Goessling *et al*., 2019; Galosi *et al*., 2023). Despite this, little has been published regarding treatment options for affected tortoises.

Mycoplasma-positive American alligators (*Alligator mississippiensis*) have been treated with oxytetracycline (10 mg/kg IM weekly for 5 wk and then biweekly for three additional months) (Clippinger *et al*., 2000). Despite a subjective improvement in their health status and no mycoplasma isolation from opportunistically sampled, previously symptomatic alligators after 4 months of antibiotic therapy, its administration did not slow death, with only 14 of 74 animals remaining 6 months after the first diagnosis.

Clarithromycin, a macrolide antibiotic, concentrates in nasal secretions and is used in treatment of human mycoplasmosis (Tagliabue *et al.*, 2011). Clarithromycin at 20 mg/kg every 2–3 days for a total of 90 days failed to suppress *Mycoplasma* spp. shedding in all but 1 of 10 *Mycoplasma* spp. PCR-positive tortoises (Rettenmund *et al.*, 2017). Nasal flushing with enrofloxacin, dexamethasone, and saline every 48–96 h for three to five treatments, combined with systemic enrofloxacin, cleared nasal discharge in 8 of 10 tortoises while they were followed posttreatment for 11–78 months; however, no diagnostic testing was done (Jarchow, 2004).

The purpose of this study was to compare positive and negative mycoplasma results before and after a nasal flushing protocol, combined with systemic enrofloxacin (Jarchow, 2004), as well as assess agreement between a next generation sequencing (NGS) test (MiDOG pet microbiome test, MiDOG LLC, Tustin, CA, USA) and PCR (Zoological Medicine Diagnostic Laboratory, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA) for *Mycoplasma* spp. An effective treatment would be beneficial to both captive and wild chelonians and facilitate conservation efforts.

Materials and Methods

Inclusion criteria included captive-owned Testudinidae species presented to the Pet Hospital of Penasquitos between September 2022 and May 2023, with nasal discharge and no history or clinical signs suggestive of nonmycoplasma causes of nasal discharge. Only tortoises with *Mycoplasma* spp. detected on either PCR or DNA sequencing were included in the study. If both PCR and DNA sequencing were negative for *Mycoplasma* spp., that patient was excluded from the study. Consent for inclusion of tortoises in this study was obtained from all owners. The sample used for PCR and DNA sequencing was fluid acquired during the nasal flush, as described below.

A thorough husbandry review was performed with each owner at first presentation, and the importance of adequate thermogradients within species preferred operating temperature zones and appropriate diets was relayed. Home visits were not performed to ensure these conditions.

During the duration of treatment, included tortoises were housed individually in stainless steel kennels (Shor-line, Kansas City, KS, USA) or high-density polyethylene plastic cages (Vision Products, Neenah, WI, USA) on newspaper in a temperature-controlled room kept at 27–29°C (80–85°F) at the Pet Hospital of Penasquitos.

Treatment consisted of five nasal flushes under sedation, administered every 48–72 h, with additional flushes performed if nasal discharge was persistent at the fifth flush. Sedation was induced using 20–30 mg/kg of tiletamine/zolazepam (Telazol, 100 mg/ml, Zoetis, Parsippany, NJ, USA) IM to assist nasal flushing, as follows. With the tortoise held head down and in dorsal recumbency, an appropriately sized cotton ball (0.5 to 3 cotton balls depending on oropharynx size) was grasped with hemostats, inserted into the oropharynx, and used to occlude and protect the glottis from aspiration during nasal flushing. Each naris was flushed with 15 ml of 0.9% saline (0.9% sodium chloride, ICU Medical, Inc., Lake Forest, IL, USA) exiting out the choanae and oral cavity. The oral cavity was cleared of any remaining mucous and fluids with fresh, dry cotton balls. Next, a modified Jarchow (2004) solution was made using 0.4 ml of enrofloxacin (Enroflox, 22.7 mg/ml, Norbrook Laboratories Limited, Newry, Northern Ireland); 0.2 ml of dexamethasone (Dexamethasone Injection, 2 mg/ml, Sparhawk Laboratories, Inc., Lenexa, KS, USA); and 2.4 ml of 0.9% saline, for a final volume of 3 ml. Next, 0.5 ml of this solution was instilled into each nostril and then 1 ml was instilled into each choanal slit and allowed to drip down and out the nares to flood the nasal sinuses. An intramuscular injection of 10 mg/kg enrofloxacin (100 mg/ml, Norbrook Laboratories Limited) was given in the pectoral muscle after each flush.

Both PCR and DNA sequencing samples were collected as previously described, with 0.9% saline at the first nasal flush, the last nasal flush, and 1 month after the last nasal flush. For PCR testing, the Zoological Medicine Diagnostic Laboratory requested one choanal swab and 2 ml of nasal flush solution, in separate preservative-free vials. Samples were frozen, sent via overnight shipping, and pooled for analysis. For DNA sequencing, MiDOG pet microbiome test requested one choanal swab and 0.5 ml of nasal flush fluid within a single lab-provided vial with proprietary preservative at room temperature via 2-day shipping. Note that a smaller volume of nasal flush fluid was sent at the request of the DNA sequencing test-kit supplier to avoid overly diluting the preservative within the test kit. In both cases, we followed established submission protocols predetermined by each laboratory.

For statistical analysis, a Cochran's Q test was used to determine whether there was a difference in mycoplasma-positive status over the treatment period. A kappa test was used to measure interrater agreement between the PCR and DNA sequencing results, with values of 0.61–0.80 considered good agreement. A $P < 0.05$ was used to determine statistical significance. MedCalc statistical software (MedCalc Software Ltd., Ostend, Belgium) was used to analyze the data.

Results

Sixteen tortoises with nasal discharge were tested for mycoplasma by PCR and DNA sequencing and included 15 positive tortoises in the study. One tortoise was excluded due to testing negative on both PCR and DNA sequencing at the first and last nasal flushes. Of the 15 tortoises included, 8 were desert tortoises, five were leopard tortoises (*Stigmochelys pardalis*), and two were African spurred tortoises (*Centrochelys sulcata*).

At the first nasal flush, all 15 tortoises were mycoplasma positive by PCR and mycoplasma was detected in 11 tortoises by DNA sequencing. There was a 73% agreement between the two tests. At the last nasal flush, three tortoises were positive on PCR and mycoplasma was detected in one tortoise by DNA sequencing, with 0% agreement between the two tests. One month after the last nasal flush, mycoplasma was detected

Table 1. Results[a] of next generation DNA sequencing (NGDS) and PCR testing for *Mycoplasma* sp. in 15 chelonian patients before their first treatment (#1), at their fifth treatment (#2), and 1 month after the final treatment (#3).

Patient no.	Species[b]	NGDS #1	PCR #1	NGDS #2	PCR #2	NGDS #3	PCR #3
1	Leopard	+	+	−	−	+	+
2	Leopard	−	+	−	+	−	−
3	Leopard	+	+	−	−	−	−
4	Leopard	+	+	−	−	−	−
5	Desert	−	+	−	−	−	−
6	Desert	+	+	−	+	−	−
7	Sulcata	+	+	−	−	−	−
8	Desert	+	+	−	−	−	−
9	Desert	+	+	−	−	−	−
10	Sulcata	+	+	−	−	−	−
11	Desert	−	+	−	−	−	−
12	Desert	+	+	−	+	+	+
13	Leopard	+	+	−	−	−	−
14	Desert	−	+	−	−	−	−
15	Desert	+	+	+	−	+	+

[a] +, positive; −, negative.
[b] Desert, desert tortoise, *Gopherus agassizii*; Leopard, leopard tortoise, *Stigmochelys pardalis*; Sulcata, African spurred tortoise, *Centrochelys sulcata*.

in three tortoises by both PCR and DNA sequencing (100% agreement). Overall, 12 of 15 tortoises that were mycoplasma positive at the beginning of the study tested negative 1 month after the last nasal flush (Table 1).

At the end of the nasal flush protocol, nasal discharge was present in 0 of 15 tortoises. Of 6 of 15 tortoises that had rechecks between 4 and 10 months after treatment, 3 of the 6 remained negative and 3 of the 6 were positive. Of these three positive tortoises, two had been negative at the 1-month recheck and one tested positive at the 1-month recheck. At the time this paper was submitted (1 yr after the end of the study), 2 have been confirmed to have had a recurrence of nasal discharge.

Per inclusion criteria, all 15 tortoises had tested positive for mycoplasma before the nasal flush protocol, whereas 26.7% were positive before the fifth nasal flush protocol and 20% were positive 1 month after the last nasal flush. There was a significant difference in the test results over time ($P < 0.001$), with the samples at the end of the nasal flush protocol and 1 month after the nasal flush protocol being significantly more likely to be negative than samples before the nasal flush protocol. There was no significant difference in the results between samples at the end of the nasal flush protocol and 1 month after the nasal flush protocol.

There was one sample that tested negative for mycoplasma by PCR, but positive by DNA sequencing, whereas seven samples tested negative by DNA sequencing, but positive by PCR. The results of the kappa test for interrater agreement found a weighted kappa of 0.64 between DNA sequencing and PCR results. Based on the standard for kappa results, the tests have good agreement.

Discussion

An *M. agassizii* infection was consistently diagnosed in tortoises with nasal discharge, in agreement with previously fulfilled Koch's postulates that determined *M. agassizii* is an etiologic agent of URTD. The outlined nasal flush protocol, modified from the Jarchow (2004) protocol, was determined to be successful based on the second and third samples being significantly more likely to be *M. agassizii* negative than the first sample, in addition to resolution of acute nasal discharge. Because mycoplasmosis decreases olfaction in tortoises, which may suppress appetite, the resolution of acute nasal discharge was considered an important measure of success for this study.

There are few reports of successful treatment of URTD in tortoises. Clarithromycin at 20 mg/kg by mouth every 2–3 days for 3 months failed to eliminate shedding in 90% of Forsten's tortoises (*Indotestudo forstenii*) and Sulawesi forest turtles (*Leukocephalon yuwonoi*) (Rettenmund *et al.*, 2017). One of the more promising treatments described a nasal flush protocol combined with systemic enrofloxacin (Jarchow, 2004), which showed that nasal discharge resolved in 80% (8/10) of treated tortoises. Enrofloxacin was chosen in that study because it is considered an effective bactericidal antimycoplasma antibiotic. Subsequent *in vitro* susceptibility testing of other reptile mycoplasmas, such as *M. alligatoris* and *M. iguanae*, have borne this out (Helmick *et al.*, 2002; Westfall *et al.*, 2006). Dexamethasone was included within the solution to reduce the overexuberant host response that contributes to the development of nasal discharge. Limitations of that study included a lack of initial diagnostics testing to confirm *Mycoplasma* spp. infections and lack of follow-up diagnostic testing to pair with clinical changes. Our nasal flushing protocol differed from Jarchow's in several ways. First, we used tiletamine-zolazepam sedation to allow better manipulation of the tortoise head for nasal flushing. Second, we flushed each naris with 15 ml of saline before instilling the medicated solution, to clean out nasal sinus mucus and debris and provide diagnostic samples. Third, we performed a minimum of five nasal flushes, with additional flushes if nasal discharge was still present at the fifth, whereas Jarchow (2004) performed three to five flushes. Fourth, we used a higher steroid dosage within the medicated solution based on a miscalculation printed on procedure protocol. And fifth, we used 10 mg/kg enrofloxacin IM every 48–72 h, whereas Jarchow (2004) used 5 mg/kg IM every 48–96 h.

Of the 16 tortoises that received the nasal flush protocol during this study (including a tortoise that tested negative for mycoplasma on both PCR and DNA sequencing, because it had nasal discharge), only 1 tortoise had a recurrence of nasal discharge 1 month after the nasal flush protocol. That tortoise had a complicating factor including an abscess with firm swelling of the left nasal region. A biopsy of that swelling was submitted to a pathology laboratory (NorthWest Zoopath, Monroe, WA, USA) approximately 1 month after the end of the nasal flush protocol. Histopathology showed nasal or sinusoidal mucosa subtended by a broad zone of fibrocartilaginous metaplasia. The specimen was ruled as a reactive process

likely associated with healing of a previous abscess or trauma, with no active inflammation or neoplasia seen. The remaining 15 tortoises did not have any nasal discharge reported 1 month after the nasal flush protocol. One tortoise had a recurrence of nasal discharge 7 months after the nasal flush protocol, 1.5 months after it underwent a transplastronal cystotomy for removal of a large bladder stone. Stress and subsequent immune suppression from this may have played a role in recrudescence, given its positive PCR and DNA sequencing results 2 wk before the surgery. At the time this paper was submitted, seven tortoises were confirmed to remain without nasal discharge. The remainder were lost to follow-up despite communication attempts. Because of many patients being lost to follow-up despite multiple contact attempts, it is difficult to report reliable statistics after the first month posttreatment.

This study demonstrates that NGS is an alternative method of testing for mycoplasma that is not only at least as effective as PCR, but may be preferable due to the ability of NGS to detect a wide range of bacterial and fungal organisms. McCready *et al.* (2024) compared NGS and PCR detection of *Mycoplasma* spp. in rabbits and showed that 5 of 10 samples were positive on NGS, but negative on PCR. This could be attributed to NGS having a higher sensitivity, but other explanations could not be ruled out, such as PCR having higher specificity or a loss of genetic material over time due to sampling storage or shipping. A 2023 paper showed that traditional culture was more likely to show no growth of bacteria by 14% and fungus by 49% compared with NGS results (Damerum *et al.*, 2023). NGS was shown to report more pathogens and commensals for both bacteria and fungi, including *Mycoplasma* spp. Damerum *et al.* (2023) also included samples from a wide variety of exotic animal species, including reptiles (three African spurred tortoises and one Russian tortoise [*Testudo horsfieldii*]).

PCR and DNA sequencing each have a minimum required DNA concentration necessary for organism detection; therefore, mycoplasma could be present in a sample below the limit of detection. One should therefore be careful when interpreting negative test results as a true lack of infection. PCR and DNA sequencing had overall good agreement with each other. Despite this, PCR seemed to have fewer negative results that disagreed with DNA sequencing rather than the other way around and therefore has been adopted by the authors as the preferred testing modality for future cases.

The two laboratories each requested different sample sizes and different sample handling. The Zoological Medicine Diagnostic Laboratory pooled DNA collected from 2 ml of nasal flush fluid and a choana swab, sent frozen in separate vials. The MiDOG pet microbiome test uses prepackaged swabs and test vials. The vials contain a preservative that remains a MiDOG LLC proprietary trade secret, in which the swab is inserted. Fluids can also be submitted, but in small amounts, so as not to dilute the preservative and lead to poor preservation of the sample. The swab was used to collect samples from the choana. A small amount (0.5 ml) of nasal flush fluid was added to the vial that contained the preservative, as well as the swab. The sample was packaged and mailed at room temperature as per test-kit instructions. The variation of sample size may also have contributed to testing performance. There was also no standardized way of distributing the nasal flush fluid for each test. Any mucous or debris was attempted to be prioritized and divided equally for each laboratory.

As mentioned, a negative result does not necessarily indicate a lack of infection. In addition to minimum DNA requirements for the testing modalities used in this study, the importance of considering lack of shedding and latency cannot be understated when interpreting negative results.

Despite an appropriate, consistent, and uniform in-hospital environment being provided to subjects in this study throughout the nasal flush protocol, at-home environments could not be standardized between patients once they returned home with their owners. Inadequate home care (including temperature, humidity, light, and diet) could lead to immunosuppression and contribute to clinical disease development, recurrence, or both (Howerth, 2019). This could have played a part in recrudescence of clinical signs or positive results. One patient was placed in an outdoor pen with another conspecific that had previously had nasal discharge, despite our instructions to keep them separately and was positive 1-month posttreatment. There is no way to know whether this was re-exposure, a treatment failure, or both. In addition, despite similar and consistent training, there was variation in personnel acquiring testing samples and performing the nasal flushes in hospital, which could lead to variability in therapeutic administration and testing sample quality.

Because of the high infectivity rate of *Mycoplasma* spp., wide distribution worldwide, and low host specificity within Testudinidae, a high-sensitivity testing method is ideal to ensure false negative animals do not transmit the infection to others. Despite the effectivity of the outlined treatment protocol at acutely resolving nasal discharge, this study failed to demonstrate a method of treatment that is effective at preventing recurrence of nasal discharge in all included tortoises. In addition, recurrences of mycoplasma detectability on NGS, PCR, or both may suggest that mycoplasma cannot be cleared from the patient, meaning that tortoises may serve as a source of infection to other tortoises despite testing negative for mycoplasma. However, this treatment protocol does show promise despite further research being certainly indicated. If a more effective protocol can be found, it could potentially lead to reduced mortality in wild and captive populations, boosting conservation.

In summary, the outlined nasal flush protocol successfully decreases detectability of *M. agassizii* in tortoises and was efficacious at acutely resolving nasal discharge. If the patient is still positive at the fifth treatment, additional nasal flushing should be considered until PCR testing is negative.

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