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PREVALENCE OF NEUROTOXIC *CLOSTRIDIUM BOTULINUM* TYPE C IN THE GASTROINTESTINAL TRACTS OF TILAPIA (*OREOCHROMIS MOSSAMBICUS*) IN THE SALTON SEA

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ABSTRACT: Tilapia (*Oreochromis mossambicus*) have been implicated as the source of type C toxin in avian botulism outbreaks in pelicans (*Pelecanus erythrorhynchos, Pelecanus occidentalis californicus*) at the Salton Sea in southern California (USA). We collected sick, dead, and healthy fish from various sites throughout the Sea during the summers of 1999 through 2001 and tested them for the presence of *Clostridium botulinum* type C cells by polymerase chain reaction targeting the C_1 neurotoxin gene. Four of 96 (4%), 57 of 664 (9%), and five of 355 (1%) tilapia tested were positive for *C. botulinum* type C toxin gene in 1999, 2000, and 2001, respectively. The total number of positive fish was significantly greater in 2000 than in 2001 ($P<0.0001$). No difference in numbers of positives was detected between sick and dead fish compared with live fish. In 2000, no significant relationships were revealed among the variables studied, such as location and date of collection.

Key words: Avian botulism, *Clostridium botulinum* type C, *Oreochromis mossambicus,* polymerase chain reaction, Salton Sea, tilapia.

INTRODUCTION

In recent years, the Salton Sea in southern California (USA) has been the site of massive mortality events involving fish-eating birds (Friend, 2002). During 1996, type C avian botulism killed over 15,000 birds, including nearly 9,000 western white pelicans (*Pelecanus erythrorhynchos*). More than 1,200 endangered California brown pelicans (*Pelecanus occidentalis californicus*) were affected or died during this outbreak as well (Rocke et al., 2004). Since 1996, much smaller epizootics have occurred every year. Type C botulism is not typically associated with fish-eating birds, although it has on occasion been diagnosed in a few white pelicans involved in waterfowl outbreaks (Rocke and McLean, in press). Type C botulism commonly affects waterfowl in wetlands throughout the US and is often perpetuated by a bird/maggot cycle (Bell et al., 1955; Lee et al., 1962; Duncan and Jensen, 1976; Reed and Rocke, 1992; Rocke and Friend, 1999). At Salton Sea, fish are the suspected source of type C toxin for fish-eating birds, although the mechanism by which fish acquire the toxin is unknown.

Mozambique tilapia (*Oreochromis mossambicus*), an African cichlid, is one of the most numerous fish species in the Sea (Gonzalez et al., 1998; Riedel et al., 2002) and has been a predominant food source for pelicans. In 1995, the Salton Sea's tilapia population experienced a dramatic recruitment of many millions of young fish (R. Riedel, pers. comm.). This large 1995 cohort dominated the population from 1996 until 2000, although it has now undergone a substantial decline (Riedel et al., 2002). In the late 1990s, fish kills involving millions of individuals were a common occurrence at the Salton Sea, as was the presence of individual sick and dead fish between episodes of mass mortality (US Fish and Wildlife Service [USFWS], unpubl. data). Several fish kills and sick tilapia observed at the time of the 1996 epizootic were associated with bacterial and parasitic infections. *Vibrio* spp. and *Amylodinium* spp. were among the pathogens

identified (Kuperman and Matey, 1999; National Wildlife Health Center [NWHC], US Geological Survey, unpubl. data). The NWHC tested live, moribund, and dead tilapia from various sites around the Salton Sea during the outbreak, as well as tilapia remains recovered from the esophagus of sick and dead pelicans, and a large percentage of them contained type C botulinum toxin (Rocke et al., 2004). Rocke et al. (2004) suspected that tilapia suffering from a variety of bacterial infections might have experienced increased susceptibility to toxin or toxin formation inside their gastrointestinal (GI) tracts because of their compromised status.

In this 3-yr study (1999–2001), our objectives were to determine whether tilapia in the Salton Sea harbored cells with the C_1 neurotoxin gene in their GI tracts. We compared prevalence in apparently healthy fish versus sick and dead fish. We also looked for spatial and temporal patterns associated with prevalence of tilapia testing positive for the type C cells.

METHODS

Collection of sick and freshly dead tilapia

In 1999, 2000, and 2001, we collected moribund and freshly dead tilapia from the Salton Sea (33°30′N, 116°3′W; 33°6′N, 115°43′W; 33°21′N, 115°44′W; 33°22′N, 116°00′W) with hand nets along shorelines and from boats before, during, and after botulism outbreaks in birds and during fish kills. Collections were made from July through October. The location of each collection was documented with a global positioning system (GPS) instrument. We immediately placed fish on cold packs or ice and stored them as soon as possible at 4 C until necropsy within 2–12 hr. Entire GI tracts were removed and placed in sterile sample bags and frozen at −20 C until analysis.

Collection of live, healthy tilapia

In 1999, we obtained GI tracts from live, apparently healthy tilapia collected at the mouth of the Alamo River (Fig. 1). These fish were captured with nylon gill nets by collaborating scientists who provided GI tracts frozen to us.

From 1 July to 1 October 2000 and 2001, we collected live, apparently healthy fish from nine locations, each recorded by GPS, around the perimeter of the Sea (Fig. 1). At depths of 1 and 3 m, we captured fish in 10-cm monofilament gill nets that were 16–50 m long. In 2000, collections were carried out at three locations per week so that a representative sample of the entire Sea could be collected every week, and all sites were sampled every 3 wk. Nets were set between sunrise and 10:00 AM for 10 min to 3 hr, depending on success of capture. All fish were placed on cold packs in coolers and then stored at 4 C until necropsy. We saved entire GI tracts in sterile plastic sample bags, which were stored at −20 C.

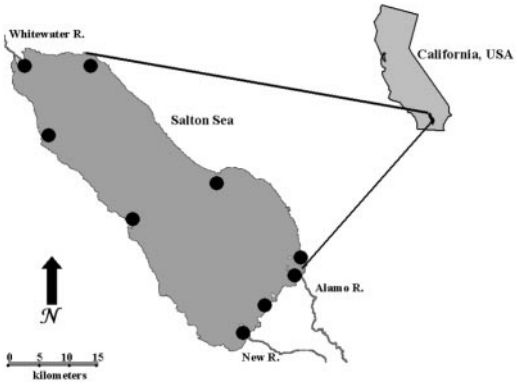

FIGURE 1. Sites in the Salton Sea at which tilapia were collected with gill nets in 2000 and 2001. Each circle represents two stations, one at 1 m and one at 3 m deep.

In 2001, we collected fish the first 2 wk of each month from 1 July through 1 October. The sites were visited at approximately 1-mo intervals. In the event that no fish were caught, we revisited some sites at a later date.

Control tilapia

We obtained Mozambique tilapia from a fish farm in Desert Hot Springs, California (33°58′N, 116°30′W), to serve as a negative control group. These fish were descended from stock that originally came from the Salton Sea. They were stored as whole carcasses at −20 C.

Detection of *Clostridium botulinum* in tilapia

Whole fish and GI samples were shipped on dry ice to NWHC for further processing. We processed and analyzed tilapia GI contents for the presence of *C. botulinum* type C with the use of preparation and DNA extraction procedures followed by a seminested polymerase chain reaction (PCR) assay, as described in Nol et al. (in press) and Williamson et al. (1999). Briefly, DNA was extracted with the UltraClean™ Soil DNA Isolation Kit (MoBio Labo-

TABLE 1. Prevalence of vegetative *Clostridium botulinum* type C in tilapia by year and by status.

Status	Prevalence			
	1999	2000	2001	Total
Live fish	1/31 (3%)	51/535 (10%)	5/318 (2%)	57/884 (7%)
Sick fish	3/46 (7%)	1/106 (0.9%)	0/30 (0%)	9/222 (4%)
Dead fish	0/19 (0%)	5/23 (22%)	0/7 (0%)	5/49 (10%)
Total	4/96 (4%)	57/658 (9%)[a]	5/355 (1%)	

[a] Significantly different from 2001 prevalence (χ^2=20.83; P<0.0001, 1 df).

ratories Inc., Solana Beach, California) with additional purification performed by hexadecyltrimethyl ammonium bromide (CTAB) extraction (Ausubel et al., 1992). The CTAB extraction effectively eliminated any detectable inhibition in the PCR assay (Nol et al., in press). This extraction method minimizes spore lysis and thus yields mainly DNA from vegetative cells. This assay therefore indirectly detects, without enhancement by culture, vegetative cells rather than spores. The PCR targets a portion of the gene that encodes the light chain of C_1 toxin and is specific to the type C gene. A negative, or no-template, control was included in every experiment. A positive control, with the use of DNA from a known strain of type C *C. botulinum* (96-SAC), was also included. We used 150 pg of DNA for the initial reaction and 0.25 μl of the initial reaction mixture in the seminested PCR. In addition, a duplicate set of initial sample reactions spiked with 150 pg of 96-SAC was run to confirm that there was no inhibition of the PCR reactions, and no inhibition was observed. Ten microliters of the initial spiked amplification reactions and the seminested amplification reactions were size fractionated through 2% agarose gels (Invitrogen, Life Technologies Corporation, Carlsbad, California) in 1× TAE buffer (40 mM Tris acetate, 1 mM ethylenediaminetetraacetic acid). Gels were stained in 0.01% Vistra Green (Amersham Biosciences, Sunnyvale, California) for 15 min, and products were visualized with the Fluorimager system (Molecular Dynamics, Sunnyvale, California).

Statistical analysis

We used logistic regression (SAS Institute, 1989) to look for relationships between the presence of neurotoxic *C. botulinum* type C in live fish from 2000 and six potential explanatory variables: date of capture, depth, site, sex, length, and weight. Backward elimination was used to eliminate variables that were not statistically significant (P>0.05) from the model. The rarity of positive fish in 2001 prevented us from performing a similar analysis with these data. Pearson chi-square tests were used to compare prevalence rates among years and between sick or dead fish and healthy fish. Comparisons between years used Bonferroni correction for multiple comparisons.

RESULTS

We tested a total of 62, 123, and 37 sick and dead fish collected in 1999, 2000, and 2001, respectively, and 31, 535, and 318 healthy fish from those three respective years. We also tested 49 control tilapia.

None of the 49 control tilapia were positive for the C_1 toxin gene; the gene, and thus neurotoxic *C. botulinum* type C cells, was detected in 4, 9, and 1% of tilapia tested each year in 1999, 2000, and 2001, respectively, and a significant difference was detected among those 3 yr (χ^2=21.97, P<0.0001, 2 df; Table 1). Prevalence of positive fish collected in 2000 was significantly greater than in fish collected in 2001 (χ^2=20.83, P<0.0001, 1 df) but did not differ significantly among other years (Table 1). Pooling the data across years, there is no evidence that prevalence differed between live and healthy fish and sick and dead fish (χ^2=2.14, P=0.144, 1 df); neither were there any differences between sick and dead fish (χ^2=1.97, P=0.160, 1 df; Table 1).

We found no statistically significant relationship between the presence of the C_1 toxin gene and any of six potential explanatory variables (date of capture, depth, site, sex, length, and weight) for live fish caught in 2000. There was suggestive evidence that the date of fish collection explained some of the variability in prevalence rates (P=0.089), with the odds of

cellular presence decreasing by 1.2% each day.

DISCUSSION

Our results demonstrate that the tilapia population in the Salton Sea harbors *C. botulinum* capable of producing neurotoxin within their GI tracts and that the prevalence of positive fish varies from year to year. This finding links tilapia to the deaths of pelicans and other piscivorous birds by type C avian botulism at Salton Sea. To our knowledge, this association has not been demonstrated in any other bird or fish population, although similar work has looked for other types of *C. botulinum* (Fach et al., 2002). The intestines of healthy fish generally would be considered inhospitable to *C. botulinum,* but Riedel and Costa-Pierce (2001) showed that Salton Sea tilapia consume significant amounts of sediment during the year. Thus, these fish likely ingest cells and spores of *C. botulinum* that are present in the sea substrate. Perhaps the tilapias' reduced foraging efforts, combined with environmental stresses, create an altered, possibly static, gut environment that is conducive to spore germination and cell replication. The occurrence of cell replication in live fish might be somewhat similar to botulism toxicoinfections in humans (Midura and Arnon, 1979), horses (Swerczek, 1980), and other animals (Minervin, 1967), although we do not know whether active toxin is actually produced by these cells and, if so, whether the tilapia themselves become sick as a result of this process. Work is currently underway in this area to verify active toxin formation in Salton Sea tilapia.

Contrary to our expectations, the prevalence of neurotoxic *C. botulinum* type C was no different between apparently healthy fish and sick and dead fish. We expected to find a higher prevalence in sick and dead tilapia because the intestines of compromised or dead fish would seem more suitable for cell replication and toxin production. A comparison of sick and dead fish also revealed no statistical differences, although it is interesting to note that the prevalence of *C. botulinum* in dead tilapia in 2000 was 22%. All five positive fish were collected during a single fish kill event, the significance of which is unknown.

Our data do not indicate any patterns or trends related to location of fish containing neurotoxic *C. botulinum* type C. The majority of sick and dead pelicans afflicted with botulism are retrieved in the vicinities of the three river deltas (NWHC, unpubl. data; Salton Sea Authority, unpubl. data); thus, we expected to find higher prevalence of positive fish from these sites. One explanation might be that, because pelicans feed and rest more frequently at the deltas, they are thus more likely to acquire toxin at the deltas, regardless of prevalence of fish with *C. botulinum.* Also, it is possible that pelicans can acquire toxin at other locations but are still able to move back to the rivers before the toxin takes effect.

Total prevalence of tilapia with neurotoxic *C. botulinum* type C was significantly higher in 2000 (9%) than in 2001 (1%). The total number of brown pelicans affected by botulism that were retrieved in 2000 was nearly 1,500, and less than 600 were retrieved the following year (Rocke et al., 2004). Although not statistically different from the other 2 yr, prevalence of positive fish was nearly 4% in 1999, which appears to correspond to the over 700 pelicans retrieved that season. It appears that the prevalence of positive tilapia from 1999 to 2001 might correlate with the severity of botulism outbreaks in pelicans over those 3 yr; however, this observation cannot be statistically supported.

The possible trend we observed regarding date of collection might reflect the apparent shift in the retrieval patterns of botulism-affected pelicans over the last 3 yr. Rocke et al. (2004) describes a shift toward August, away from September as the month of greatest bird collection, which could correspond to the gradual, albeit statistically insignificant, decrease in *C. botulinum* detection in tilapia from July to

September, although this is based on only 1 yr of data. In addition, the changes in peak times of pelican mortality are very likely influenced by bird migration and use patterns at the Salton Sea.

Although it does not appear that sick and dead tilapia, as opposed to healthy tilapia, were the predominant source of botulinum toxin for birds from 1999 to 2001, we do not know whether these findings reflect what happened in the 1996 epizootic. Many factors can influence the epizootiology of avian botulism outbreaks (Kalmbach and Gunderson, 1934; Quortrup and Holt, 1941; Bell et al., 1955; Rocke et al., 1999; Rocke and Samuel, 1999). Pelican mortality during this study period was much lower than in 1996. In light of changes that have occurred in the status of the fish and bird populations since then, it will be difficult to determine whether these differences, as well as differences in environmental conditions, might have set 1996 apart from other years.

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