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Reference Baseline Data for Gastric Cytology in Healthy Bottlenose Dolphins (*Tursiops truncatus*) Under Human Care

Letizia Fiorucci,[1] Francesco Grande,[2] Carla Flanagan,[3] Joana Silva,[3] Nuno Urbani,[3] Jose Sampayo,[4] and Roberto Macrelli[5]

[1]*Division of Animal Histology and Pathology, Institute of Animal Health, University of Las Palmas de Gran Canaria, Trasmontaña 35013, Las Palmas, Spain*
E-mail: letiziafiorucci@gmail.com
[2]*Zoomarine Italia, Torvaianica, Pomezia, Rome, Italy*
[3]*Mundo Aquático S.A. – Zoomarine – Est. Nacional 125, Cortelhas, Guia 8201-864, Albufeira, Portugal*
[4]*Veterinary Endoscopy Support, Madrid, Spain*
[5]*Department of Basic Sciences and Foundations, University of Urbino, Urbino 61029, Italy*

Abstract

Preventative medicine of the digestive system in dolphins is fundamental to ensure the welfare of these animals under human care. This study aimed to establish a reference baseline data for normal cytological findings (e.g., pH, epithelial cells, leukocytes) in gastric samples of healthy bottlenose dolphins (*Tursiops truncatus*), following, for the first time, the "Guidelines for the Determination of Reference Intervals in Veterinary Species" issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012).

Twenty-seven bottlenose dolphins kept under human care were examined and sampled. Sampling was carried out from January to December 2010. Results show that no correlation was found between pH and the epithelial cell values (r_s = 0.292, p = 0.1657), between pH and the leukocyte values (r_s = 0.168, p = 0.4321), and between epithelial cells and WBC (r_s = 0.076, p = 0.7240). Also the *t*-test (assuming equal variances) did not find a significant difference for weight (p = 0.6739), pH (p = 0.7707), epithelial cells (p = 0.6385), or leukocytes (p = 0.6968) between sexes.

Key Words: bottlenose dolphin, *Tursiops truncatus* cytology, endoscopy, reference intervals

Introduction

The collection of clinical samples is the cornerstone of preventative medical care and early detection of disease processes in cetaceans. Cytological examination is a common, inexpensive, and readily available diagnostic tool that constitutes a valuable part of a medical evaluation for both terrestrial and aquatic species (Sweeney & Reddy, 2001). Several species, including marine mammals, frequently do not show clinical signs of illness early in various disease processes (Sweeney & Reddy, 2001). The identification of cytological abnormalities combined with hematology and serum chemistry could serve as a useful tool with which to determine the presence of illness prior to the exhibition of clinical signs (Sweeney & Reddy, 2001). In cases of severe gastric inflammation, clinical signs in dolphins have been reported to be similar to those in other mammals and may include abdominal pain, anorexia, vomiting, regurgitation, and depressed mentation (Harper et al., 2000). Without medical treatment, the progression of gastric inflammation could potentially result in complications, including intestinal erosion-ulceration, perforation, and subsequent death (Goldstein et al., 2012).

Dolphins in a managed care or rehabilitation facility exhibiting moderate to severe cytological evidence of gastric inflammation are often treated for potential gastric ulceration. However, an increased number of inflammatory cells in gastric fluid may not be indicative of gastric ulceration (Goldstein et al., 2012). Without the use of advanced sampling methods, such as endoscopic examination of the cetacean stomach combined with the histological examination of stomach tissue, there is no definitive way to diagnose gastric disease (Goldstein et al., 2012).

The gastrointestinal system of cetaceans is complex in its anatomical structure but is similar to many terrestrial animals (Harrison et al., 1970). Collection of a gastric sample may be performed with a variety of gastric tubes commonly used in equine medicine. Diagnostics that are commonly performed on gastric samples include

cytology, pH, and microbial cultures (Sweeney & Reddy, 2001). Endoscopic examination allows the direct visualization of the gastric mucosa and the removal of biopsy specimens.

The main goal of this study was to establish a reference baseline data for normal cytological findings in gastric fluid samples of healthy bottlenose dolphins (*Tursiops truncatus*) based, for the first time, on the "Guidelines for the Determination of Reference Intervals in Veterinary Species" issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012). Reference intervals (RI) are an integral component of laboratory diagnostic testing and clinical decision-making and represent estimated distributions of reference values (RV) from healthy populations of comparable individuals. Because decisions to pursue diagnoses or initiate treatment are often based on values falling outside RI, the collection and analysis of RV should be approached with diligence. Newer topics include robust methods for calculating RI from small sample sizes and procedures for outlier detection adapted to data quality. Because collecting sufficient reference samples is challenging, these guidelines also provide recommendations for determining multicenter RI and for transference and validation of RI from other sources (e.g., manufacturers). Advice for use and interpretation of subject-based RI is included as these RI are an alternative to population-based RI when sample size or inter-individual variation is high. Finally, the generation of decision limits, which distinguish between populations according to a predefined query (e.g., diseased or nondiseased), is described. Adoption of these guidelines by the entire veterinary community will improve communication and dissemination of expected clinical laboratory values in a variety of animal species and will provide a template for publications on RI in order to promote quality laboratory practices in laboratories serving both clinical and research veterinarians (Friedrichs et al., 2012).

One purpose of this work is also to assess the existence of a correlation between healthy dolphins in the values of pH and epithelial cells, including pH and WBC, between epithelial cells and WBC.

Moreover, in this study, for the first time, cytology was combined with the endoscopic and histological examination of stomach tissue. For this reason, endoscopy should also be performed to confirm the medical state of the examined animal. Endoscopy can provide supporting data and gives the clinician the information necessary to evaluate the animal when a gastrointestinal disorder is suspected.

Methods

Twenty-seven bottlenose dolphins kept under human care were examined and sampled. The average water temperature in the different facilities was 23° C. The dolphins were fed a diet of the following frozen fish: capelin (*Mallotus villossus*), spratt (*Sprattus sprattus*), herring (*Clupea harengus*), mackerel (*Scomber scombrus*), horsemackerel (*Trachurus trachurus*), smelt (*Atherina boyeri*), squid (*Loligo opalescens*), blue whiting (*Micromesistius poutassou*), and sandeel (*Hyperoplus lanceolatus*). Sampling was carried out from January to December 2010. Samples were collected and immediately analyzed.

The following criteria were used for inclusion in the study:

- All animals considered clinically healthy based upon physical exam and laboratory evaluation (complete blood count and serum chemistry, urinalysis, and fecal examination)
- Endoscopy of the forestomach and the fundic stomach of all animals
- Histopathological evaluation of the biopsy of the forestomach and the fundic stomach of all animals

Criteria for exclusion of individuals from the study included the following:

- Lack of good health or presence of ongoing disease
- Lack of endoscopic evaluation of the subjects
- Pregnancy

Among these examined dolphins, 24 were considered clinically healthy. This group consists of 10 females and 14 males; the average age of the group was 16 y (Min.-Max.: 6 to 36 y); and the average weight was 187.95 kg (Min.-Max.: 146.20 to 266.70 kg) (Table 1).

Endoscopy was carried out as a component of the annual general clinical examination, which was included in the veterinary preventative medicine program established at each institution. No additional endoscopic evaluations were specifically required for the purpose of this particular study. Bottlenose dolphins were restrained in a pool that features a lifting platform and were examined individually. For gastroscopy, standard endoscopic procedure for cetaceans were followed (Dover et al., 2001). All animals were fasted at the time of the examination. The dolphin was placed over a close pore foam mattress in sternal recumbency and maintained in place by trainers on each side of the animal. The distal scope tip was passed over the dolphin's tongue and

Table 1. Summary statistics for age, weight, pH, volume, epithelial cells, and WBC from gastric samples collected from clinically healthy bottlenose dolphins (*Tursiops truncatus*) (n = 24); SD = standard deviation, Min.-max. = minimum-maximum values.

Statistic (n = 24)	Mean ± SD	Min.	Max.	D'Agostino-Pearson test
Age (y)	16.00 ± 8.60	6.00	36.00	p = 0.0526
Weight (kg)	187.95 ± 36.04	146.20	266.70	p = 0.1733
pH	1.83 ± 0.92	1.00	3.00	p = 0.1757
Epithelial cells (Epithelial cells/hpf)	3.77 ± 1.46	2.00	7.00	p = 0.3255
WBC (WBC/hpf)	2.42 ± 0.69	1.50	4.00	p = 0.4756

into the oropharynx, through the esophagus, and then into the forestomach. Air insufflation was required during the whole procedure to obtain a good image. The forestomach has thick squamous epithelium-lined rugal folds that appear light pink in color (Figure 1), and the fundic portion has a mucosal surface that is deep pink to red in color (Dover et al., 2001) (Figure 2). The ostium between the forestomach and the second chamber is located cranially in the left ventral quadrant of the first compartment (Figure 3). The third chamber is the pyloric region, which has a thin mucosal lining and mucous glands (Dover et al., 2001). This chamber is impossible to evaluate by endoscope.

A moderate amount of fluid was present in the forestomach of each animal; this fluid was collected by aspiration into a 120 ml polypropylene container with screw cap (LP Italia, Milan, Italy) and analyzed (3 to 8 mL) (Mitchell et al., 2008).

The pH of the fluid was measured by color fixed indicator strips (pH-Fix 0-14; Macherey-Nagel, Duren, Germany). The samples were

Figure 1. Thick squamous epithelium-lined rugal folds of the forestomach in a healthy bottlenose dolphin (*Tursiops truncatus*)

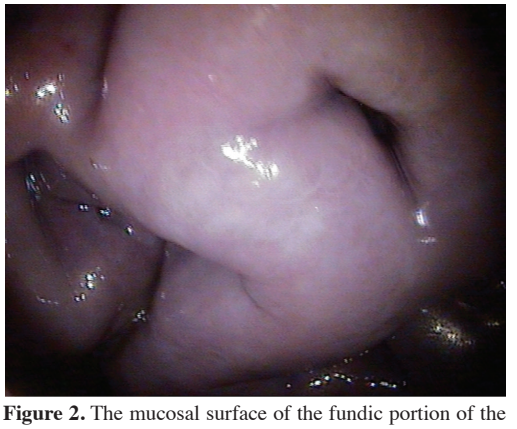

Figure 2. The mucosal surface of the fundic portion of the stomach in a bottlenose dolphin

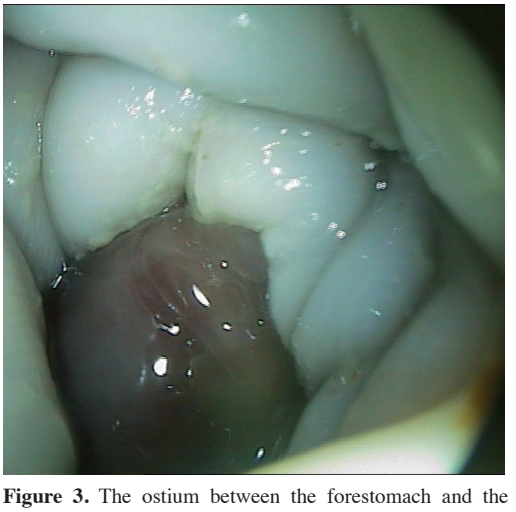

Figure 3. The ostium between the forestomach and the second chamber, located cranially in the left ventral quadrant of the first compartment of a healthy bottlenose dolphin

Table 2. Reference intervals (RI) determined using parametric methods for gastric samples collected from clinically healthy bottlenose dolphins ($n = 24$); CI = confidence interval.

RI ($n = 24$)	Lower limit	90% CI	Upper limit	90% CI
Epithelial cells (Epithelial cells/hpf)	0.91	0.05-1.77	6.63	5.77-7.49
WBC (WBC/hpf)	1.07	0.67-1.48	3.76	3.56-4.17
pH	0.00	0.0-0.6	3.60	3.1-4.2

examined after being stained with New methylene blue (NMB) and Dif-Quick (Bio-Optica, Milan, Italy). Cytologic review of the slides followed standard procedures (Sweeney & Reddy, 2001).

Results

Medcalc, Version 11.6.0.0, was used to analyze the data, and $p = 0.05$ was used to determine statistical significance. Reference values for pH, epithelial cells (half-sum between max. and min. values of epithelial cell), and leukocytes (WBC) (half-sum between max. and min. values of WBC) were calculated from clinically healthy dolphins ($n = 24$). The "Guidelines for the Determination of Reference Intervals in Veterinary Species" issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012) was used to calculate the RI. The Gaussian distribution for the values of pH, epithelial cells, and WBC was tested using D'Agostino-Pearson's test. Outliers were examined and excluded using Horn's algorithm (Turkey's fences interquartile). A Spearman's correlation coefficient (r_s) was calculated to examine any correlations, biologically relevant (Sweeney & Reddy, 2001), between pH and epithelial cells, pH and WBC, and epithelial cells and WBC. The *t*-test, assuming equal variances (more robust than Mann-Whitney in parametric statistical method), was used to compare weight, pH, epithelial cells, and WBC between sexes.

Summary statistics for age, weight, pH, volume, epithelial cells, and WBC were calculated for clinically healthy bottlenose dolphins ($n = 24$) and presented in Table 1. There were no outliers noted in the analysis. RI were determined using "parametric method" as recommended by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012) for $20 \leq n < 40$ reference samples when the data follow Gaussian distribution (Table 2). In fact, the ASVCP protocol for establishing RI recommends parametric statistical method if Gaussian normality can be established with 90% confidence interval (CI) for reference limits (Friedrichs et al., 2012). No correlation was found between pH and the epithelial cells values ($r_s = 0.292$, $p = 0.1657$), pH and WBC ($r_s = 0.168$, $p = 0.4321$), and epithelial cells and WBC ($r_s = 0.076$, $p = 0.7240$). The *t*-test between the sexes (female, $n = 10$; male, $n = 14$), assuming equal variances, did not find a significant difference for weight ($p = 0.6739$), pH ($p = 0.7707$), epithelial cells ($p = 0.6385$), or WBC ($p = 0.6968$).

Endoscopy showed no morphological abnormalities. All the full-thickness biopsy samples were considered histologically normal: the forestomach showed a keratinized squamous epithelium; and the fundus consisted of mucosa, submucosa, muscular layer, subserosa, and serosa. The mucosa had a simple cylindrical epithelium forming the gastric crypts where there are glands composed of parietal cells and chief cells.

Discussion

Three types of epithelial cells are usually reported in gastric samples: (1) squamous epithelial cells from the esophagus and fundic stomach, (2) columnar epithelial cells from the second stomach, and (3) basal cells from necrosis in the first and the second chamber (Sweeney & Reddy, 2001). Only squamous epithelial cells were reported during the cytological examination of the gastric fluid of healthy animals (Sweeney & Reddy, 2001). The presence of a small number of leukocytes (< 20 cells/hpf) in the forestomach is a normal occurrence in both captive and free-ranging dolphins (Fair et al., 2006; Varela et al., 2007). However, the finding of moderate numbers of leukocytes (> 20 cells/hpf) may lead clinicians to suspect the presence of a mild gastritis (Varela et al., 2007).

The current study shows that the pH value of the gastric sample from a fasted dolphin ranges from 1.0 to 3.0. The presence of bacterial overgrowth in marine mammals typically results in increased gastric pH secondary to bacterial phagocytosis of the stomach mucosa; this is in contrast to the response in humans, which results in a lowered pH (Zhu et al., 2006). Furthermore, individual differences can influence the results. Thus, it seems important to set up an individual baseline, preferably by taking gastric fluid samples on a routine basis as a part of a routine medical examination. The results found in this study correspond with

previous research (Fair et al., 2006; Goldstein et al., 2006; Varela et al., 2007; Mitchell et al., 2008). The main goal of this study was to establish a reference baseline data for normal cytological findings in gastric fluid samples of healthy bottlenose dolphins based, for the first time, on the "Guidelines for the Determination of Reference Intervals in Veterinary Species" issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012). Adoption of these guidelines by the entire veterinary community will improve communication and dissemination of expected clinical laboratory values in a variety of animal species and will provide a template for publications on RI in order to promote quality laboratory practices in laboratories serving both clinical and research veterinarians (Friedrichs et al., 2012). Cytologic examinations can provide a snapshot of potential illness prior to systemic disease (Cowell et al., 1999); elevated leukocytes along with increased epithelial cells, particularly basal cells and or erythrocytes, might suggest ulceration (Mitchell et al., 2008).

Endoscopy has traditionally been the most useful tool to examine and evaluate the upper gastrointestinal (GI) tract (Dover et al., 2001). Lesions in the forestomach and crianial portions of the fundic stomach can be visualized by endoscopy. The dolphin stomach has three divisions: (1) forestomach; (2) fundic stomach; and (3) pyloric stomach, which joins the duodenal ampulla. The forestomach has keratinized and stratified squamous epithelium, and it is the only nonglandular portion of the dolphin's stomach. The fundus consists of neck cells, chief cells, and parietal cells, whereas the pyloric portion has columnar mucous cells and argentaffin cells. The duodenal ampulla is an extension of the duodenum from which the common bile duct exits from the liver (Ridgway, 1968, 1972; Harrison et al., 1970). Disturbances of the GI tract are encountered in both wild and under human care dolphins, and damage to the gastric mucosa, including ulcers, has been reported (Harper et al., 2000). The study showed the importance of including endoscopic evaluation of the gastric chambers and histological assessment of the mucosa of the forestomach and fundic to give more scientific value to the RI found. In addition, this study underlines the importance of including endoscopy in routine testing as a procedure to complement clinical and cytological examination of the animal to monitor gastric compartments, considering that animals can be trained for such an examination.

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