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On-site classification of pansteatitis in Mozambique tilapia (*Oreochromis mossambicus*) using a portable lipid-based analyzer†

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While no pansteatitis-related large-scale mortality events have occurred since 2008, the current status of pansteatitis (presence and pervasiveness) in the Olifants River system and other regions of South Africa remain largely unknown. In part, this is due to both a lack of known biological markers of pansteatitis and a lack of suitable non-invasive assays capable of rapidly classifying the disease. Here, we propose the application of a point-of-care (POC) device using lipid-based test strips (total cholesterol (TC) and total triglyceride (TG)), for classifying pansteatitis status in the whole blood of pre-spawning Mozambique tilapia (*Oreochromis mossambicus*). Using the TC strips, the POC device was able to non-lethally classify the tilapia as either healthy or pansteatitis-affected; the sexes were examined independently because sexual dimorphism was observed for TC (males $p = 0.0364$, females $\chi^2 = 0.0007$). No significant difference between healthy and pansteatitis-affected tilapia was observed using the TG strips. This is one of the first described applications of using POC devices for on-site environmental disease state testing. A discussion on the merits of using portable lipid-based analyzers as an in-field disease state diagnostic tool is provided.

Introduction

The search for a simple, rapid, and cost-effective disease state diagnostic tool capable of providing on-site environmental health assessments resulted in the evaluation of a handheld/portable point of care (POC) analyzer. Excellent reports are available that discuss the merits of personal handheld POC devices in helping manage human disease, largely centered on eliminating the need for expensive, technically-involved, lengthy, and/or labor-intensive laboratory methods. To date, POC devices have been used in a variety of human studies and have generally focused on comparing the device to established lab procedures in the overall assessment of population-based disease risk and health status in humans[1–6] and to a lesser extent other mammals.[7–9] In laboratory-based animal models, POC devices have been used to examine the effects of drugs and diet on health outcomes[10–13] and monitoring disease status and recovery.[14,15] Despite its noted advantages, application of POC devices for in-field measurement of whole blood biomarkers for assessing environmental health has not been fully explored. For environmental applications, wherein specific metabolites are a key target for interrogation, portable analyzers could hold considerable promise as approaches capable of quickly and non-invasively surveying disease status at the population level. In addition, POC devices have noted qualities that lend to being suitable as on-site diagnostic tools, such as the rapid nature of data collection, minimal operating cost, portability of device, variety of test strips, minimal sample volume required, and the extended stability of test strips. This study is one of the first described applications focused on exploring the use of a portable analyzer for on-site environmental disease testing.

An environmental form of the disease termed pansteatitis has afflicted the Olifants River region in South Africa to various degrees for almost a decade, most notably in the Nile crocodile (*Crocodylus niloticus*) population, as well as several fish species, such as the African sharptooth catfish (*Clarias gariepinus*), Rednose labeo (*Labeo rosae*), and Mozambique tilapia (*Oreochromis mossambicus*).[16–21] Large-scale mortality events of the crocodile population occurred in Kruger National Park (from

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2007 to 2009), as well as several other noted locations to a lesser extent, including Lake Loskop, where there is also a high prevalence of pansteatitis in tilapia.[21–24] Current methods used to determine pansteatitis status require necropsy of the individual for certainty of diagnosis (*e.g.*, gross anatomy, tissue inspection, histology), which precludes translation to population-wide surveys. While the major hallmark and current diagnostic medium of pansteatitis is the presentation of inflamed adipose tissue, the investigation of non-lethal biological matrices (*e.g.*, whole blood, plasma) could allow the ability to perform population-wide assessments. Since pansteatitis has been traditionally characterized in case reports by the infiltration of inflammatory cells in adipose tissue,[25–27] and because the storage, synthesis, and transport of both cholesterol and triglycerides in adipose tissue has been described to be in a dynamic equilibrium either directly or indirectly with circulating plasma concentrations,[28,29] measurement of lipids in whole blood by a POC lipid-based analyzer could provide a rapid classification of disease status. Further considering this aspect and the fact that pansteatitis largely manifests and disrupts cellular membranes in lipid-rich adipose tissue,[20,30] it is proposed that lipids (specifically, cholesterol and triglycerides) are viable candidates as pansteatitis markers using whole blood and/or plasma.

Here, we examined the use of circulating lipids as potential markers for detecting the presence of pansteatitis in Mozambique tilapia at Lake Loskop, Mpumalanga, South Africa. To achieve this, a commercially available POC lipid-based analyzer was evaluated in its ability to classify healthy and pansteatitis-affected tilapia ($n = 42$) on-site using whole blood and total cholesterol (TC) and total triglyceride (TG) test strips. During the on-site analysis of both TC and TG, the lipids of several individuals were measured in triplicate to assess on-site reproducibility. Accuracy of the device was examined at a later date (in the laboratory) using a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1950 Metabolites in Human Plasma, which has noted certified plasma values for both cholesterol and triglycerides. Beyond discussion of the ability to classify pansteatitis in tilapia using measurement of lipid concentrations, the merits of the non-lethal diagnostic device as a suitable approach to provide immediate heath/disease assessment on environmental populations is discussed.

Experimental

Pre-spawning Mozambique tilapia ($n = 42$) were collected using 8-inch mesh gill nets from Lake Loskop, Mpumalanga, South Africa (21 males/21 females). Blood was taken *via* syringe immediately upon removal from the net from the lateral line of the fish (caudal venipuncture) into 10 mL lithium heparin tubes (BD Medical, Franklin Lakes, NJ). The blood was stored briefly on ice undisturbed until 50 μL of the whole blood was removed and transferred to a 1 mL microcentrifuge tube using an Eppendorf pipette. This aliquot was stored briefly on ice until analysis by the POC device. A CardioChek analyzer (Polymer Technology Systems (PTS), Indianapolis, IN) was used as directed by the user guide. In brief, the appropriate MEMo chip, which contained an internal calibration for each analyte, was installed (TC or TG) and a test strip was inserted. Next, 15 μL of whole blood was added to the strip using an Eppendorf pipette and the analysis commenced. The measured values for both TC and TG for each animal are shown in ESI Table 1.† To assess the reproducibility of using the POC lipid-based device for tilapia whole blood analysis on-site, several animals were selected to undergo consecutive repeat measurements using the TC and TG test strips and the measured values (with calculated relative standard deviation (RSD)) are provided in ESI Tables 2 and 3,† respectively.

The same operating procedure was repeated for the analysis of frozen human plasma (NIST SRM 1950) in the laboratory (plasma was thawed and vortexed prior to strip application) using both TC and TG test strips. A total of 17 and 15 replicate analyses of SRM 1950 (for TC and TG, respectively) were analyzed over three days to provide an initial assessment of the accuracy of the POC device. The measured TC and TG values were compared to the certified values on the Certificate of Analysis (COA) noted for SRM 1950 (http://www.nist.gov/srm/).

Post blood collection, the fish were rapidly transported to the onsite laboratory, where they were kept in a cold water bath. The fish were decapitated using a sharpened filet knife, which was immediately followed by pithing of the brain. Upon dissection, the tilapia were scored healthy or diseased (pansteatitis) upon visual examination of lesions in adipose tissue by veterinarian collaborators on-site during necropsy. The scoring system was based on a 0 to 5 scale (zero indicating no disease). Due to the subjectivity of disease scoring, a healthy/diseased assignment was made (for initial data handling): fish with score < 1 were classified healthy and fish with a score ≥ 1 were classified diseased. Additional fish measurements included sex, weight, and total length, as shown in ESI Table 4.† The project proposal (ES 6/1) was peer-reviewed by Mpumalanga Tourism and Parks Agency (MTPA) scientists. Animals were maintained in accordance with the South African National Standard: The Care and Use of Animals for Scientific Purposes (SANS 10386: 2008), and approval and ethical clearance was granted by the Research and Development Committee of the MTPA.

Two-tailed *t*-tests and Mann–Whitney *U* tests determined significance (p or $\chi^2 < 0.05$, respectively) between healthy (vet score < 1) and pansteatitis-affected tilapia (vet score ≥ 1) using JMP software (v.11.2.1, SAS, Cary, NC). Normality was tested using a Shapiro–Wilk test and equal variances were tested using a Levene test. Sexual dimorphism of the measured lipid values (TC and TG) using the portable lipid-based analyzer was investigated (using either *t*-test or Mann–Whitney *U* test). Since sexual dimorphism was observed for both TC and TG measurement, significance was determined for each sex independently.

Results and discussion

The fundamentals of measurement for most POC devices that employ test strips generally center on the detection of a color change once the blood is applied to the strip. Often this color

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change is the result of an enzymatic reaction and in the case of both the TC and TG test strips, the final concentration value for each lipid is determined using the Trinder method.[31–33] This approach has been noted to be highly specific for both lipids, thus application of this device beyond traditional mammalian analyses has merit, which is further supported by previous studies that have used the Trinder method to measure cholesterol and triglycerides in fish.[34–37] However, one caveat of using this POC device with lipid strips in non-mammalian (or non-human) subjects is that the TC and TG measurement dynamic range (100–400 mg dL^{-1} and 50–500 mg dL^{-1}, respectively) has to be suitable for the subject in question. In this study, it should be noted that several tilapia, healthy and diseased ($n = 2$ and $n = 9$, for TC and TG, respectively), had measured concentration values above the device ranges for both TC (noted on device as >400 mg dL^{-1}) and TG (noted on device as >500 mg dL^{-1}) strips. It should also be noted that for the purpose of this examination, when performing statistics using these individuals, the values were set to the maximum value (400 and 500, for TC and TG, respectively). Due to the fact that over 20% (9/42, mostly female) of individuals examined had TG levels outside the device range, it appears that the TG test strip may not be suitable to classify pansteatitis in this fish species, at least during this seasonal period. While further validation is needed to validate the use of these test strips for non-model species, it appears preliminarily that TC could be measured effectively within the framework of this study.

The whole blood of both healthy and pansteatitis-affected tilapia was examined using a POC lipid-based analyzer. The measured TC and TG values for each tilapia on-site ($n = 42$) are shown in ESI Table 1.† Scatter plots, displaying the measured TC and TG values with respect to sex and pansteatitis status, are shown in Fig. 1 and 2, respectively. As shown, TC values in whole blood demonstrated promise as an on-site marker for classifying pansteatitis. The device was able to detect statistically significant differences between healthy and pansteatitis-affected individuals in both male ($p = 0.0364$) and female ($\chi^2 = 0.0007$) tilapia sub-populations during this seasonal period. The

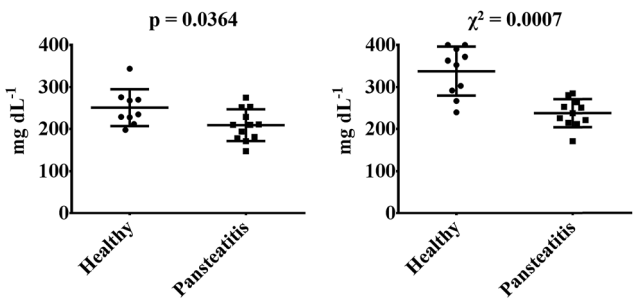

Fig. 1 Scatter plots for male (left) and female (right) TC values and statistical examination, with respect to healthy and pansteatitis-affected status in Mozambique tilapia whole blood. Significance was determined by obtained p or χ^2 values shown (p or $\chi^2 < 0.05$). The average (in mg dL^{-1}), SEM, and sample number (n) for each subpopulation was 251 ± 15 ($n = 9$), 209 ± 11 ($n = 12$), 338 ± 18 ($n = 10$), and 238 ± 10 ($n = 11$), for healthy males, pansteatitis-affected males, healthy females, and pansteatitis-affected females, respectively.

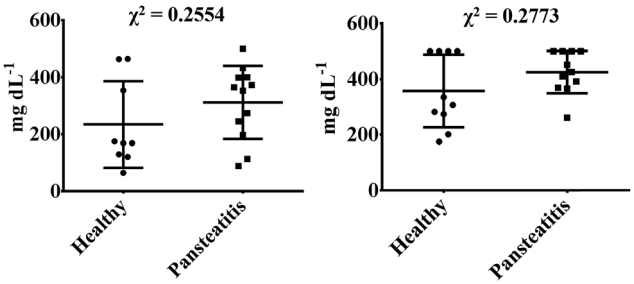

Fig. 2 Scatter plots for male (left) and female (right) TG values and statistical examination, with respect to healthy and pansteatitis-affected status in Mozambique tilapia whole blood. Significance was determined by obtained χ^2 values shown ($\chi^2 < 0.05$). The average (in mg dL^{-1}), SEM, and sample number (n) for each subpopulation was 235 ± 51 ($n = 9$), 312 ± 37 ($n = 12$), 357 ± 41 ($n = 10$), and 425 ± 23 ($n = 11$), for healthy males, pansteatitis-affected males, healthy females, and pansteatitis-affected females, respectively.

means (with standard error of the mean, SEM) using each test strip (TC and TG) are also provided in Fig. 1 and 2, respectively, for each sex analyzed independently (with disease classification), as sexual dimorphism was observed for both TC and TG measurements. It was important to consider sexual dimorphism in TC and TG measurement during this pre-spawning period. Healthy ($\chi^2 = 0.0296$) and pansteatitis-affected ($\chi^2 = 0.0221$) females exhibited higher concentrations of plasma TG when compared to male counterparts. In contrast, females had higher plasma concentrations of TC when healthy ($p = 0.0019$), but the diseased females did not exhibit sexual dimorphism ($p = 0.0681$) when compared to diseased males. In general, the healthy tilapia had higher TC concentrations when compared to pansteatitis-affected counterparts. No significant difference in plasma concentrations of TG between healthy and pansteatitis-affected tilapia (males $\chi^2 = 0.2554$, females $\chi^2 = 0.2773$) was observed.

In vertebrate species, cholesterol plays an integral role in several physiological and pathophysiological processes, which include cell membrane structure and function, digestion, reproduction, stress/immune response, metabolism, as well as being the main precursor to bile acids and steroid hormones.[38–41] The role cholesterol exhibits in both human health and disease has been well documented;[41–44] however, the role of cholesterol in fish health and disease is less known, as is its role in seasonal changes associated with altered ambient temperature. One important caveat that must be noted is that we have examined tilapia at only one time point – early pre-spawning period – in a seasonal cycle (reproductive). Tilapia were collected in early spring, when gonadal recrudescence has just begun.

In this examination, diseased tilapia exhibited a reduced level of TC when compared to the healthy counterpart. In non-fish species, reduced cholesterol levels have been associated with malnutrition,[45,46] age/stress,[47,48] and exposure to contaminants (*e.g.* perfluorooctane sulfonic acid).[49,50] In fish, reduced cholesterol has been observed after exposure to heavy metals,[51] stress,[52] organophosphorus pesticides,[53] pulp mill effluent,[54]

Table 1 TC and TG measurement in NIST SRM 1950 using the portable lipid-based analyzer in laboratory with comparison to certified value

		Portable lipid-based analyzer value			NIST certified value		
Plasma examination	n	Average		Standard deviation	Mass concentration		Expanded uncertainty
TC (mg dL^{-1})	17	143	±	14	151.4	±	3.3
TG (mg dL^{-1})	15	74	±	8	99.0	±	2.1

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and microcystins.[55] It has also been reported that pansteatitis-affected fish are in pain (leading to a compromised ability to move) and are capable of surviving long periods without feeding,[30] which suggests that a lack of food intake could be a contributing factor to the lower levels of TC observed. However, it is premature to attribute the changes in TC to any noted trigger of pansteatitis at this given time. The purpose of this study was to demonstrate a proof of concept application using a POC device for a real environmental disease. On-site TC measurement, using the portable lipid-based analyzer, could prove to be a useful approach for classifying pansteatitis, and potentially other diseases, with tilapia or possibly other affected species.

On-site analysis of TC in whole blood allowed for a nearly real-time assessment that could be used to differentiate healthy from pansteatitis-affected tilapia. By providing close to real-time measurements, issues of sample stability for lipid measurement can be mitigated, especially when sampling at remote sites. Initial efforts to evaluate the on-site feasibility of the POC device focused on measurement reproducibility. To measure the reproducibility (and robustness) of the POC device in-field, several animals were randomly selected to be analyzed in triplicate using both TC ($n = 22$) and TG ($n = 31$) test strips. The mean, standard deviation, and RSD for each animal using both TC and TG test strips are shown in ESI Tables 2 and 3,† respectively. The POC device performed well, as the average RSD for TC and TG was 4% and 5%, respectively. The accuracy of the POC device was examined later in the laboratory using a commercially available plasma-based reference material, NIST SRM 1950, another suitable matrix for lipid measurement using the test strips. While not a direct comparison to on-site fish whole blood measurement, the laboratory examination did demonstrate preliminarily that the measured plasma TC values using the device were within the 95% confidence interval noted on the COA, as shown in Table 1, though the RSD was 10% (as compared to expanded uncertainty on the COA, which was 2%). The method did not perform as well for TG measurement, as it was not within the 95% confidence interval and it had a percent difference of approximately −29% in comparison to the noted value listed for NIST SRM 1950, as shown in Table 1, thus again limiting its potential as an on-site diagnostic tool for tilapia.

Conclusions

Before an effective management plan for fish exhibiting pansteatitis can occur in the Olifants River region, better survey information is needed regarding the prevalence, pervasiveness, and current status of the disease. This information remains unclear throughout many parts of the Olifants River region and other watersheds in southern Africa. Using the POC device, a significant difference was observed in whole blood TC concentrations between healthy and pansteatitis-affected tilapia for both males ($p = 0.0364$) and females ($\chi^2 = 0.0007$), resulting in the ability to rapidly classify pansteatitis status. As with recent reports with the heath of human populations, the noted advantages of handheld POC devices present a promising application for use in assessing environmental health on-site on a population-wide level. However, further studies are warranted to fully examine the capabilities of screening pansteatitis with higher sample numbers across sites, seasons, species, trophic levels and sample matrices. Future efforts are also needed to examine the accuracy and precision of the device using whole blood and/or plasma for non-traditional species.

Disclaimer

Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology; nor does it imply that the materials or equipment identified are necessarily the best for the purpose.

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