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Loop-mediated isothermal amplification (LAMP) as a confirmatory and rapid DNA detection method for grey seal (*Halichoerus grypus*) predation on harbour porpoises (*Phocoena phocoena*)

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ABSTRACT

An increasing number of harbour porpoises along the coastlines of northern Europe are victims of attacks by preying grey seals. To confirm grey seal attacks at a molecular level, a fast loop-mediated isothermal amplification (LAMP) method was developed, based on the amplification of the *cytochrome b* target gene. With this method, saliva residues within bite marks, presumably originating from grey seal attacks, can be detected on-site, using a swab-based method in combination with a portable real-time fluorometer (Genie® II). The developed LAMP assay included an internal amplification control and was validated for the sensitivity, specificity, limit of detection and a spiking experiment was performed with saliva samples from two grey seals. Finally, three stranded harbour porpoises, with injury patterns presumably originating from grey seal attacks, were analysed. Grey seal DNA was successfully determined in a bite mark of one harbour porpoise with the LAMP method. For direct on-site molecular biological investigations without the opportunity to perform more time-consuming and equipment intensive PCR investigations, the developed rapid LAMP assay offers the possibility to confirm that injuries, probably caused by a grey seal, indeed resulted from a grey seal attack.

1. Introduction

During the last few years, an increasing number of stranded harbour porpoises (*Phocoena phocoena*) with massive loss of tissue ['strips of blubber removed or hanging loose; sometimes muscle partly removed' (Haelters et al., 2016)] were found along the coastlines of northern Europe. Initially, it was assumed that those animals were victims of fishery bycatch or a ship's propeller (Camphuysen & Siemensma, 2011; IAMMWG et al., 2015). However, Bouveroux et al. (2014) observed and photographed grey seal (*Halichoerus grypus*) attacks on harbour porpoises on the coast of northern France. Stringell et al. (2015) also provided evidence of the phenomenon of a grey seal predation on a harbour porpoise in Wales with the aid of photo and video material. Thus, there was the presumption that these serious injuries, which led to death, might be attributed to grey seal attacks (Haelters et al., 2012; Jauniaux et al., 2014; Leopold et al., 2014; van Bleijswijk et al., 2014; Stringell et al., 2015). To date, assessing carcasses mainly relies on macroscopic inspection and the resulting judgment of the researcher, posing the risk of respective occasional misjudgment (Hanson et al., 2017). In some areas, grey seal predation was held responsible for the death of at least 17% of stranded deceased porpoises, representing one of the major causes of death in the southeastern North Sea (Leopold et al., 2014). The reasons why grey seals attack harbour porpoises are not fully clarified. Stringell et al. (2015) assumed that harbour porpoises function as food because blubber and skin were consumed by an attacking grey seal and did not preclude a competition for food between both species. On the other hand, Jauniaux et al. (2014) and Haelters et al. (2012) cannot rule out aggressive behaviour of grey seals. To confirm the assumption of grey seal attacks, researchers analysed bite marks of harbour porpoises for residues of grey seal DNA via PCR and successfully determined DNA of grey seals in harbour porpoise carcasses (Jauniaux et al., 2014; van Bleijswijk et al., 2014). The issue of frequently occurring severely injured or dead harbour porpoises on the coastline of northern Germany raised the question whether these

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wounds also originated from grey seal attacks. Therefore, a rapid detection technique, which can be used during forensic field studies, was needed. The highly sensitive and fast loop-mediated isothermal amplification (LAMP) method is a relatively new and rapid molecular biological method requiring six different primers, binding on eight target regions, which results in high sensitivity and specificity (Notomi et al., 2000; Nagamine et al., 2002). The easy-to-use method can be performed with a real-time fluorometer (Genie® II, Optigene, Horsham, United Kingdom), which allows the parallel testing of 16 samples. Furthermore, the reaction process can be observed in real-time and after 30 min the entire run is finished. The LAMP technology has already been used in numerous research projects, for example, for detecting human blood-borne infectious disease like HIV (Vanjari et al., 2014) or hepatitis B (Moslemi et al., 2009). Moreover, it has been used to provide evidence of foodborne pathogens like *Staphylococcus aureus* or *Listeria monocytogenes* (Wang et al., 2010; Sheet et al., 2016) as well as animal species identification of ostrich, pork or horse in meat (Abdulmawjood et al., 2014; Cho et al., 2014; Yang et al., 2014). Furthermore, the detection of red fox (*Vulpes vulpes*) DNA in bite marks of a harbour porpoise carcass was possible with the LAMP technology (Heers et al., 2017). The latter case is related to the issue of grey seal predation on harbour porpoises as the bite lesions of red foxes displayed a similarity to bite marks of grey seals and both can hardly be differentiated when relying solely on macroscopic assessment (Heers et al., 2017). Heers et al. (2017) showed that by using a certain swab technique, a DNA purification step is not needed prior to LAMP, which accelerates and simplifies the procedure considerably. This swap technique was also used in the present study. The aim of the study was the development of a rapid and sensitive method to detect grey seal DNA in stranded harbour porpoises directly on the beach. Based on the *cytochrome b* sequence, six specific LAMP primers were created and for the internal amplification control, six common LAMP primers were generated. The method was validated and subsequently tested on three stranded harbour porpoises, showing typical injury patterns of grey seal predations. Using this assay, it was feasible to detect predator DNA residues of a grey seal in a bite lesion of one harbour porpoise. The LAMP assay is therefore of enormous benefit for wildlife researchers. Not only is it time-saving and field testing can be carried out with little equipment, but it also enables researchers to detect DNA traces of grey seals in bite marks of harbour porpoises and clarify forensic issues.

2. Material and methods

2.1. Design of LAMP primer sets

By using the software PrimerExplorer V4 (Eiken Chemical Co., Japan), six specific LAMP primers (forward outer primer HG-F3, backward outer primer HG-B3, forward inner primer HG-FIP, backward inner primer HG-BIP, forward loop primer HG-LoopF and backward loop primer HG-LoopB) were designed. The *cytochrome b* sequences of grey seal and harbour porpoise were compared with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (Maryland, USA). For grey seals (*Halichoerus grypus*) the accession number GU167293.1 and for harbour porpoises (*Phocoena phocoena*) the accession number U72039.1 were used (Fig. 1). To select a species-specific primer set, the differences in the *cytochrome b* sequences between grey seals and harbour porpoises were considered. To rule out false negative results, an internal amplification control (IAC) with six common primers was designed (forward outer Primer F3-IAC, backward outer primer B3-IAC, forward inner primer FIP-IAC, backward inner primer BIP-IAC, forward loop primer LoopF-IAC and backward loop primer LoopB-IAC), to amplify the DNA of grey seals as well as the DNA of harbour porpoises. This was conducted with the 16S sequences of harbour porpoise (accession number AB481401.1) and grey seal (accession number ×72004.1).

All primers were produced by Eurofins Genomics (Ebersberg, Germany). The detailed primer sequences of the specific primers are listed in Table 1 and the sequences of the common primers (IAC) are displayed in Table 2.

2.2. Positive and negative control material

The DNA of the positive and negative control material was isolated from muscle samples. The tissue samples were taken from stranded marine mammal carcasses during routine necropsy by the Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation. The samples were collected in the frame of the states' stranding network funded and permitted by the responsible Ministry of Energy Transition, Agriculture, Environment, Nature and Digitalisation of Schleswig-Holstein (MELUND), Germany. As positive control, material muscle samples of grey seals ($n = 8$) and as negative control material muscle samples of harbour porpoises ($n = 24$) were extracted by employees of the aforementioned institute.

2.3. Swab method

The swab system was used to forego a previous DNA purification step and utilise the DNA directly in the LAMP reaction. To select the best swab type with the highest sensitivity, a preliminary test with three commercially available swab types (MSwab™, Copan, Italy; eSwab™, Copan, Italy; hyplex® LPTV, Amplex BioSystems GmbH, Gießen) was performed (data not shown). In the swab validation experiment, the MSwab™ swab (Copan Italia S.p.A., Brescia, Italy) yielded the fastest amplification times, therefore it was used for further research work. The swab was stroked across the muscle tissue and subsequently submerged in the associated medium (1 ml MSwab™ medium contains Tris HCl, EDTA, TRIS Base, Dimethyl Sulfoxide (DMSO), Bovine Serum Albumin and distilled water). Finally, the reaction tube was shaken by hand and 8 µl of the MSwab™ medium was used directly in the LAMP reaction.

2.4. DNA isolation

The DNA of the muscle samples was extracted with the commercial DNeasy® blood and tissue kit (Qiagen, Darmstadt, Germany), in accordance with the manufacturer's instructions set forth in the protocol “Purification of Total DNA from Animal Tissues (Spin-Column Protocol)”. In brief, 25 mg of muscle tissue, 200 µl lysis buffer and 20 µl proteinase K were mixed and incubated subsequently. The following steps were processed in accordance with the manufacturer's instructions.

To increase the sensitivity of the swab method (mentioned above), it is also possible to extract the DNA from the MSwab™ medium. Therefore, 100 µl of the MSwab™ medium was used and isolated in accordance with the protocol “Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)”.

Subsequently, after DNA isolation, the DNA concentration was determined, using the spectrophotometer NanoDrop2000c (Thermo Fisher Scientific, Dreieich, Germany).

2.5. LAMP assay

The loop-mediated isothermal amplification assay was performed with a real-time fluorometer Genie® II, which is a battery-operated, portable device with two heating blocks, allowing a total number of 16 samples to be amplified simultaneously. The amplification process can be monitored in real-time on screen.

In accordance with the manufacturer's instructions, 15 µl Isothermal Master Mix ISO-001 (Optigene, Horsham, United Kingdom) was used with 0.4 µM of the F3-Primer and the B3-Primer for each sample. Furthermore, 0.8 µM of the LoopF-Primer and the LoopB-Primer as well as 1.6 µM of the FIP-Primer and BIP-Primer were added. Finally, a