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Exploratory Research into the RNA Transcripts Present within Beluga Whale Blow Samples

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Introduction

Beluga whales (*Delphinapterus leucas*) are an Arctic species that are currently being affected by the melting sea ice. This allows for increased vessel traffic and human presence in their environment, which have the potential to release pollutants or toxins thus negatively impacting the physiology of the whale. To understand more about how pollutants affect the beluga's overall health it is important to look at their cellular function. This mainly occurs through measuring gene expression, which is the change in mRNA transcription rates. This is dependent on either the animal's physiology or the environment surrounding them. To notice if something is wrong with health of the animal looking at immune function via cytokine gene expression is a good way to gain some insight.

Cytokine genes have many different influences in the body pertaining to immune function. TNFα, IL-12, and TGFβ have previously been amplified from leukocytes collected from blood samples of bottlenose dolphins (*Tursiops truncatus*), Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) and beluga whales.[1] TNFα (tumor necrosis factor) is produced during acute inflammation by many different immune cells. It also possesses antitumor activity but at the same time cause cell proliferation and differentiation.[2] There are many different roles for this one gene to play but is clearly critical to immune function and the health of the animal. When pathogens are detected in the body antigen presenting cells such as macrophages produce IL-12. When produced its function is to help with the development of T-helper cells and promote inflammation.[3] This is one of the key responses in the immune system making this gene important to the health of the animal. TGFβ is produced by many different types of cells as well and is vital to maintaining homeostasis within the body.[4] It plays a role in inhibition of differentiation and proliferation of T cells to avoid excess production.[5] In doing so it helps control the immune response and contributes to the animal's health just as the other two genes.

An example of one of the toxins that has been determined to affect killer whales (*Orcinus orca*) is an anthropogenic or manmade toxin called polychlorinated biphenyl or PCB. Contamination by PCBs has been correlated with an increase in IL-10 mRNA transcripts in killer whales. This causes a disruption in cellular function affecting the animal's immune response.[1] In order to be able to do this research an invasive process using darts to get skin and blubber samples has been the main route for collection.[6] This can lead to possible infections and behavioral changes in the whales causing them to swim away from boats when spotted making collection of samples a difficult process.

Using a non-invasive process such as collecting blow (exhale) samples is a newer and better option for these whales. Exhalation is a natural behavior for these whales and is extremely forceful. Each exhale carries an abundance of epithelial cells that contain the RNA necessary in order to perform these experiments. In a previous study an RNA isolation protocol established and housekeeping genes were used to verify the presence of RNA.[7] The hypothesis for this study is that cytokine genes are present in blow samples. The specific objective of this study is to determine if TNFα, IL-12, and TGFβ can successfully be amplified from RNA isolated from beluga blow samples.

Materials and Methods

Sample Collection

Beluga whales from Mystic Aquarium have been trained to exhale on command. Samples from 3 different whales were collected, 2 males and 1 female, in the size of either 1 or 3 exhales per sample. The samples were collected into a 50ml conical tube held inverted over the blowhole. One ml of RNAlater was added, and the tube was rolled to cover the inner surface of the tube with RNA Later. The tube was then centrifuged at 3000 RPM for 8 minutes. Then the sample was transferred to a 1.5ml microcentrifuge tube and stored on ice for transportation, then in a -20°C freezer until use.

RNA Isolation

The samples previously stored in the -20°C freezer were centrifuged at 12,000 rpm for 10 minutes. If a pellet was formed all of the supernatant was removed. If there was no pellet then about 50µl was left in the 1.5ml collection tube. From this point the cells were lysed and DNA was removed following an adapted version of the RNA isolation protocol of fibrous tissue from the Qiagen RNeasy Micro Kit (Item #74004, Valencia, CA). In the RNeasy Micro Handbook step two of the fibrous tissue protocol calls for excision of tissue from the sample however this step is not necessary with a blow sample. Step 3a is used however only the first part is followed due to not needing to use a TissueRuptor when working with blow samples. From this point on the rest of the protocol was followed accordingly until step 11 when instead of adding the DNase to the Buffer RDD, the Buffer RDD was carefully added to the DNase because it is a very sensitive enzyme. Then from this point in the protocol everything was followed accordingly right to the end. The RNA was then analyzed via Nanodrop to determine the concentration and purity of the sample. Samples were stored at -70°C if not used right away for the next reaction.

Reverse Transcription – Polymerase Chain Reaction

The Quantitect Reverse Transcription Kit (Qiagen, Item #205311) was used to convert the isolated RNA into cDNA. The incubation period for this reaction was about 30 minutes. From here the cDNA was either stored at -20°C for later or used right away in a polymerase chain reaction which went through 1 minute denaturation at 94°C, 1 minute annealing at 55°C, 2 minute extension at 72°C for 40 cycles with a final extension period of 8 minutes at 72°C. The genes that were tested to be amplified are RPl8 as a housekeeping gene, TNFα, IL-12, and TGFβ. The primers can be seen in the table below. The PCR products were mixed with 50ul of 1000x Gel Red and loaded into a 2% agarose gel to perform gel electrophoresis. Resulting bands were excised and the cDNA extracted via QIAquick Gel Extraction Kit (Qiagen, Item #28704).

Gene	Forward Primer	Reverse Primer
RPl8	5'-GCGGACGGAGTTGTTCAT-3'	5'-TTTGTCTCAGGGTTGTGGG-3'
TNFα	5'-CCAGAGGGAAGAGTTCCCAACTG-3'	5'-GAGCACTGAGGTTGGCTACAACAT-3'
IL-12	5'-GGAGATGCTGGGCAGTACACCTG-3'	5'-GGGCTCTTTCTGGTCCTTTAAGATATC-3'
TGFβ	5'-AATGATTCCTGGCGCTACCTC-3'	5'-CGAAAGCCCTCTATTTCCTCTC-3'

Sequence Analysis

These were sent for sequencing at the Rhode Island Genomics and Sequencing Center (RIGSC). When the results for the sequencing were received two programs, BLAST and BLAT provided online by NCBI, were used to determine if the sequences were from the correct gene and of RNA origin and not genomic. First the sequence received from the RIGSC was put into BLAST to make sure it was from the correct gene. If it was then this sequence was put into BLAT to determine if it was exon and also to find its line number within the gene. Then the accession number of the gene used to create the primers was put into BLAST to get the full gene sequence. It was downloaded in either the FASTA or GenBank form and then copied and pasted into BLAT. Within this sequence the forward primer was found. To find the reverse primer it needed to be inverted and reversed then looked for in the BLAT of the entire gene sequence. Within BLAT if the primers were intron (lowercase letters) spanning then part of them was at the end of one exon (uppercase dark blue letters) and the beginning of the next. The light blue uppercase letters were just to mark the point at which the exon or intron ends and the other begin. Once the primers were found the sequence from the RIGSC that was put into BLAT was also found in the big gene sequence. If it was between the primers and only made of exon then it was of RNA origin. If it was made of intron as well then it was of genomic origin.

Results

A total of 18 exhale samples have been collected. Half of the samples were 3-exhales and the other half were 1-exhale with average RNA concentrations of 5.418ng/µl and 4.547ng/µl, respectively. The average 260/280 absorbance of,the 3-exhale and 1-exhale were 1.498 and, 1.644, respectively. These values are represented in figures 1 and 2 below. There were nine samples that showed positive amplification for the RPl8 housekeeping gene. 8/9 from the 3-exhales amplified and 1/9 from the 1-exhale samples amplified RPL8. From the 3-exhale samples one did not positively amplify due to a failed PCR. From the 1-exhale samples three did not amplify for this gene and five had failed PCRs due to contamination. Contamination was detected by the appearance of bands in the RT-PCR or PCR negative control lanes. The samples that did amplify for RPl8 were used to test for TNFα, IL-12, and TGFβ. There was amplification of TNFα in 5/6 samples. IL-12 had positively amplified in 4/6 samples. There were seven PCRs performed for TGFβ however they all failed. The TNFα and IL-12 samples that had positive amplification were sent for sequencing. There were seven bands from TNFα that ended up going to sequencing even though there were only five samples with amplification because two of the lanes each had an extra band at the 400bp mark. This was indicative of some type of genomic contamination. These extra band sequences were also put into BLAST and determined to be of the Family Dysteriidae. They are zooplankton from the South Atlantic Ocean and have symbiotic relationships with marine life. The rest of the sequences from the bands at the 100bp mark were matched to be TNFα and determined to be of RNA origin. The IL-12 gels did not have any genomic contamination and four of the lanes that showed amplification at the 100bp mark were sent for sequencing. The results all matched IL-12 and were determined to be of RNA origin.

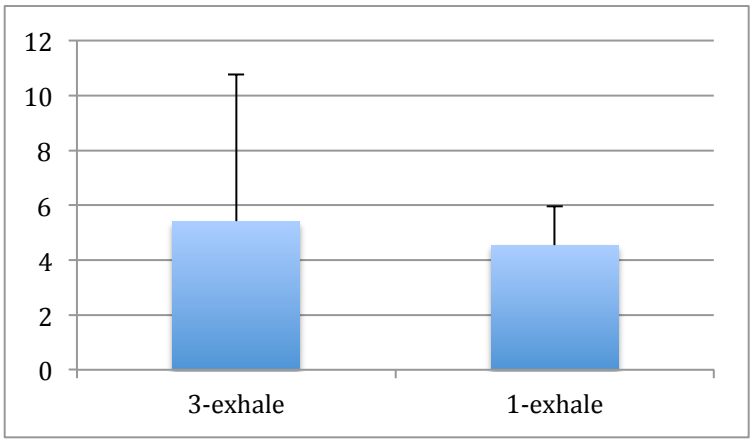

Figure 1. Average concentration (± SD) of RNA present in the 3-exhale and 1-exhale blow samples.

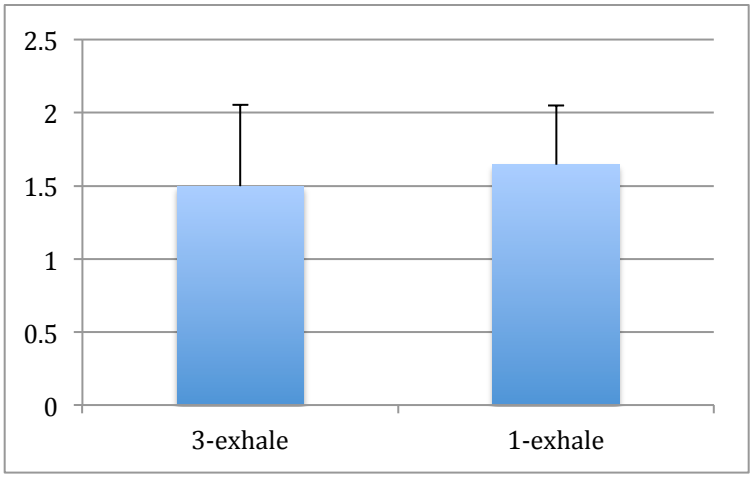

Figure 2. Average 260/280 absorbance (± SD) of RNA present in the 3-exhale and 1-exhale blow samples.

Discussion

The 3-exhale samples overall had better amplification of RPl8 than the 1-exhale samples due to the fact that there were three samples form the 1-exhale category that just did not amplify for the RPl8. There is no correlation between amplification of a sample and the concentration or purity levels because they were different each time whether it be a high or low number and still samples would amplify. These samples that did not amplify had concentrations of 5.573ng/µl, 5.01ng/µl, and 1.889ng/µl. Their 260/280 absorbance levels were 1.93, 1.3, and 1.28, respectively. The reason for no amplification of the RPl8 could be that there was an RNase present during the RT-PCR and got into those samples. The contamination for most of the samples can be concluded to be from human error. The contamination and overall failure of the TGFβ PCRs is thought to be from possible contamination of the primer source. In order to wipe out any further contamination if one reaction showed up with it then all of the aliquots of reagents were remade. The same reagent aliquots were used for each PCR even with other primers and those did not show any sign of contamination. However, once the primer for TGFβ was used with the same reagents there was contamination present.

The sequencing results for TNFα and IL-12 revealed that the experiments were a success in amplification of the mRNA transcripts of each gene from the blow samples. The cells that express these genes are mainly white blood cells that play a role in the immune response implying that blow samples contain not only epithelial cells but leukocytes as well. This is the first time that immune markers have been amplified from the blow of beluga whales. This will allow future researchers to continue to determine other genes that are present and to use that knowledge to then quantify the level of expression of these genes. With these immune markers present in blow samples a non-invasive procedure is available to learn the health status of the whale being studied. This can lead to future research in correlating the health status of the animal with any pollutants or toxins in the water.

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1. Sitt, T., Bowen, L., Blanchard, M.T., Smith, B.R., Gershwin, L.J., Byrne, B.A., Stott, J.L. (2008). Quantitation of leukocyte gene expression in cetaceans. Developmental and Comparative Immunology, 32, 1253-1259.
2. Ordas, M.C., Costa, M.M., Roca, F.J, Castejon, G.L, Mulero, V., Meseguer, J., Figueras, A., Novoa, B. (2007). Turbot TNFα gne: Molecular characterization and biological activity of the recombinant protein. Molecular Immunology, 44, 389-400.
3. Wang, T., Husain, M. (2014). The expanding repertoire of the IL-12 cytokine family in teleost fish: Identification of three paralogues each of the p35 and p40 genes in salmonids, and comparative analysis of their expression and modulation in Atlantic salmon *Salmo salar*. Developmental and Comparative Immunology, 46, 194-207.
4. Maehr, T., Costa, M.M., Vecino, J.L.G., Wadsworth, S., Martin, S.A.M., Wang, T., Secombes, C.J. (2013). Transforming growth factor- β1b: A second TGF- β1 paralogue in the rainbow trout (Oncorhynchus mykiss) that has a lower constitutive expression but is more responsive to immune stimulation. Fish and Shellfish Immunology, 34, 420-432.
5. Arjaans, M., Munnink, T.H.O., Bosscha, H.T., Reiss, M., Walenkamp, A.M.E., Lub-de Hooge, M.N., de Vries, E.G.E., Schroder, C.P. (2012). Transforming growth factor (TGF)-β expression and activation mechanisms as potential targets for anti-tumor therapy and tumor imaging. Pharmacology and Therapeutics, 135, 123-132.
6. Buckman, A.H., Veldhoen, N., Ellis, G., Ford, J.K.B., Helbing, C.C., Ross, P. S. (2011). PCB-Associated Changes in mRNA Expression in Killer Whales (*Orcinus orca*) from the NE Pacific Ocean. Environmental Science and Technology, 45, 10194-10202.
7. Schultz, K. 2015. Molecular Analysis of mtDNA Haplotypes and RNA Transcripts from Single-Exhale Blow Samples in the Beluga Whale (*Delphinapterus leucas*). University of Rhode Island Honors Project.