PROJECT COMPLETION REPORT

00-3 "Surveillance of Infectious Salmon Anemia Virus (ISAV) in the Northeast"

Progress Report Period: August 1, 2000—July 31, 2003

NRAC Total Funding: $134,650 (August 1, 2000—July 31, 2003)

Principal Investigators: John T. Singer

Participating Investigators/ Cooperative Agencies:

Bruce L. Nicholson	University of Maine, Orono	Maine
Carol H. Kim	University of Maine, Orono	Maine
Deborah A. Bouchard	Micro Technologies, Inc	Maine
Eric D. Anderson	University of Maine, Orono	Maine
H. Michael Opitz	University of Maine, Orono	Maine
Paul R. Waterstrat	Maine Department of Marine Resources	Maine

REASON FOR TERMINATION:

Major objectives completed and funding period terminated.

PROJECT OBJECTIVES

Objective 1: Develop and optimize reverse-transcriptase polymerase chain reaction (RT-PCR) assay (completed) and molecular padlock assays (continuing with other funding) for the detection and identification of infectious salmon anemia virus (ISAV).

Objective 2: Conduct surveillance program for ISAV in Atlantic salmon aquaculture sites in the State of Maine that are at high risk of becoming ISAV-positive (completed).

ANTICIPATED BENEFITS

We are using a progressive approach to aid in the avoidance, control, and containment of ISAV in the Northeast. This will be accomplished by having available and using preemptively, existing-, new-, and improved technologies. Our work will contribute to: (1) Development of several PCR assays as rapid and sensitive diagnostic assays for ISAV, (2) Development and implementation of methods for molecular epidemiology to initiate a pilot program to definitively track the transmission and identify possible reservoirs for the dissemination of ISAV, and (3) Development of an integrated, comprehensive surveillance and diagnostic program for ISAV. Our work will contribute to the development of an isothermal amplification assay as a rapid and sensitive diagnostic test for ISAV. In addition, we are developing instrumentation that will provide improved sensitivity and ease of use. These technologies will be used by diagnostic, veterinary, vaccine, biotechnology, and academic laboratories. The salmon aquaculture industry will benefit by maintaining good fish health status (ISAV free fish stocks) and having information available for informed decision making if the virus is diagnosed at a site in Maine. The salmon aquaculture industry will benefit economically from the technologies by help in avoiding and containing ISAV. In a worst-case scenario, the detection of ISAV at a grow-out site, the possible quarantine or destruction of fish and fallowing of production sites would be based on accurate information gathered through diagnosis and molecular epidemiology. This information hopefully will limit the extent of such extreme measures.

PRINCIPAL ACCOMPLISHMENTS:

Anderson Group: We generated ISAV cDNA libraries composed of 768 pGEM/cDNA plasmid clones. The individual
plasmids were grouped, by nucleic acid hybridization, into ten distinct genetic groups that represent the entire ISAV viral genome. Three of the genetic groups have been found to correspond to ISAV viral segments 2, 5, and 8. The remaining groups were "screened" by nucleic

acid sequencing and Northern blot analysis. The complete cDNA translation revealed that 47 and 42-kDa polypeptides were the P3 and HA proteins encoded by segments 5 and 6, respectively. P3 shared 99.6%, 84.0% and 83.1% amino acid sequence identity with ISAV isolates from Maine, Scotland, and Norway, respectively. DNA vaccines were constructed using full-length cDNAs of 5 ISAV virus segments. DNA vaccines were used to vaccinate rainbow trout and Atlantic salmon. Studies focused on their ability to provide cross-protection, persistent immunity, and a humoral immune response. Trivalent ISAV DNA vaccine provided only moderate protection in Atlantic salmon when compared with currently available whole killed vaccine, and none of the vaccines elicited a pronounced antibody response.

Kim Group: Specific padlocks and synthetic targets Based on ISAV RNA sequences were designed and synthesized. Approaches for padlock ligation in the presence of synthetic DNA and ISAV RNA were optimized. Fluorescence methods for quantitative analysis of rolling circle amplification (RCA) products using OliGreen nucleic acid stain were developed and RCA with synthetic DNA targets (splints) in the presence of fish tissue RNA was evaluated. RCA of extracted ISAV RNA was carried out and quantified using the OliGreen assay procedure. Oligonucleotides were used to immobilize padlock probes to both gold and SiO_2 substrates. In addition to serving as a tether between the substrate and the padlock, these oligonucleotides also serve as primers for rolling circle amplification. Further amplification via hyperbranching is initiated by the inclusion of additional primer oligonucleotides. Amplification of specific target nucleic acid sequences increases the amount of nucleic acid associated with the QCM increasing the mechanical mass, elasticity, and viscosity, as well as in electrical conductivity and polarization of the film, all of which were measured as a shift in resonant frequency. Since the measurement of conductivity should yield the highest sensitivity, experiments were carried out to maximize interaction between the HRCA product and the QCM surface. Ultimately, the combination of molecular padlock probe (MPP) amplification and QCM detection will be developed and compared with current diagnostic procedures. These experiments are continuing with additional funds from other sources.

Nicholson Group: A new nested RT-PCR assay for detection and identification of ISAV was developed based on sequences of viral genome segment 2 which encodes the putative polymerase protein. Results indicate that this new RT-PCT assay was more sensitive than our previously developed RT-PCR assay. Also, we developed an RT-PCR assay for ISAV based on recent sequence data for genome segment 7 which encodes the viral matrix (M) protein. We used DNA sequence data from the Anderson group, as well as other published sequence data on the hemaglutinin (HA) and nucleoprotein (NP) genes to develop RT-PCR assays based on these genes. We used our original RT-PCR assay to identify the first ISAV outbreak in farmed Atlantic salmon in the USA (Cobscook Bay, Maine). Furthermore, we determined partial sequences of the HA gene, genome segment 2 (PB1 polymerase gene), and genome segment 8 of the first Maine ISAV isolate and compared these sequences to published sequences of ISAV isolates from New Brunswick, Canada, Norway, and Scotland. These sequence comparisons showed that the Maine ISAV isolate was identical to ISAV isolates from New Brunswick, Canada, and distinct from ISAV isolates from Norway and Scotland. Based on the available genomic sequences of ISAV isolates from North America, Norway and Scotland, we developed primer sets that specifically recognize sequences unique to the North American strains of ISAV and the strains found in Norway and Scotland. We used these primer sets to develop a single RT-PCR assay that would identify all strains of ISAV but, at the same time, distinguish North American isolates from European ISAV isolates. PCR primers T1 and 2 were used to test wild fish collected by the NMFS. Sixty samples each of herring and mackerel from Cobscook Bay were collected, processed, and kidney samples were prepared in RNA Later. Using primers T1 and 2, only one sample out of the 120 tested resulted in a possible positive reaction for ISAV. Repeated PCR reactions were negative.

MicroTechnologies: Micro Technologies has received and continues to receive a fair volume of diagnostic samples for ISAV screening. Because of this, there was an immediate need for a direct tissue RT-PCR assay to be developed and Micro Technologies developed a direct tissue RT-PCR technique using the primer set established by Bruce Nicholson (really Sharon Blake) that worked well for ISAV diagnostics. We participate in the Canadian QA/QC program and find that our results are very consistent and accurate. Over the last year, however, we have begun working on optimization of the direct tissue procedure and the RT-PCR in general. Essentially, we want to establish an environmentally friendly protocol that is both specific and sensitive. The following two paragraphs outline the first developed direct tissue assay and our present assay. **Former procedures:** 30-50 mg of kidney tissue was collected and placed in

RNAlater. A 20 mg piece of tissue was excised from the original sample and extracted with Trizol. The tissue was ground in a 1.5 ml microfuge tube using a nuclease free pellet pestle. Initially the Access RT-PCR System (Promega), then the Ready-to-go RT-PCR Beads system (Amersham and Promega) and ISAV 1D/2 primers were used for amplification. **Present procedure:** Tissue is collected as before and placed in RNAlater. Extraction is done using either the Sigma GenElute Mammalian Total RNA Miniprep kit or the Qiagen RNeasy kit. We decided against Trizol in part due to its toxicity to users and waste handling and in part due to the ease of use of the Sigma and Qiagen extraction kits. The tissue piece is homogenized with glass beads (710-1180 µm) in the supplied lysis buffer and RNA extracted following protocols indicated with the respective kits. Amplification is done using the EZ rTth RNA PCR kit (Perkin Elmer) and ISAV 1D/2 primers. This amplification kit worked equally as well as those listed above. It was chosen over the two former kits due to lower licensing fees. Our sensitivity with this procedure is presently at or below 0.1 pg using kidney tissue. SYBR Green is being used as an alternative to ethidium bromide for staining gels in order to reduce hazards to users and eliminate hazardous waste handling. Also, in addition to the ISAV 1D/2 primers, the FA-3 / RA-3 primer set is also used for RT-PCR in certain instances. Micro Technologies has been continuing testing for ISAV using the RT-PCR assay system described above. Results remain consistent. Efforts towards the optimization of the RT-PCR assay have continued. A new primer is being developed to replace the ISAV 2 primer utilized in the ISAV D1/2 primer set. The new primer shows reduced background and promises a more robust assay.

Throughout the granting period MicroTechnologies Inc.'s (MTI) research focused on optimizing the RT-PCR assay and an initial evaluation of sample sources for ISAV screening. Optimizing the RT-PCR reaction first involved examining testing parameters such as amplification programs, annealing temperatures, and salt concentrations. The experimental strategy was to vary the cycling parameters and buffer components in order to identify a set of conditions that would decrease nonspecific amplification without reducing the sensitivity of the assay to detect ISAV in a sample. The results of the experiments on PCR reaction parameters indicated that the nonspecific background bands observed are probably not a consequence of sub-optimal conditions but rather associated with the quality and the condition of the sample (ie. length of time samples are collected postmortem, condition of specimen prior to collection). The second phase involved the examination of the performance of the Nicholson primers with sub-optimal samples and the development of an alternative primer (PM41) to replace the ISAV 2 primer. The results showed that the PM41 primer performed better with the sub-optimal samples but had a lower sensitivity than the ISAV 2 primer. It was concluded that the PM41 primer could be used instead of the ISAV 2 primer with sub-optimal samples while the ISAV 2 primers would be utilized routinely. The third phase of the optimization involved comparing the Nicholson primers and the Nylund primers. Some researchers have suggested that the Nylund RT-PCR protocol and primers result in better sensitivity for ISAV detection than the Nicholson protocol. To test this, experiments were carried out similar to the PCR parameter optimization experiments using the published Nylund and Nicholson primers, and compared the ISAV detection and non-specific background levels of the two primer sets. No consistent differences in sensitivity between the Nicholson and Nylund RT-PCR primer sets were found. Near the limit of detection, variations in the positive and negative RNAs seemed to have a greater affect on the threshold than either PCR protocol. Further confirmation of reliability and sensitivity of the optimized RT-PCR assay and the primer set sensitivity was tested by performing a double blind study involving MTI and a Canadian laboratory. MTI used the Qiagen RNeasy kit for extraction, the EZ rTth RNA PCR kit and the Blake primers for amplification while the Canadian laboratory used trizol extraction, and the Ready-to-go RT-PCR Beads and the Nylund primers. Results of the double blind study indicated that both procedures performed equally. Since this work, the US Fish and Wildlife service has adopted MTI's ISAV RT-PCR protocol as its standard operating procedure. MTI has tested over 5,000 direct tissue samples using the optimized procedure. Another aspect of MTI's research involved evaluation of sample sources to determine the optimal tissue submission conditions for the detection of ISAV. This also allowed for the comparison of lethal versus non-lethal sampling. Trials have been performed comparing blood, kidney, and mucus for ISAV detection by RT-PCR and by viral culture. Mucus was dropped out of the comparison testing early. It was clear based on results that there was likely a PCR inhibitor and ISAV detection by RT-PCR from mucus was inconsistent. Blood proved to be an excellent sample source for screening by RT-PCR and proved to be better than kidney tissue. Kidney performed the same as blood for detection of ISAV by cell culture methods. The above research has passed along usable and reliable procedures to other laboratories including the US Fish and Wildlife service, as well as,

USDA/AHPIS National Veterinary Diagnostic Laboratory. The optimized RT-PCR assay for the detection of ISAV is presently employed in the current mandatory ISAV surveillance program sponsored by the USDA/ APHIS under the jurisdiction of the Maine Department of Marine Resources.

IMPACTS:

This study resulted in an optimal RT-PCR assay for the detection and identification of ISAV.

The optimized RT-PCR assay for the detection of ISAV is presently employed in the current mandatory ISAV surveillance program sponsored by the USDA/APHIS under the jurisdiction of the Maine Department of Marine Resources.

Blood proved to be an excellent sample source for screening by RT-PCR and proved to be better than kidney tissue (no lethal sampling required).

The complete nucleotide sequences of 4 ISAV isolates were obtained, indicating that the Maine and Canada isolates are highly related, but are epidemiologically distinct from the European strains of ISAV.

Prototypic DNA vaccines for ISAV failed to protect salmon better than conventional killed ISAV vaccines, indicating that optimization of traditional killed vaccines should be explored.

The ISAV surveillance on fish samples and biosecurity programs involving environmental monitoring will allow the salmon aquaculture industry to develop ISAV management strategies designed to control and possibly eliminate disease outbreaks.

RECOMMENDED FOLLOW-UP ACTIVITIES:

With additional funding from NOAA-Sea Grant and DOD we are 1) quantifying the density and distribution of surface-coupled oligonucleotides on gold and SiO_2 surfaces, 2) modifying the QCM electrode geometry, 3) evaluating the target-initiated amplification by primer-immobilized padlock probes, and 4) testing the padlock probes on QCM devices. Using the developed PCR assay we are continuing to monitor ISAV in wild fish and to confirm potential positives detected in other laboratories. MTI will continue environmental monitoring programs for the early detection of ISAV. Studies are continuing that are designed to detect ISAV from concentrated water samples, sediment, fomites, and invertebrates. Filtration techniques are being used for water samples. Examination of sample size and various extraction procedures will continue on fomites and invertebrates.

The Department of Biochemistry, Microbiology and Molecular Biology at University of Maine has in place a Stratagene Mx4000 Multiplex Quantitative PCR System and accompanying Agilent Technologies Bioanalyzer that was purchased for fish disease diagnostics and that is available for use by investigators in the Northeast region. Please contact Dr. Greg Mayer (207-581-2852; gmayer@maine.edu) for the particulars on using the instrument.

PUBLICATIONS, MANUSCRIPTS, PAPERS PRESENTED:

Publications in Print:

Ahne, W., S. Blake, S. Essbauer, and B. L. Nicholson. 2003. Isolation and identification of aquatic birnaviruses from flounder (*Pseudopleuronectes americanus*) and mummichog (*Fundulus heteroclitus*) in the Chesapeake Bay, Virginia, USA. Diseases of Aquatic Organisms. In press.

Altmann, S. M., M. T. Mellon, M. C. Johnson, B. H. Paw, N. S. Trede, L. I. Zon, and C. H. Kim. 2003. Cloning and characterization of an Mx gene and its corresponding promoter from the zebrafish, Danio rerio. Developments in Comparative Immunology In Press.

Altmann, S. M., M. T. Mellon, D. L. Distel, and C. H. Kim. 2003. Molecular and functional analysis of an interferon gene from the zebrafish, Danio rerio. Journal of Virology 77:1992–2002.

Anderson, ED, Engelking, MH, and Kurath, G. 2000. Molecular epidemiology of IHN virus within an Oregon study site reveals emergence of a new virulent virus strain and transmission from wild salmon to hatchery fish. J Aquat Anim Health 12:85-99.

Blake, S., J-Y. Ma, D. Caporale, S., S. Jairath and B. L. Nicholson. 2001. Phyogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of cDNA of genome segment A. Diseases of Aquatic Organisms 45:89–102.

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Chiou PP, CH Kim, P Ormonde, JC Leong. 2000. Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and induces morphological changes of apoptosis in cell cultures. J Virol. 74:7619-27.

Clouthier, S. C., T. Rector, N. E. C. Brown and E. D. Anderson. 2002. Genomic organization of infectious salmon anemia virus. Journal of General Virology 83:421–428.

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Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine. 18:2817-24.

Trobridge, G.D., C.H. Kim, S. Lapatra and J.C. Leong. 2000. Mx mRNA expression and RFLP analysis of rainbow trout Oncorhynchus mykiss genetic crosses selected for susceptibility or resistance to IHNV. Dis Aquat Organ.40:1-7.

Johnson, M.C., B.E. Simon, C.H. Kim, and J.C. Leong. 2000. Production of recombinant snake head rhabdovirus (SHRV); the NV protein is not required for viral replication. J Virol. 74:2343-50.

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Lapatra, SE., Corbeil, S., Jones, RG., Shewmaker, WD., Lorenzen, N., Anderson, ED., and Kurath, G. 2001. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 19(28–29):4011–4019.

Leong, J. C., D. Brown, P. Dobos, F. S. B. Kibenge, J. E. Ludert, H. Muller, E. Mundt, and B. Nicholson. 2000. Family *Birnaviridae*. In M. H. V. vanRegenmortel, C. M. Fauget, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.) Virus Taxonomy: Seventh Report of the International Committee on the Taxonomy of Viruses, Academic Press, pp. 481-490.

Nicholson, B. L. 2000. Fish diseases in aquaculture. University of Maine College of Natural Sciences, Forestry and Agriculture White Paper Series 3, #8.

Opitz, H.M., Bouchard, D., Anderson, E., Blake, S., Nicholson, B., and Keleher W. 2000 A comparison of methods for the detection of experimentally induced subclinical infectious salmon anae-mia in Atlantic salmon. Bull. Eur. Ass. Fish Pathol. 20(1):12–22.

Opitz, H. M., D. Bouchard, E. Anderson, S. Blake, B. Nicholson, and W. Keleher. 2000. A comparison of methods for the detection of experimentally induced subclinical infectious salmon anemia in Atlantic salmon. Bulletin of the European Association of Fish Pathologists, 20(1): 12-22.

Ortega, C., R. M. de Oca, D. Groman, C. Yason, B. Nicholson, and S. Blake. 2002. Case Report: Viral infectious pancreatic necrosis in farmed rainbow trout from Mexico. Journal of Aquatic Animal Health 14:305–310.

Papers Presented:

Altmann, S. M., M. T. Mellon, R. E. Cashon, M. C. Johnson, and C. H. Kim. 2003. Characterization of the structure and function of zebrafish interferon and the MX promoter. International Society for Developmental and Comparative Immunology, June 29–July 4, St. Andrews, Scotland.

Clouthier, S., S. LaPatra, B. Glebe, W. Young-Lai and E. Anderson. 2002. Humoral immune reponse to ISAV, p. 34. Tenth Annual New England Farmed Fish Health Management Workshop, April 4.

Genbank Accession Numbers for ISAV isolate CCBB: Segments 1, AF40437; 2, AF404346; 3, AF404345; 4, AF404344; 5, AF404343; 6, AF404342; 7, AF404341; and 8, AF404340.

Genbank Accession Numbers for ISAV isolate Norway: Segments 5, AF429987; 7, AF429990. Genbank Accession Numbers for ISAV isolate Scotland: Segment 5, AF429988. Genbank Accession Numbers for ISAV isolate ME/01: Segments 5, AF429986; 6, AY059402; 7, AF429989.

Mellon, M. E., S. Pelsue, C. Sullivan, P. Phelan, K. Shaw, S. Altmann, J. Fernandez, A. Hermann, M. Moody, M. Pressley, and C. H. Kim. 2001. Indentification of Toll-like receptor gene homologues in the developing zebrafish, Danio rerio. New England Immunology Conference, Woods Hole, MA.

Millard, P., H. Gordon, L. Clepper, K. Boettcher, and C. Kim. 2000. Improving the performance of molecular biosensors with photoinitiated fluorescence signal amplification. 8th International Meeting on Chemical Sensors,p. 395.

Nicholson, B. L. 2000. Applications of Molecular Biological Tools in Diagnostics and Epidemiology of Viral Pathogens in Aquaculture, Jan. 13-14, San Diego, CA.

Nicholson, B. L. 2000. Use of genomic sequencing in investigations of epidemiology, virulence markers and phylogenetic relationships of aquatic birnaviruses. Agricultural Microbes Genome I Conference, San Diego, CA.

Nicholson, B. L. and S. Blake. 2001. Identification and characterization of infectious salmon anemia virus (ISAV) in Atlantic salmon in

Maine, USA. International Symposium of the European Society of Fish Pathologists, Dublin Ireland

Nicholson, B. L. 2001. Applications of Molecular Biological Tools in Diagnostics and Epidemi ology of Viral Pathogens in Aquaculture, 5th International Marine Biotechnology Confer ence, Sept. 28-Oct. 4, Townsville, Australia.

Pressley, M. E., S. Blake, B. L. Nicholson, and C. H. Kim. 2002. Viral and bacterial challenge studies of the zebrafish, Danio rerio. Interna tional Society for Developmental and Com parative Immunology, New Orleans, LA.

Pressley, M. E., S. Blake, N. Stasilus, E. Witten, B. Nicholson, and C. H. Kim. 2002. Infectious disease models for the zebrafish Danio rerio. International symposium on aquatic animal health, September 1–5, New Orleans, LA.

Zhang, C., C. H. Kim, P. J. Millard, and J. F. Vetelino. 2001. An acoustic wave sensor for monitoring ammonium in water. Electrochemical Society Meeting, San Francisco.

TECHNICAL ANALYSIS AND SUMMARY:

The complete RNA genome segment order, nucleotide sequence, and putative protein sequence for ISAV isolate CCBB was determined from DNA sequencing of 10 groups of pGEM/cDNA plasmid clones selected from the 768-clone library. Complete nucleic acid sequence was obtained for eight segments encoding proteins P1, PB1, NP, P2, P3, HA, P4/P5, and P6/P7. Virion protein identifications and gene assignments were confirmed by Western blotting with Atlantic salmon ISAV antiserum and by N-terminal sequencing of cDNA translation products. Seven virion proteins from 25–72 kDa were detected by SDS-PAGE, and a 42-kDa and 72-kDa polypeptide reacted with ISAV antiserum from Atlantic salmon. The 72-kDa polypeptide was identified as the NP protein encoded by segment 3. NP shared 96.6% amino acid sequence identity with NP of a Scottish ISAV isolate. N-terminal amino acid sequencing and cDNA translation revealed that 47 and 42-kDa polypeptides were the P3 and HA proteins encoded by segments 5 and 6, respectively. P3 shared 99.6%, 84.0% and 83.1% amino acid sequence identity with ISAV isolates from Maine, Scotland, and Norway, respectively.

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men prior to parameters indicated that the nonspecific background bands observed are probably not a consequence of sub-optimal conditions but rather associated with the quality and the condition of the sample (i.e. length of time samples are collection). The second phase involved the examination of the performance of the Nicholson primers with sub-optimal samples and the development of an alternative primer (PM41) to replace the ISAV 2 primer. The results showed that the PM41 primer performed better with the sub-optimal samples but had a lower sensitivity than the ISAV 2 primer. It was concluded that the PM41 primer could be used instead of the ISAV 2 primer with sub-optimal samples while the ISAV 2 primers would be utilized routinely. The third phase of the optimization involved comparing the Nicholson primers and the Nylund primers. Some researchers have suggested that the Nylund RT-PCR protocol and primers result in better sensitivity for ISAV detection than the Nicholson protocol. To test this, experiments were carried out similar to the PCR parameter optimization experiments using the published Nylund and Nicholson primers, and compared the ISAV detection and non-specific background levels of the two primer sets. No consistent differences in sensitivity between the Nicholson and Nylund RT-PCR primer sets were found. Near the limit of detection, variations in the positive and negative RNAs seemed to have a greater affect on the threshold than either PCR protocol. Further confirmation of reliability and sensitivity of the optimized RT-PCR assay and the primer set sensitivity was tested by performing a double blind study involving MTI and a Canadian laboratory. MTI used the Qiagen RNeasy kit for extraction, the EZ rTth RNA PCR kit and the Blake primers for amplification while the Canadian laboratory used trizol extraction, and the Ready-to-go RT-PCR Beads and the Nylund primers. Results of the double blind study indicated that both procedures performed equally. Since this work, the US Fish and Wildlife service has adopted MTI's ISAV RT-PCR protocol as its standard operating procedure. MTI has tested over 5,000 direct tissue samples using the optimized procedure. Another aspect of MTI's research involved evaluation of sample sources to determine the optimal tissue submission conditions for the detection of ISAV. This also allowed for the comparison of lethal versus non-lethal sampling.

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