ISAC
INVASIVE SPECIES ADVISORY COMMITTEE

U.S. Department of the Interior
Office of the Secretary
1849 C Street NW
Washington, DC 20240

Validation of PCR-Based Assays and Laboratory Accreditation for Environmental Detection of Aquatic Invasive Species

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"Validation is the bridge between research and regulatory decisions!"
(Anything else is jumping across the abyss of unknowns to any possible conclusion!)

This white paper provides:

a) Background information on the use, accuracy and reliability of PCR-based assays such as environmentally sampled DNA (eDNA) for early detection of aquatic invasive species (AIS) and;
b) Recommendations for establishing a system for validating assays and accrediting laboratories that report on the presence or absence of AIS.

This white paper was developed by the members of ISAC and discusses the need for developing validation requirements for Polymerase Chain Reaction (PCR) and other DNA-based molecular assays that are increasingly being used to detect AIS. It does not provide a simplified checklist for evaluation of their ability to detect AIS. Rather, it is intended to demonstrate the need for a required and regulated framework to validate these molecular assays. A regulated framework for validation would greatly increase confidence in the utility of DNA-based assays and better enable decision-makers and managers regarding AIS detection, prevention, monitoring and control.

ISSUES

Aquatic invasive species can have major environmental, economic, and in some cases human health impacts. The National Invasive Species Council's (NISC) bureaus and agencies have a responsibility to make the most appropriate decisions possible and take timely action. However, traditional visual methods for the early detection and identification of invasive species are difficult and time-consuming to conduct in aquatic systems and maybe inadequate to support effective and timely actions. Delays, data gaps, and inaccurate information can be costly and allow an invasive species to become too well established and widespread to apply effective rapid response and eradication plans.

Molecular assays based on PCR can amplify tiny amounts of DNA in water samples (i.e., environmental DNA or eDNA) and detect the presence of AIS at high levels of sensitivity and specificity (Blanchet 2012, Darling and Mahon 2011, Jerde et al. 2011). This approach is currently being used to detect Asian carp species and zebra and quagga mussel larvae in water systems. There is increasing interest in the development of additional PCR-based assays for these and other AIS.

These factors, coupled with the increasing availability of rapid molecular assay systems (kits) are greatly expanding the use of PCR-based technologies to detect AIS. Due to their relative sensitivity, the use of molecular assays is causing major paradigm shifts in the way that AIS are detected, monitored, and controlled.

The successful application of molecular technologies will increase the speed and number of samples that can be analyzed, making early AIS detection more likely and increasing the probability that AIS populations will be contained and eradicated. However, there are numerous concerns regarding the reliability of these assays which were originally developed for research applications rather than to inform regulatory and/or management decisions (e.g., Longshaw et. al. 2012). They have been conducted without appropriate validation of methodology or definition of minimum laboratory requirements. These concerns are especially important when molecular tests are the only means available/possible to detect AIS because "traditional" methods cannot be used. However, the consequences of trusting an assay that has not been validated could be far more damaging, destructive, and long-lasting in loss of agency credibility or harm to non-target species than the damage caused by the arrival and establishment of an invasive species. Due to their potential negative regulatory, economic, and ecological impacts, one may question why managers or agencies would attempt to make decisions regarding AIS based on results from assays that have not been validated and/or conducted by unaccredited laboratories.

Regardless of what assays are used, making authoritative public announcements and appropriate regulatory decisions requires a suitable number (statistically valid) of certifiable samples to be collected under strict protocols.

The establishment of well regulated sample collection, sample custody, and analyses protocols will allow NISC agencies and their partners to provide authoritative public announce-

ments and make appropriate regulatory decisions in order to avoid wasteful use of regulatory resources, unnecessary public confusion or unrest, national and international commercial damage, and legal remediation.

To ensure that decision makers can make appropriately informed decisions and most effectively use these powerful new techniques, they need to be assured that the information generated by assay results is reliable via high analytical specificity and diagnostic specificity for the target species in a tested water body. However, decision-makers often have little information concerning the accuracy or reliability of the various DNA detection methods being used or the performance quality of the various laboratories conducting them. In addition, commercial assay kits used by some laboratories are protected from public release of specific data concerning their contents and internal protocols that are considered confidential commercial business information. While a method may meet the needs for research applications and be published in a peer-reviewed journal, this does not equate to an assay being judged or accepted as validated for other applications. Decision-makers may initiate rapid response efforts based solely on eDNA evidence with little assurance of its quality or limitations. Currently, there is no formal process for approving sampling and testing protocols. Ultimately, this reduces the effectiveness of efforts to combat the introduction and spread of AIS.

Although there is increasing use of PCR assays to detect AIS in aquatic systems and increasing reliance on them for making critical regulatory and management decisions, there is no formal organizational process for approving sampling and testing protocols and questions concerning their effectiveness remain.

Each of the AIS-detection assay/sampling protocol systems that are developed requires validation. They must be evaluated to ensure that the protocols used yield results that are: specific to the target organism (specific), can detect low concentrations of eDNA (sensitive), consistent over time (reproducible); provide results that are within acceptable limits of variation from replicate samples obtained from both within and among locations (precise); able to yield similar results under differing environmental and sample conditions (robust), and consistent with positive and negative control samples (accurate).

A new assay needs to be evaluated against an established "gold-standard" or compared diligently to a long accepted methodology; validated for their specificity, sensitivity, precision, accuracy, robustness, and reproducibility; and, laboratories conducting the assays need to be accredited.

Moving from traditional visual identification methods to molecular detection assays involves complex paradigm shifts which have great importance for decision-makers. It is a shift from the identification of organisms at a specific location and time to the detection of the current and/or past presence of an organism. It is also a paradigm shift from direct detection (i.e., collecting a specimen) to indirect detection (i.e., collecting DNA shed from an organism).

These paradigm shifts have enormous import for managers and require a correlation between "traditional" and "newer" approaches. Decision makers must have a clear understanding of the strengths and weakness of all the methods used.

The terms "validation" and "accreditation" have been defined by several quality assurance organizations and have been standardized domestically and internationally in support of trade and other agreements. However, these definitions have not been uniformly applied to the discussion of PCR-based assays for AIS detection in environmental samples ("validation" in this white paper is defined as "the systemic and scientific evaluation of an assay to accurately define its usefulness, robustness, accuracy, specificity, sensitivity, and repeatability."). A lack of clear and consistent terminology has led to confusion and can hinder the progress of AIS detection or control efforts.

The clear and consistent use of standard terminology is critical to avoiding confusion and understanding and effectively communicating the information used to make decisions.

No assay is 100% accurate and consistent. The utility of PCR-based AIS early detection methodologies for decision-makers would be greatly increased if decision-makers and the public had clear measures of the specificity, sensitivity, precision, and accuracy of reported results. Increased confidence in eDNA detection would allow regulators to make more informed decisions and take scientifically based actions at the earliest possible stage of invasion when rapid response and eradication efforts have the highest likelihood of success. It would also greatly augment public communication efforts. Similarly, independent performance testing, and eventually laboratory accreditation could direct decision-makers to high performing laboratories that consistently generate trustworthy results that can be tracked over time and among locations.

The eventual outcome of evaluating laboratory performance would be the establishment of a national reference laboratory fully capable of meeting international requirements and standards.

Application of the concepts of assay validation and laboratory accreditation are urgently needed. For example, a lack of certainty and confusion regarding DNA-based detections has led some agencies to require, separate and independent verification of initial assay results before taking action (Darling and Mahon 2011). The degree of confidence that regulatory officials and private and public stakeholders have in the specificity, accuracy, and robustness of current eDNA assays for correctly informing AIS decisions could be greatly increased by establishing systems for performance testing, validation and accreditation to benchmark both methodological and laboratory performance.

Asian carp species currently threaten the Great Lakes. The use of eDNA evidence indicating that Asian carp could be in Lake Michigan has been the subject of heated controversy (Jerde et al., 2011) and extensive review (see below). Currently, litigation is shackling Asian carp control because of a lack of convincing correlations between visual traditional methods (i.e., having captured fish at a specific location and time) versus PCR detection of carp DNA in water samples. In early 2010, the Solicitor General informed the U.S. Supreme Court, in part, that the use of a PCR eDNA assay for detecting invasive Asian Carp as:

"the best information available… the government has not rejected any option… compelled by the facts… Nothing in federal law warrants second-guessing its expert judgment that the best information available today does not yet justify the dramatic steps Michigan demands."[1]

Again, in February 2010 testimony to the US House of Representatives stated:

"Because eDNA is a new approach to assessing the presence of Asian carp and is being applied operationally before standard independent scientific review could occur, the Corps (U.S. Army Corps of Engineers) continues to collaborate with the University of Notre Dame to determine what eDNA does and does not tell us and continues to research how to improve the usefulness of this technology to inform management decisions."[2]

More recently in May 2011, a U.S. Army Corps of Engineers expert gave testimony in the U.S. Appeals Court stating:

"Efforts to corroborate eDNA results with traditional methods of capturing fish have not been successful thus far."[3]

Perhaps the most compelling testimony that eDNA is an emerging technology and not validated is from the U.S. Army Corps of Engineers before the U.S. Supreme Court dated January 2010:

"Scientific research typically follows a process that includes a hypothesis regarding a topic, predictions about experimental or observational results based on the hypothesis, gathering of data, analysis of data, assessments of prediction accuracy, revision of the hypothesis, conclusions, and iterations if necessary. This process allows for revision and fine-tuning of hypotheses as predictions are tested and more information becomes available, and allows for an increasingly better understanding about the phenomenon or topic of interest. Hypotheses regarding the robustness and information content associated with positive eDNA detections are currently being formulated by Notre Dame.… In scientific research processes and terminology, this would involve further gathering and analysis of data to be used in testing predictions and assessing hypotheses regarding the inferential power of the eDNA method. This is a critical process in making sure that strong scientific conclusions are made and appropriate management actions undertaken."[4]

Could the "best information available" be devastatingly wrong if there is a deficiency of solid science (still in research mode) or a lack of validation of the assay or accreditation of the laboratory before it is applied in a real life situation? Indeed, because of the regulatory, interstate commerce, and legal concerns regarding use of eDNA to detect the presence of Asian carp, the methodology and laboratory which developed it have undergone an extensive independent review process (Battelle Memorial Institute 2010, United States Environmental Protection Agency 2010, Asian Carp Regional Coordinating Committee 2012). The laboratory audit reviewed and reported on: 1) staff qualifications, training and quality assurance roles, 2) laboratory facilities, 3) field sampling practices, 4) eDNA methodology, 5) PCR methodology, and 6) quality assurance systems (United States Environmental Protection Agency 2010). This audit may be an initial step for future eDNA assay validation and laboratory accreditation.

At the very least, laboratories using eDNA technology for early detection and monitoring of AIS should be offered the opportunity for independent performance testing as has been done for dreissenid mussel PCR detection (Frischer et al. 2011) with public access to performance results so that entities seeking the laboratories' services can be confident of their accuracy, reliability, and capacity to detect target species' DNA. The availability of such independent performance testing could be a step in the eventual development of comprehensive eDNA methodology validation and laboratory accreditation systems.

RECOMMENDATIONS

To encourage the development of a validation/accreditation system for AIS eDNA detection methodologies and laboratories, ISAC recommends that the NISC member Departments and Agencies and their partners consider adoption of the following recommendations.

- Encourage and develop funding for the National Academy of Sciences to undertake a review of the reliability and effectiveness of PCR and other DNA-based applications for detecting AIS, focusing on establishment of appropriate validation processes and a framework and standards for this new and potentially invaluable tool in the early detection, eradication, prevention and control of AIS.
- Establish and fund an ongoing independent performance testing program for laboratories utilizing DNA-based AIS detection methodologies such as that recently undertaken for evaluating laboratory performance in PCR detection of dreissenid mussel larvae (Frischer et al. 2011). Testing

1 U.S. Memorandum in Opposition, January 2010, to the US Supreme Court hearing Michigan's renewed petition for closure of the Chicago Area Waterway System to prevent Asian Carp species from entering the Lake Michigan from the Illinois River system.

2 Statement of: Major General John Peabody, Commander, Great Lakes and Ohio River Division, U.S. Army Corps of Engineers, Before: Subcommittee on Water Resources and Environment Committee on Transportation and Infrastructure, United States House of Representatives on Asian Carp and the Great Lakes, February 9 2010

3 Slater. U.S. Army Corps of Engineers, US Court of Appeals, 7 Circuit, May 5, 2011.

4 Declaration of Dr. Elizabeth C. Fleming, Senior Executive Service, Director of the Environmental Laboratory, and Civil Works Business Area Lead at U.S. Army Engineer Research and Development Center. App. 30a http://www.supremecourt.gov/specmastrpt/us_appendix_to_renewed_opp.pdf

results should be made public so that managers may make informed decisions about the accuracy and reliability of a laboratory's performance when including an eDNA component in an AIS monitoring and early detection system.
- Utilize lessons learned in establishing a laboratory performance testing system to fully develop a validation/accreditation program(s) for other invasive species eDNA methodologies and laboratories.

BACKGROUND

Molecular PCR-based assays amplify trace amounts of DNA by orders of magnitude. Using short highly specific segments of DNA called primers; these primers are a critical component of the assays that can detect the presence of target organisms' DNA in water samples. This approach has been used in attempting to monitor the spread of quagga mussels (*Dreissena rostriformis bugensis*) by detecting DNA from their larvae in plankton net tow samples in the western United States (Hosler 2011, Turner et al. 2011) and the free DNA of Asian carp (i.e., environmental DNA or eDNA) in water samples from the Chicago Area Waterway System (Jerde et al. 2010, 2011). A large proportion of DNA is "conserved" among species. Only a small amount is unique to a species. Isolating specific and stable primers to bind to "i.e., probe" a target DNA sequence is difficult. Primer selection and PCR protocols can profoundly alter the results obtained. Primers must be highly specific to the target species (Darling and Mahon 2011).

The need to rapidly detect AIS has led to the recent development of numerous PCR-based and other molecular detection assays for the analysis of environmental samples (Darling and Blum 2007, Li et al. 2011, Darling and Mann 2011, Mahon et al. 2011, Blanchet 2012). Numerous molecular assays have been published for detection of aquatic organisms including microbial pathogens (i.e., viruses, bacteria, protozoa and helminthes) (Toze 1999); bivalves (Claxton and Boulding 1998); fish (Jerde et al. 2011); and amphibians (frogs and salamanders) (Goldberg et al. 2011). However, the various molecular assays that have been used to detect a target organism's DNA in water samples obtained from the field (United States Environmental Protection Agency 2010) have not been standardized using validated assays conducted in accredited laboratories. Only one report of a laboratory performance evaluation that examined 11 laboratories' performance in identifying zebra mussel larva (veligers) DNA is available (Frischer et al. 2011). This "double-blind, round robin" evaluation using standardized low target organism density water samples found that PCR techniques *were the least reliable detection method.* The traditional visual microscopic examination under polarized light was most reliable and accurate (75.8% versus 96.3% accuracy in determining presence/absence). This finding has led to legitimate concerns regarding the accuracy and reliability of eDNA for early AIS detection.

Of 11 laboratories tested, the most common error was failure to detect eDNA (i.e., false negative test result) in samples known to contain veliger DNA. There was also considerable variation (lack of precision) among laboratories. The average precision of more "experienced laboratories" as defined by the study was 86.9% while that for laboratories with less experience with these assays was considerably lower at 62.4% (Frischer et al. 2011). This round-robin conclusion will not be totally known until the information on the diagnostic sensitivity and specificity become known with assay validation.

As with all assays, a major concern is positive test results that do not reflect the true presence of the AIS at a location (i.e., false positives). This may be due to sample contamination, problems with the assay, DNA from dead target organisms, and/or only the target DNA and not the organism itself being present. DNA may last 14–25 days in the water column (Dejean et al. 2011) and can be carried by water currents far from the actual range of the target species. For example, it is not clear if Asian carp DNA in areas of the Chicago Area Waterway System was a "false positive" finding (Jerde et al. 2011). In addition, carp DNA could have been released into waterways from rinse water from fish markets and/or from ice melt-water used to store harvested carp (Asian Carp Regional Coordinating Committee 2012). There are several possible sources of target species DNA, such as 1) sewage discharge, 2) discarding remains of target species in slaughter and processing activities, 3) dead individuals captured elsewhere and transported by humans or wildlife and, 4) uptake of water from a target species habitat by boats or barges followed by discharge into an area outside the target species' range (Darling and Mahon 2011, Asian Carp Regional Coordinating Committee 2012).

Conversely, false interpretations may occur due to insufficient test sensitivity or because, even if target species is present, AIS DNA may not be in the water sample collected or the concentration is below the limits of detection of the assay (Darling and Mahon 2011).

Four potential sources of error in eDNA testing are:

1. lack of assay sensitivity and/or specificity,
2. insufficient laboratory quality assurance, i.e., sample contamination, failure to follow protocols, and misinterpretation of results,
3. ineffective sampling design and protocols to maximize potential for discovering the target species DNA, and
4. lack of understanding of the relationship between a detection of a target species' DNA and actual target species presence, including DNA persistence and opportunities/vectors for its transport outside the range of the target species (Darling and Mahon 2011, Asian Carp Regional Coordinating Committee 2012).

Existing Validation Requirements for DNA-based Detection Assays

Currently, at least two federal agencies have some level of regulatory control regarding PCR assays developed and validated for marketing in the United States. The Federal Drug Administration (FDA) is responsible for enforcement of the Federal Food, Drug, and Cosmetic Act that covers *in vitro* diagnostic devices which are a subset of medical devices "intended for use in the diagnosis of disease and other conditions, including

determination of the state of health, to cure, mitigate, treat, or prevent disease or its sequel." The Animal and Plant Health Inspection Service through the Center for Veterinary Biologics regulates the licensing and sale of diagnostic kits used in detecting animal diseases under the authority of the Virus Serum Toxin Act. Both agencies are involved in assuring that commercially available kits for running assays are safe, effective, reliable, and truthful in their label claims.

Existing Accreditation of Laboratories Offering DNA-based Detection Systems

Currently, there is no required independent or regulatory oversight of laboratories conducting and performing DNA-based AIS assays when using in-house primers, reagents, protocols, and technologies. There are numerous "quality" concerned organizations which orient their policies and philosophies towards globally standardized laboratory quality and analytical assay validation. These "quality" associations/organizations are voluntary. Membership brings recognition of a laboratory's effort to conform to quality standards in several areas important to reliable and reproducible laboratory operations and outcomes.

Generally "inspections" (i.e. audits, reviews, verifications, etc.) by quality organizations are conducted by a team of experts from member laboratories. Each team member can be specialized in some area of concern to the quality standards being verified. Typically areas reviewed, observed and audited are facilities, equipment, personnel qualifications, protocols, references, mechanisms of internal control and direction, etc.

There are areas of exceptional standards in some regulatory programs for the prevention, control, and eradication of animal disease where participation may need to meet mandatory standards for facilities, equipment, and personnel. Many protocols in these regulated laboratories are standardized in accordance with international trade agreements or other legally binding documents. Personnel must follow the various "Uniform Methods and Rules" used when testing for animal pathogens of commercial and economic significance. It appears that human and animal health is well on its way to utilizing reliable, validated assays for information regarding disease. There is also a system in place for plant health certification by way of testing for plant pathogens. These programs could serve as models for development of validation of protocols and accreditation of laboratories providing DNA-based AIS detection systems.

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