CANADIAN WILDLIFE HEALTH COOPERATIVE

CWHC NATIONAL OFFICE
Western College of Veterinary Medicine
52 Campus Dr, Saskatoon, SK S7N 5B4
PH. 1.306.966.5099 EM: info@cwhc-rcsf.ca

Evolving approach to white-nose syndrome diagnostic standards in Canada

Prepared by: Scott McBurney[1], Hugh Cai[2], Jordi Segers[1], Patricia Bell-Rogers[2] and Tomy Joseph[3]

[1] Canadian Wildlife Health Cooperative
[2]Animal Health Laboratory, University of Guelph
[3]Animal Health Centre, Government of British Columbia

Introduction

The Canadian Wildlife Health Cooperative (CWHC) delivers Canada's national program for wildlife health and is the leading voice for wildlife health in Canada. Every bat submitted to the national white-nose syndrome (WNS) surveillance program or for health assessment is sent to one of the six regional nodes of the CWHC that are embedded in a veterinary college or provincial diagnostic laboratory. Standard diagnostic categories as developed and agreed upon by the United States (U.S.) and Canadian WNS diagnostic working groups are used to report cases of WNS and the environmental presence of the causative fungal agent, *Pseudogymnoascus destructans* (Pd). To ensure quality control and quality assurance for the interpretation of diagnostic tests as they relate to WNS surveillance and Pd detection across Canada, the CWHC provides Federal, Provincial, and Territorial partners with a standardized approach to the analysis of the diagnostic findings in each case so that the wildlife agency receiving the results can have confidence in them and develop appropriate wildlife management responses and strategies to recover and protect those bat species affected by WNS.

Diagnostic definitions

A bat submitted for WNS surveillance is defined as positive for WNS when histologic lesions of WNS are present AND the presence of Pd is confirmed with a quantitative real-time polymerase chain reaction (qPCR) or fungal culture. The same qPCR is used to test environmental samples for Pd. In Canada, samples collected for Pd detection are sent for qPCR either to the Animal Health Laboratory (AHL) at the University of Guelph or the Animal Health Centre (AHC) at the Government of British Columbia. To ensure the highest level of quality control and quality assurance, AHL and AHC are accredited by the American Association of Veterinary Laboratory Diagnosticians.

A qPCR reaction has a threshold line which is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The cycle threshold (Ct) or crossing point (Cp), collectively represented as Cq (quantificaton cycle), is the intersection between the predetermined amplification curve of the qPCR and a threshold line at the point of exponential increase of PCR product from the specific gene region being targeted by the qPCR. When the qPCR amplification happens (Cq $\geq$ 1), the Cq value obtained is usually inversely and exponentially proportional to the concentration (or

amount) of the target template gene region present in the specimen being tested (*i.e.,* a qPCR with a low Cq result indicates that there is a large concentration of the target template gene region in the specimen being tested). The reverse is also true, but while a qPCR with a high Cq result is compatible with the possibility of a low concentration (or amount) of the target template gene region being present in the tested specimen, usually the higher Cq results of the late amplification cycles of the qPCR are considered less reliable and possibly the result of artifacts such as degradation of the qPCR's probe-based fluorophore, cross contamination of the specimen or nonspecific amplification of background nucleic acids. Therefore, for most qPCR reactions, laboratory diagnosticians set a cut-off Cq value based on validation experiments above which the Cq results are considered as potentially negative.

Initially, the AHL was the only Canadian laboratory using a molecular technique to test for Pd, and in the 2011-12 WNS surveillance season, a SYBR Green qPCR was used with a standard operating protocol that specified any reaction crossing the threshold baseline prior to 40 cycles and that had the expected melting temperature was considered positive (*i.e.,* cut-off Cq < 40). The TaqMan qPCR technique for Pd described by Muller *et al.* (2013) was adopted as the U.S. and Canadian standard diagnostic test for WNS and Pd surveillance, and the methodology described for this technique indicated that any reaction that crossed the threshold baseline within 40 amplification cycles was considered positive (*i.e.,* cut-off Cq $\leq$ 40). However, after the AHL validated the technique on their platform, it was determined that if a sample crossed the threshold baseline within 35 amplification cycles it was positive (*i.e.,* cut-off Cq $\leq$ 35). Therefore, subsequently the Muller *et al.* (2013) TaqMan qPCR was used consistently through the 2012-13 to the 2016-17 Canadian WNS surveillance seasons with a cut-off Cq $\leq$ 35. During the 2017-18 WNS surveillance season, a new qPCR universal cycling protocol was validated by the AHL that used different cycle conditions, and it was adopted as their standard operating protocol in May 2018. While this new protocol has proven more sensitive, analysis demonstrated that a positive sample still crossed the threshold baseline with 35 amplification cycles (*i.e.,* cut-off Cq remained $\leq$ 35). The AHC began participating in Pd and WNS surveillance during the 2012-13 surveillance season by using the Muller *et al.* (2013) TaqMan qPCR on their platform beginning in April 2013. They have used this technique consistently from April 2013 – March 2018, but with a cut-off Cq $\leq$ 36. Very recently in April 2018, the AHC formally changed their cut-off Cq from $\leq$ 36 to $\leq$ 35, standardizing the interpretation of Pd qPCR results in Canada. Notably, the AHC's first positive Pd qPCR results only occurred in the 2017-18 Canadian WNS surveillance season, and all seven positive samples had Cq values < 32.4 which is significantly lower than the cut-off Cq of $\leq$ 36 that was in their standard operating protocol at the time. However, it is important to recognize that not all laboratories in North America using qPCR for detection of Pd interpret the Cq results in a similar conservative manner to the AHL and AHC because some may choose a higher cut-off Cq value for positive samples while others consider a specimen positive as long as the Cq is $\leq$ the terminal amplification cycle of the qPCR (*i.e.,* cut-off Cq $\leq$ 40).

CWHC NATIONAL OFFICE
Western College of Veterinary Medicine
52 Campus Dr, Saskatoon, SK S7N 5B4
PH. 1.306.966.5099 EM: info@cwhc-rcsf.ca

Canadian Interpretation of qPCR results for Pd

As mentioned previously, the interpretation of qPCR for Pd has been relatively consistent in Canadian diagnostic laboratories since the initiation of the WNS surveillance program. At both the AHL and AHC, a specimen's qPCR result for Pd has always been negative if Pd DNA is not detected within 40 amplification cycles. Irrespective of the protocol used at the AHL since the 2012-13 Canadian WNS Surveillance season, a positive Pd qPCR result is given for a specimen if the Cq $\leq$ 35 (*i.e.,* cut-off Cq $\leq$ 35). Additionally, as a conservative measure, the majority of positive samples with a Cq = 35 at the AHL were retested to confirm a Cq $\leq$ 35 prior to confirming them as positive for Pd. At the AHC, the interpretation of positive Pd qPCR results was similar from the 2012-13 through the 2017-18 Canadian WNS surveillance seasons, but with the higher cut-off Cq of $\leq$ 36 that was subsequently changed to a cut-off Cq of $\leq$ 35 in April 2018, standardizing the cut-off Cq value in Canada. Lastly, a specimen has always been considered suspicious/equivocal/inconclusive if it had a Cq > 35 but $\leq$ 40 consistently at the AHL and as of April 2018 at the AHC. At the AHC from April 2013 through March 2018 a specimen was considered suspicious/equivocal/inconclusive if it had a Cq > 36 but $\leq$ 40 based on the cut-off Cq $\leq$ 36 that was their standard at that time. At both Canadian laboratories, a specimen with suspicious/equivocal/inconclusive Cq results may receive additional testing to determine if it is actually positive, especially if it was taken from a species not previously diagnosed with WNS or a location not yet confirmed as Pd positive. Additional testing might include sequencing the qPCR product to confirm the presence of Pd DNA or retesting with qPCR for Pd in triplicate in which case the specimen would only be considered positive if one or more of the three runs had a positive Cq value (*i.e.,* $\leq$ 35) or negative if a Cq was not obtained within 40 cycles. If after retesting in triplicate the specimen remains as suspicious/equivocal/inconclusive, the jurisdiction responsible for submitting the specimen would be encouraged to sample more bats of that species or submit additional environmental samples from that site to establish a more definitive positive or negative result, but specimens remaining in the suspicious/equivocal/inconclusive category are not used to contribute to a positive or suspect status for a geographical area on the Canadian WNS surveillance map. Information on the diagnostic categories for reporting positive, suspect and negative cases of WNS and categories of detection for Pd, present or not detected, can be found in the Canadian Bat White-nose Syndrome Necropsy Protocol (CWHC, 2014 – http://www.cwhc-rcsf.ca/docs/Canadian%20Bat%20WNS%20Necropsy%20Protocol.pdf).

Management implications of diagnostic test interpretation

The CWHC and collaborating Canadian laboratories have maintained a standardized approach to the methodology and the interpretation of the qPCR results for Pd so wildlife managers and researchers in our country can have confidence in the results and use them to determine appropriate management strategies and thresholds for action. There are other laboratories in the U.S. that offer qPCR for Pd for research, WNS surveillance and environmental Pd detection purposes. However, it is important to recognize that the qPCR methodologies they use may not be consistent with the Canadian approach and the qPCR results for Pd from these laboratories are not necessarily interpreted in the same consistent manner as those from Canadian laboratories are. Therefore, individuals choosing to use these

CWHC NATIONAL OFFICE
Western College of Veterinary Medicine
52 Campus Dr, Saskatoon, SK S7N 5B4
PH. 1.306.966.5099 EM: info@cwhc-rcsf.ca

laboratories for WNS surveillance and Pd detection should seek to understand any differences in their qPCR methodology for Pd and interpretation of the results as compared to the standardized Canadian approach to qPCR for Pd. This recommendation is cautionary and made to ensure the CWHC's partners and collaborators do not implement resource-intensive management actions based on qPCR results for Pd not considered as reliable by our standardized approach to this diagnostic test. Currently, this seems especially true when qPCR results for Pd fall into the suspicious/equivocal/inconclusive category as there are many differing approaches to the interpretation of these results.

A standardized or harmonized diagnostic approach to qPCR for Pd

Recognizing the importance of standardizing or harmonizing diagnostic tests for the partners and collaborators of the CWHC, we believe establishing a network of laboratories for WNS surveillance and Pd detection testing is highly desirable so that managers understand that any laboratory participating in the network can be relied upon for a high degree of quality control and quality assurance when it comes to their test methodologies and diagnostic results. In other words, management decisions based on results from such laboratories can be made with confidence. To this end, the CWHC and the Canadian laboratories performing qPCR for Pd are collaborating with the United States Geological Survey, National Wildlife Health Centre to develop a network of laboratories that would participate in WNS surveillance and Pd detection using a standardized or harmonized diagnostic approach to the methodology of qPCR for Pd and interpretation of the test's results. There are also goals to quantify limits of detection for the current qPCR for Pd and understand the role that contamination might play in relation to suspicious/equivocal/inconclusive test results. This information will help develop appropriate test interpretation standards for those laboratories choosing to participate in the network.