SIMULTANEOUS DETECTION AND DISCRIMINATION OF ECHINOCOCCUS GRANULOSUS AND ECHINOCOCCUS MULTILOCULARIS USING REAL-TIME POLYMERASE CHAIN REACTION AND HIGH-RESOLUTION MELTING ANALYSIS

by

Kathryn Mary Olson

A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

in

Laboratory Medicine and Biomedical Science

Department of Pathology

The University of Utah

August 2016

The University of Utah Graduate School

STATEMENT OF THESIS APPROVAL

The thesis of **Kathryn Mary Olson**

has been approved by the following supervisory committee members:

Carl T. Wittwer	, Chair	**June 1, 2016** Date Approved
Robert Schlaberg	, Member	**June 1, 2016** Date Approved
Robert Palais	, Member	**June 1, 2016** Date Approved

and by **Peter E. Jensen** , Chair/Dean of

the Department/College/School of **Pathology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Echinococcosis is a zoonotic disease caused by the tapeworm *Echinococcus*. The two most common species are *E. granulosus* and *E. multilocularis*. They cause infections in humans called cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. Due to current epidemiological trends, there is a growing need for a sensitive and specific assay that can distinguish between the two infections. The purpose of this research was to design a multiplex PCR assay for serum that will be able to simultaneously identify and distinguish between *E. granulosus* and *E. multilocularis* via high-resolution melting analysis (HRMA).

A primer set was designed to amplify the mitochondrial ND5 gene of *E. multilocularis* and a previously designed and tested primer set was used to amplify a genomic repeat in *E. granulosus* known as EgG1 Hae III. Human DNA was used as the positive internal control along with previously designed primers targeting the CFTR gene. All templates and primer sets were combined into a multiplex reaction. Optimization was achieved by varying the primer concentrations to achieve equal amplification of all targets.

Serial dilution of all three templates was carried out. Each concentration of *Echinococcus* template was tested individually in combination with each concentration of control DNA to establish a limit of detection for each organism and an appropriate amount of control DNA to be used in the assay.

To eliminate bias from the interpretation of results, 20 blind samples were tested. Each consisted of one of four concentrations of either *Echinococcus* template or water. Results were reported as *E. multilocularis* positive, *E. granulosus* positive or negative. The samples were de-blinded and compared to the results obtained. Eighteen out of 20 results were identified correctly. The two samples that were not identified correctly were called negative, but had very low concentrations of either *E. granulosus* or *E. multilocularis* template.

Further research should be conducted to find a more suitable positive control due to its preferential amplification. However, this assay shows promise in its ability to detect very low levels of *Echinococcus* DNA and may have clinical use in the future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to thank the members of my thesis committee and especially Dr. Carl Wittwer for his expertise, guidance and patience. I would also like to thank the members of the Wittwer lab for their invaluable help and their willingness to share their knowledge.

A special thanks goes to my family for their unfailing support, understanding and encouragement.

INTRODUCTION

There are nine species of *Echinococcus*, but only four are currently known to cause disease in humans. The two most common are *E. granulosus*, which causes cystic echinococcosis (CE) and *E. multilocularis*, which causes alveolar echinococcosis (AE).[1-4] Both species require mammalian intermediate and definitive hosts to complete their lifecycles. In the case of *E. granulosus*, a number of species can serve as intermediate hosts, but most are ungulates such as moose, elk, cattle and sheep. The definitive hosts are canids such as wolves, foxes and dogs. With *E. multilocularis*, intermediate hosts are rodents. Definitive hosts are typically foxes, dogs and cats. Humans can serve as aberrant intermediate hosts for both species. When an intermediate host ingests an egg, it hatches in the stomach. The hatched egg, known as an oncosphere, penetrates the wall of the small intestine and finds access to the vascular system, which transports it into organs such as the liver and lungs. Although less common, they may be transported to other organs such as the brain, spleen or kidneys.[5] Once the oncosphere has been deposited into an organ, it will develop into the metacestode stage. The definitive host is infected when it ingests the organs of an infected intermediate host. The parasite will develop into the mature tapeworm once it has reached the intestine of the definitive host. The sexually mature tapeworm will produce eggs, which will be shed in the feces of the definitive host. The intermediate host is typically infected by ingesting food that has been contaminated with the feces of an infected definitive host.

The major difference between *E. granulosus* and *E. multilocularis* lies in how they each develop inside an intermediate host. For each, the metacestode stage involves the development of a germinal layer, from which the asexual production of multiple protoscoleces occurs via budding. Thousands of protoscoleces can potentially be produced, each with the ability to develop into a mature adult worm. When an *E. granulosus* oncosphere reaches its destination, it develops into the metacestode stage by producing a fluid-filled sac known as a hydatid cyst, which is why infection with *E. granulosus* is known as cystic echinococcosis or CE. Cysts may occur singly or in clusters. Daughter cysts may also develop inside primary cysts. When intact, cysts are essentially walled off from the host. Consequently, many infected individuals show no symptoms of infection and the spread of the parasite to different locations in the body is slow or nonexistent. *E. multilocularis* metacestode germinal layers develop very differently from that of *E. granulosus*. Instead of forming a fluid-filled cyst, the germinal layer forms solid protrusions that spread into surrounding tissues. Germinal cells can also detach and spread to other organs in a metastatic fashion. As the germinal layer proliferates, it produces small multiple vesicles, in which develop the protoscoleces. The small vesicle makeup of the germinal layer causes it to take on an alveolar structure, which is why infection with *E. multilocularis* is known as alveolar echinococcosis or AE.

The aberrant infection of humans with *Echinococcus* has become a major health concern worldwide. CE is common in communities where sheep herding is the prominent profession. Sheep ingest *E. granulosus* eggs by grazing in areas contaminated with feces of infected wild canids or herding dogs. When sheep are slaughtered or die, the uncooked internal organs or offal of the sheep are often fed to the herding dogs, in

turn infecting the dogs with the parasite. Shepherds can become infected by ingesting food or water that is contaminated with dog feces or by handling an infected host. AE is more common in areas with large wild fox populations. Foxes, dogs and cats are exposed when they consume an infected rodent. Humans are infected in much the same way as they are with CE, by consuming contaminated food or water and by handling infected hosts. Patients infected with CE may have no symptoms for many years. Enlarging cysts can cause symptoms such as abdominal pain, a palpable hepatic mass and obstruction of the biliary duct. If there are cysts present in the lungs, symptoms can include coughing, chest pain and asthma-like symptoms. A serious complication of CE is anaphylactic shock associated with a leaking or ruptured cyst. If not treated immediately, this can lead to rapid death.[6] The symptoms of AE are often nonspecific and include weight loss and right upper quadrant pain due to enlarged liver. Although AE infection proceeds like a slow-growing tumor, its course advances more quickly than that of CE. The morbidity rate of patients with untreated AE is greater than 90% within 10 years of infection, whereas CE is usually asymptomatic unless the cyst is ruptured, causing an infection or anaphylactic reaction.[1,4]

CE is present on all continents and is endemic to the Mediterranean, Middle East, central Asia, western China, South America and Africa.[3-5,7-14] The areas of highest endemicity are South America and western China, with reports of infection rates of up to 10% of the population.[4,7] One area of note is the Tibetan Plateau in China where rates of infection are on the rise and up to 6.8% of the population is reported to be infected with CE.[7,8] Neighboring central Asia has also reported infection rates as high as 3.4%.[9] Both regions have high populations of herding and farming communities, increasing the risk of

infection. The prevalence of infection in sheep and dogs of Tibetan communities has been reported to be up to 82% and 67%, respectively.[7,10] Despite the successful implementation of control programs in a few Chinese provinces, prevalence remains high in several regions, including the Tibetan plateau.[15]

AE is found almost exclusively in the northern hemisphere and is endemic to central Europe, China, central Asia, Eurasia and parts of North America.[4,5,7-14,16-18] A few cases of AE have also been documented in Turkey and Iran.[19,20] Recently, central Europe has seen an upswing in AE cases due to an unexpected surge in the population of red foxes, one of the primary hosts of *E. multilocularis*. This increase in population is causing foxes to move into urban areas, posing an even greater risk to humans. In addition to an increase in red foxes in central Europe, the population has spread to the North, West and especially to the East, now populating countries in the Baltic region and extending into central Asia. In the Naryn region of Kyrgyzstan, a recent study found a 65% *E. multilocularis* infection rate in wild foxes and an 18% infection rate in domestic dogs.[16,17,21] Human incidence has increased from a reported 0 – 3 cases in 1996 to more than 60 in 2011.[9] Despite the sharp increase in cases in central Asia, the number of cases of AE in western China, and especially the Tibetan plateau, is much higher. Tibetan foxes have been reported to have up to a 19% infection rate while up to 23% of dogs are infected. One study reported a human infection rate as high as 6.2% in western China in 2005, making it one of the most endemic regions in the world.[8]

As shown, both CE and AE are endemic to central Asia and western China. Co-infection has even been reported in some patients on the Tibetan plateau.[10,14,22] Both *E. granulosus* and *E. multilocularis* have been found in the fox populations of central Asia

and western China.[7] The population surge and subsequent spread of the red fox throughout Europe and central Asia is resulting in an increase of both forms of echinococcosis in humans. It also raises the question as to whether CE will spread west into Europe through the migration of the red fox between these areas. The World Health Organization recognizes both AE and CE as neglected tropical diseases.[4] Continued neglect of echinococcosis in Europe and Asia may result in the increase of both CE and AE in these areas.

The sensitivity and specificity of current methods of diagnosis of both CE and AE are highly variable. Currently, the most common method of diagnosis is by a combination of ultrasound and serologic or immunologic testing. The gold standard for the diagnosis or confirmation of echinococcosis is ultrasound.[1,23] It has a sensitivity of 90% – 95%, is easy to perform and is relatively inexpensive. This makes portable ultrasound machines useful in population screening. The hydatid cysts of CE appear as fluid-filled sacks. Separation or inner folding of the hydatid membrane from the cyst wall can be seen as well as hydatid sand, which is composed of protoscoleces and hooklets of the parasites. AE lesions appear as irregular, tumor-like shapes that lack a well-defined boundary. There is generally an area of central necrosis and there may be areas of calcification in the walls.[1] Even with the high sensitivity of ultrasound, however, hydatid cysts may be misdiagnosed as things like pseudocysts if located in the pancreas. They may also be mistaken for lipomas, ovarian cysts or hepatic hematomas. AE lesions can be misdiagnosed as cancerous tumors.[1,23] Another type of imaging that has an even higher sensitivity than ultrasound at 95% - 100% is computed tomography or CT.[20] Although CT is useful for urban areas, it is impractical for more rural areas and for

population screening due to inaccessibility and cost. Imaging, especially ultrasound, is generally confirmed by serological testing such as ELISA, indirect hemagglutination assay (IHA) or Immunoblot. One option for the confirmation of CE is biopsy. Fluid can be aspirated from potential hydatid cysts to look for the presence of structures such as hooklets and protoscoleces. However, there is a risk of causing leakage when the cyst wall is penetrated, which can cause anaphylaxis and a spread of the infection.[1,6]

ELISA is considered the most specific and sensitive of serologic tests for CE and AE. It has been reported to have a sensitivity as high as 96.7% and a specificity of up to 97.5%.[23] Dot-ELISA used in field-testing in two studies showed a sensitivity and specificity of between 96% - 100% and 89.1% - 98%, respectively.[1,23] IHA and immunoblot are also popular serologic tests for the diagnosis of AE and CE, although sensitivity and specificity are below that of ELISA. Despite the success of some assays to accurately diagnose CE and AE, results are inconsistent. Immunodiagnosis displays a high rate of false negative and false positive results. It has been suggested that this is due to lack of standardization, especially when it comes to the source of antigen used.[1] Reiter-Owona et al. found that with both CE and AE, high antibody titers were necessary to achieve good detection. They reported ELISA false negatives in 30.3% of patients, most of them coming from patients with low antibody titers.[24] Another study reported 15% false negatives by ELISA.[25] These false negatives may be due in part to patients having intact cysts or cysts that are small, in extrahepatic locations or calcified. A high rate of false positive results have also occurred from cross-reactions when patients are infected with other parasites, especially cestodes, and also in healthy subjects.[23] Cross-reactivity between *E. multilocularis* and *E. granulosus* has also been reported due to the

similarity of their antigens.[20,24,26] It has also been shown that sensitivity and specificity tend to be better in clinical settings as opposed to field settings, bringing into question the utility of serological tests for population screening.

The most common source for antigens used in serological testing for CE is hydatid cyst fluid (HCF), which is obtained by extraction from surgically removed hydatid cysts. HCF is a mixture of host and parasite. Sensitivity using crude, unpurified HCF is good but specificity varies from 30% - 90%. Better results are obtained when specific antigens are purified from HCF. One of the most studied antigens from *E. granulosus* is antigen 5 (Ag5), despite the fact that it has a high rate of cross-reactivity with healthy controls or non-CE patients. Some studies show that purified and enriched Ag5 performs much better, but no large studies confirm this.[23] Another highly studied antigen is antigen B (AgB), which is the main antigen found in HCF. It is highly immunogenic, which makes it a good candidate for diagnosing patients with lower titers of Ag5. Although it is more specific than Ag5, it has high genetic variability. The quality of AgB is important and probably explains the high variability in the sensitivity and specificity of AgB-based immune testing. It is specific for echinococcal infections but not very specific to *E. granulosus* as opposed to *E. multilocularis*, which produces its own AgB.[1,23] Mamuti et al. found that a subunit of AgB obtained from each *E. multilocularis* and *E. granulosus* cross-reacted with both species on a Western blot. The *E. multilocularis* AgB subunit produced reactions in 81% of CE patients and 40.6% of AE patients. The *E. granulosus* AgB subunit produced reactions in 86% of CE patients and 42% of AE patients.[26] Given this level of cross-reactivity and lack of specificity, AgB based testing would not give reliable results in areas with co-endemic CE and AE.

Serologic tests achieve better results for AE than CE. Sensitivity and specificity for AE testing have been reported to be between 95 – 100%. This is due to the nature of AE infection and the way it spreads. Common antigens used to test for AE are Em2 and Em10, and occasionally Em18. Some studies have reported that Em2 ELISAs, which are the most commonly used tests for AE, can differentiate between AE and CE, but Reiter-Owana et al. showed that Em2 ELISA cross-reacted with CE in 23.5% of patients and that Em10 ELISA produced no cross-reactivity. Although Em2-based testing is likely to identify AE infection, like serological CE tests, it would not be a reliable test to distinguish between AE and CE in areas where the population is at risk for both infections.

Using serological tests to evaluate patients for continued disease or relapse after treatment is problematic. Anti-CE antibodies may remain in serum for several years after treatment, making them unsuitable to evaluate possible relapse. Although testing for the presence of antigens in serum instead of antibodies is much less sensitive and is rarely used for diagnosis, it has been suggested that it may be a better option in postsurgical monitoring since the presence of antigens is more conclusive than lingering antibodies.[23] However, for CE, detection of antigens even with a known active infection is too insensitive for antigen testing to be reliable. Serological testing that distinguishes between subclasses of antibodies may help with distinguishing between active and past disease. IgE, IgG1 and IgG4 have been shown to be associated with active infection.[27] It has also been shown that igG2 is a good marker to indicate active infection and total IgG better for detection of relapse.[28] Antibodies to *E. granulosus* proteins may remain in circulation for less time. One of these proteins is P29, which only remained in circulation

in 10% of patients after three years vs. 25% for HCF antibodies. However, P29 is not very reliable for initial diagnosis, so these results may be questionable.[23] For AE, Em18 or Em2 ELISA may be useful for postsurgical follow-up, since negative results are often achieved within four years of surgery and become positive again in cases of relapse.[1] However, given that Em2 can cross-react with CE, this may not be ideal in areas where a patient is in remission for AE, but has the possibility of acquiring CE.

Another option for the diagnosis of echinococcosis is by using polymerase chain reaction (PCR)-based assays. PCR has been used for coprodiagnosis and differentiation of *Echinococcus* and other parasites in canid hosts with reports of 100% specificity.[29,30] Tissue and cystic samples have also been subjected to PCR in order identify the species via genotyping. One study used PCR and restriction fragment length polymorphism (RFLP) method to identify dual infection of *E. multilocularis* and *E. granulosus* in animal hosts using tissue samples from infected definitive hosts. The study was able to differentiate between the two species due to polymorphisms in the large ribosomal subunit in mitochondrial DNA. This was based on fragment length via electrophoresis after digestion of PCR products with enzymes.[31] There has also been success in genotyping using different genomic or mitochondrial DNA targets and electrophoresis.[29,30,32] Other studies have used PCR combined with high-resolution melting analysis (HRMA) in order to distinguish between different species of *Echinococcus*.[33,34] These studies have almost exclusively used tissue samples from intermediate hosts or fecal samples from definitive hosts. As previously stated, biopsy from cysts or the disruption of infected tissue may result in an anaphylactic reaction or the spread of the infection, making these testing methods impractical for routine use on

patients.

Very few studies have used PCR-based methods on serum samples in order to detect *Echinococcus* and those that have show varying degrees of success. One study attempted to detect *E. granulosus* in the serum of patients who had been confirmed positive for CE after surgical removal of cysts.[32] It showed moderate success in detection with 75% sensitivity, with a specificity of 100%. Another study used serum and urine samples from either surgically or ultrasonographically confirmed CE-positive patients in an attempt to diagnose CE.[35] They found that only 50% of patients who were surgically confirmed to have CE tested positive via PCR when using serum and none of the patients who did not have surgery tested positive. *E. granulosus* was also not detected in any of the urine samples via PCR. Due to the nature of CE infection, it is not surprising that it is difficult to detect in serum. Because it is walled off from the host, very little genetic material is likely to be circulating in a patient's blood. It is also noteworthy that in the study conducted by Chaya et al., the only patients who produced a positive result from serum were those that had cysts surgically removed. There would certainly be disruption of the parasitic tissue during removal, making it more likely that it would be released into the patient, whereas those who did not have surgery tested negative 100% of the time. Serum testing to detect *E. multilocularis* has almost exclusively used serologic testing since it is a reliable method with a high sensitivity. However, due to the growing problem of co-endmicity, it is becoming increasingly necessary to find a test that can distinguish between *E. granulosus* and *E. multilocularis*. Since serologic tests have shown high levels of cross-reactivity between the two organisms, a PCR-based test would be ideal to distinguish between them. The life cycle

of *E. multilocularis* means that there would likely be circulating parasitic DNA in the blood of patients. It is likely, therefore, that it would not be difficult to detect AE infection with a PCR-based serum assay. This is not the case with CE infection. If there is any parasitic DNA circulating, it would probably be in very small amounts. The specificity of PCR assays used to genotype *Echinococcus* species is almost always near 100%, which is the area that serologic tests are lacking. Where PCR-based assays are currently lacking, especially when it comes to *E. granulosus*, is sensitivity.

In order to design an assay that can detect low levels of circulating DNA, it is important to choose an appropriate genetic target. Ideally, this would be a target that occurs in many copies throughout the genome of the parasite. For this reason, mitochondrial DNA, at between 1000 – 2000 copies per cell, is almost always the target when genotyping *Echinococcus* species.[36] Mitochondrial genes that are the most conserved and which have been targeted most often include NADH dehydrogenase subunits one and five (ND1, ND5), cytochrome c oxidase subunit one (COX1) and the 12S subunit of the mitochondrial ribosome.[29,32,35,37] While the targeting of a mitochondrial gene would most likely be adequate for the detection of *E. multilocularis*, that may not be the case for *E. granulosus*, as shown by the negative results found in the study conducted by Chaya et al. Another possible target is a 269 basepair-long genomic repeat known as EgG1 Hae III.[30] This repeat is estimated to occur approximately 6900 times in the *E. granulosus* genome, which is estimated to be 150 million basepairs long. This means there is a greater chance of detecting this repeat than there would be of detecting mitochondrial DNA from the same amount of circulating parasitic cells.

In addition to choosing an appropriate target, it is essential to have a simple assay

design if it is to be useful in field settings. Because infected individuals are often in rural areas, designing an assay that involved the need for a high skill level or expensive and complicated equipment would be impractical. This would make it wise to eliminate methods that involved the need for electrophoresis or specialized probes. The simplest method would be to use an intercalating dye and perform genotyping based on the differences in melting temperatures. To simplify the testing process as much as possible, it is also essential that the assay be able to detect and differentiate between *E. granulosus* and *E. multilocularis* simultaneously, which would require a multiplex design. The advantage to using a molecular assay to detect *Echinococcus* is that specificity is nearly 100%; the challenge is to increase the sensitivity. While efforts continue to make serological testing more specific, the answer to finding an effective means of diagnosis and monitoring of AE and CE may not lie with serological testing, but with a more sensitive PCR-based assay.

MATERIALS AND METHODS

The first phase of the research plan involved choosing appropriate genetic targets and designing primer pairs that would successfully amplify the targets using real-time PCR. The amplified sequences also needed to have different and distinct melting temperatures or T_m, which is the temperature at which half the double-stranded DNA has become denatured into single strands. These different melting temperatures would be used to identify the targets in a multiplex reaction using high-resolution melting analysis (HRMA). Targets for *E. multilocularis* and *E. granulosus* were chosen from regions of mitochondrial or genomic DNA that would be unique to each organism. Candidate targets were checked in BLAST® (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for similarity between the two organisms as well as human DNA. The chosen targets were found to have no matches in the human genome or that of the other parasite. Due to lack of available parasitic tissue or extracted DNA, gBlocks® Gene Fragments (Integrated DNA Technologies) synthetic DNA was used as parasite DNA. Targets were designed using the published sequences in GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) or from previously conducted research.[30] Primers were synthesized by the DNA/Peptide Facility, part of the Health Sciences Center Cores at the University of Utah. Target sequences and primers were received lyophilized. After reconsititution, concentrations were determined and adjusted with the NanoDrop 2000 spectrophotometer (Thermo Scientific) and LabVIEW 7.1 (National Instruments) designed program CHECKOLIGO version 4. All

primer candidates were tested individually via PCR to determine the success of target amplification and suitable melting temperature. After the chosen targets and the primer pairs were individually tested, the three sets of primers were checked for the possibility of primer dimer formation during the multiplex reaction using Multiple Primer Analyzer software (ThermoFisher Scientific). Primer dimer formation was found to be unlikely or low-grade, which confirmed the chosen primers as suitable for the multiplex reaction.

The second phase consisted of all primer pairs and template DNA being combined into a multiplex reaction. In order to optimize the multiplex reaction, template DNA for each organism and the control were each added at 14,000 copies/µl. Primer concentrations were adjusted to achieve an optimized amplification of all targets. All amplicon melting conducted in preliminary primer testing and multiplex optimization was done on the LightCycler® 2.0 (Roche).

The third phase was to systematically reduce the concentration of template copies of each organism in order to establish a limit of detection. This was accomplished by tenfold serial dilution of template copies of *E. multilocularis* and *E. granulosus.* Each concentration was tested individually in combination with the control DNA. The control DNA template was then diluted in the same manner to establish if high copy numbers of control DNA would wash out low-level detection of either or both organisms. The goal of varying the quantity of control DNA was to find a concentration that would be sufficient to serve as a reliable internal control but not so high as to prevent the detection of low levels of *E. multilocularis* or *E. granulosus*. HRMA was done on all reactions conducted in the dilution series on the HR-1 analyzer (BioFire Defense).

The fourth phase was to conduct 20 blind tests. When the amount of template

DNA in each reaction was known and specific peaks were being looked for, there was a possibility that it could bias the interpretations of the HRMA results. The blind tests were conducted to eliminate that bias. Each randomly chosen template DNA sample was de-identified and assigned a number 1 – 20. Each contained 14 copies/µl, 140 copies/µl, 1400 copies/µl or 14,000 copies/µl of *E. multilocularis* or *E. granulosus* DNA templates or water. Each was tested in triplicate. Samples 1 – 10 were tested with control DNA at 140 copies/µl. Samples 11 – 20 were tested with control DNA at 14 copies/µl. Samples 1 – 10 with ambiguous results were repeated singly with control DNA reduced to 14 copies/µl. Samples 11 – 20 with ambiguous results were repeated singly with no control DNA added. The PCR reaction and HRMA were conducted and results were reported as positive for *E. multilocularis*, positive for *E. granulosus* or negative (only control DNA present). Some results were also reported as positive or negative, but with a desire to retest due to ambiguous results.

Primer Design

E. multilocularis

The target for *E. multilocularis* was the mitochondrial gene, ND5, which has distinct differences from the ND5 gene of *E. granulosus*. Three primer sets were designed using the Primer3 software at http://bioinfo.ut.ee/primer3-0.4.0/. Primer set 1, with a forward sequence of 5'-TGGTAGTGGTGGTTCTCAAGC-3' and a reverse sequence of 5'-AACCAAGGACCACAGCAAAC-3', produced a 94-basepair amplicon with a melting temperature of 79.3°C. Primer set 2, with a forward sequence of 5'-GGG CCCCTACTCCAGTTAGT-3' and a reverse sequence of 5'-ACCAAACACCAGCAGC AACT-3', produced a 63-basepair amplicon with a melting temperature of 81.3°C.

Primer set 3, with a forward sequence of 5'-GTTAGTATCGTCTCGATTTG-3' and a reverse sequence of 5'-GGAAATACCCCACTATCC-3' produced an 81-basepair amplicon with a melting temperature of 79.1°C. Primer set 1 was chosen for inclusion in the multiplex reaction due to its melting temperature and strong performance during initial testing (Figure 1).

E. granulosus

The target for *E. granulosus* was a repeated genomic sequence known as EgG1 Hae III. This repeat is estimated to constitute approximately 1.25% of the *E. granulosus* genome, making it a more abundant target than mitochondrial DNA sequences. A primer set (EGG1) designed by Abbasi et al., with a forward sequence of 5'-GAATGCAAGCA GCAGATG-3' and a reverse sequence of 5'-GAGATGAGTGAGAAGGAGTG-3', was tested and performed well.[30] It produces a 133-basepair amplicon with a melting temperature of 85.8°C (Figure 1).

Control DNA

Human DNA from a single donor and previously designed primers targeting exons 3 and 10 on the cystic fibrosis transmembrane conductance regulator (CFTR) gene were tested for use as internal controls based on their projected melting temperatures.[38,39] Primers targeting exon 3, with a forward sequence of 5'-TTTGCACATGCAACTTATTG G-3' and a reverse sequence of 5'-CAAATGAGATCCTTACCCCTAAA-3', produced a 160-basepair amplicon with a melting temperature of 82.2°C. Primers targeting exon 10, with a forward sequence of 5'-ACTTCTAATGGTGATTATGGG-3' and a reverse sequence of 5'-ACATAGTTTCTTACCTCTTC-3', produced a 201- basepair amplicon

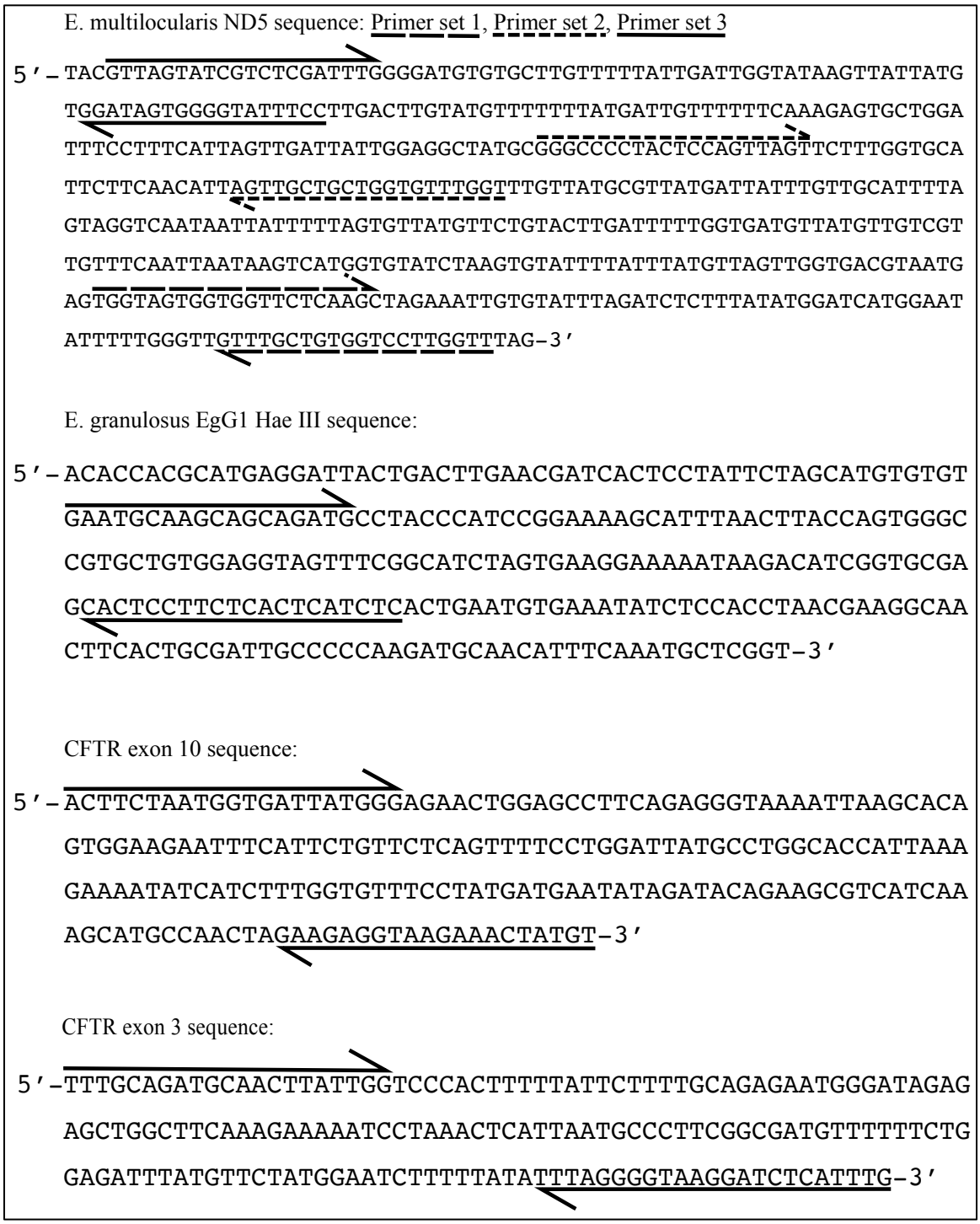


Figure 1. All templates and primer sets tested for *E. multilocularis*, *E. granulosus* and the human CFTR gene.

with a melting temperature of 82.3°C. Both performed well in preliminary testing, but the second primer set performed better in multiplex testing, so it was chosen for use in the multiplex reaction (Figure 1).

Multiplex Optimization

In order to optimize the multiplex reaction, template DNA for each organism and the control were added in equal amounts at 14,000 copies/µl. All templates and primer pairs were individually evaluated with forward and reverse primer concentrations at 0.5 µmol/L. Therefore, the multiplex reaction was first tested with all primer concentrations set to 0.5 µmol/L. Optimized amplification of all targets was achieved by making adjustments to primer concentrations. The goal was to achieve strong distinction between melting peaks and avoid unbalanced fluorescence intensity between the three peaks. Primer concentrations producing the best results were *E. multilocularis* at 0.1 µmol/L, Control at 0.5 µmol/L and *E. granulosus* at 0.25 µmol/L.

PCR Mixture

The multiplex reaction mixture in 10 µl volumes with optimized primer concentrations included 0.1 µmol/L forward and reverse *E. multilocularis* primers, 0.5 µmol/L forward and reverse control primers, 0.25 µmol/L forward and reverse *E. granulosus* primers, 0.4 U KlenTaq1™ (Ab Peptides), 3 mmol/L $MgCl_2$, 50 mmol/L Tris (pH 8.3) and 2.5 mg/L BSA (Sigma). Also included were 1X LCGreen® Plus dye (BioFire Defense), 0.2 mmol/L each deoxynucleoside triphosphate and 14,000 copies/µl of each template DNA. The multiplex reaction mixture was used for all reactions in the dilution series and blind testing with varying numbers of template DNA added.

Reactions for individual primer testing used the same reaction mixture except only primer sets being tested were included and at a concentration of 0.5 μmol/L.

PCR Amplification and Preliminary Melting Protocol

PCR was performed in closed capillary tubes in a LightCycler® 2.0 with an initial denaturation of 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 0 s, annealing at 58°C for 4 s and extension at 72°C for 4 s. Transition rate between temperatures was 20°C/s and fluorescence was acquired at the end of each extension step. Melting for preliminary primer set and multiplex optimization was achieved by denaturing PCR products at 95°C for 0 s, annealing at 70°C for 10 s, then raising the temperature to 95°C at a transition rate of 0.2°C/s with continuous fluorescence monitoring.

Dilution Protocol

Template DNA for *E. granulosus*, *E. multilocularis* and control were each serially diluted from 14,000 copies/μl to 1400 copies/μl, 140 copies/μl and 14 copies/μl. *E. granulosus* and *E. multilocularis* templates were tested individually at each of the four concentrations with each of the four concentrations of the control DNA. HRMA was done on each reaction with 1400 copies/μl or less of control DNA.

High-Resolution Melting

Following amplification on the LightCycler® II, the multiplex reaction with final primer concentrations and each reaction in the dilution series was subjected to high-resolution melting in the HR-1 analyzer. Melting was started at 65°C and the temperature was increased to 90°C at a transition rate of 0.3°C/s. LED power was

automatically adjusted to 90% fluorescence.

Data Analysis

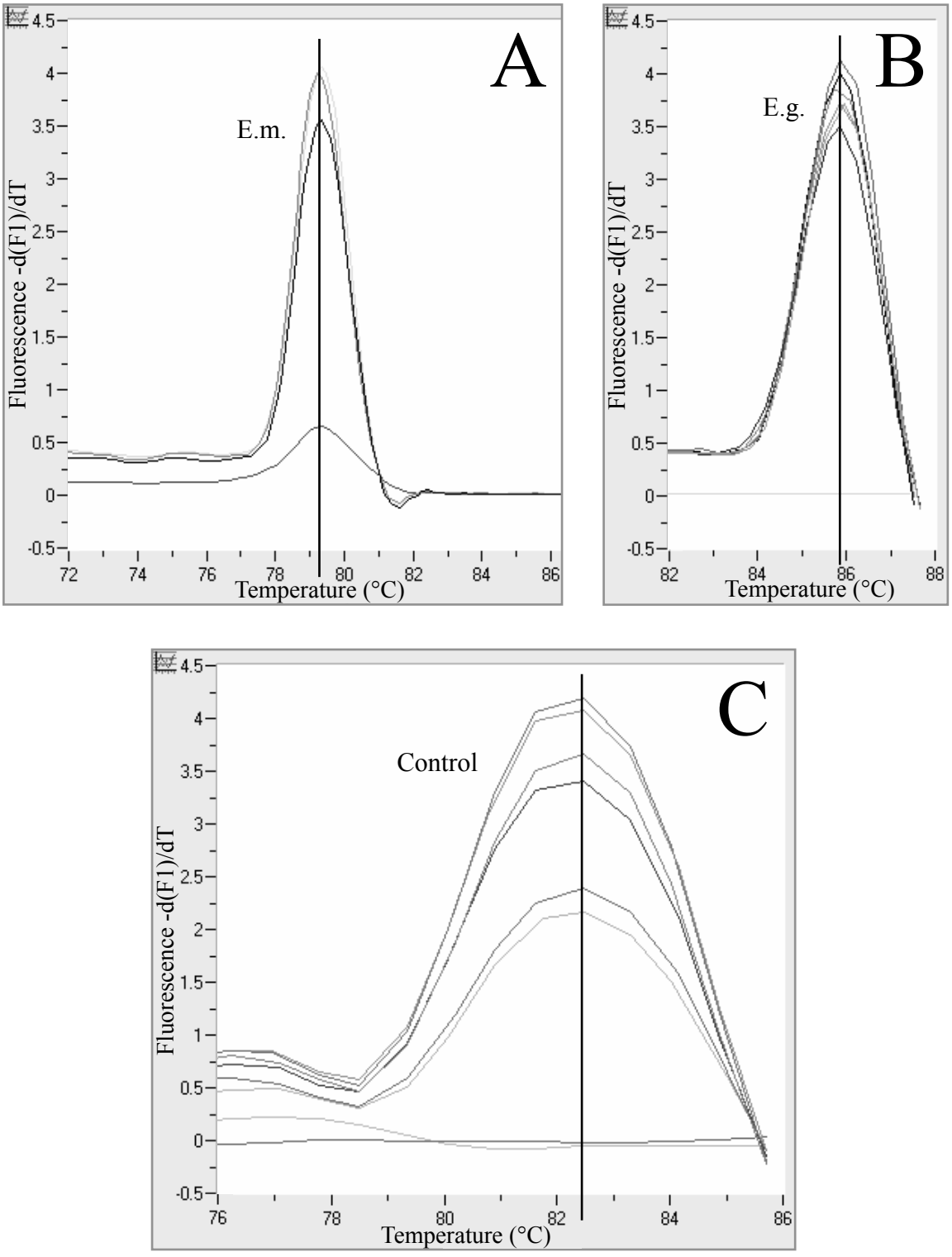
High-resolution melts were analyzed with MeltingWizard6 software (University of Utah). Each melt was converted into a negative first derivative curve and displayed as the negative first derivative of fluorescence with respect to temperature vs. temperature in the form of melting peaks. Each melting peak plot from the dilution series and blind samples was compared to that of the multiplex reaction to determine if there was detectable amplification of either or both organisms or the control DNA.

RESULTS

After combining the three DNA templates and the chosen primer sets into a multiplex reaction and comparing melting curves obtained on the HR-1, the melting temperatures increased for all three template/primer pairs as compared to when they were tested individually. *E. multilocularis* increased from 79.3°C to 80.2°C, *E. granulosus* increased from 85.8°C to 86.7°C and the control increased from 82.3°C to 82.8°C. When the reactions included either *E. multilocularis* or *E. granulosus* and control templates, melting temperatures decreased back down close to original melting temperatures when each template was evaluated alone on the LightCycler 2.0 (Figure 2). These observations were used during blind testing to determine if template DNA for either organism was present.

When templates of *E. multilocularis* or *E. granulosus* were used in 1400 copies/µl, 140 copies/µl and 14 copies/µl concentrations, it became quickly apparent that leaving the control DNA template concentration at 14,000 copies/µl would wash out any low-level detection of *E. multilocularis* or *E. granulosus*. Concentrations of 1400 copies/µl, 140 copies/µl and 14 copies/µl of the control DNA were tested with each of the four concentrations of *E. multilocularis* and *E. granulosus* template and HRMA was conducted.

Figure 2. Illustrates the shift in melting temperatures of the three DNA templates when run alone vs. in combination. (A) The melting peak of *E. multilocularis* when run alone. (B) The melting peak of *E. granulosus* when run alone. (C) The melting peak of the control DNA when run alone. (D) The melting peaks of *E. multilocularis* and the control DNA when run together. (E) The melting peaks of *E. granulosus* and the control DNA when run together. (F) The melting peaks of all three templates when combined in a multiplex reaction. A – C produced on the LightCycler 2.0; D – F produced on the HR-1.


A
E.m.
Fluorescence -d(F1)/dT
Temperature (°C)
B
E.g.
Fluorescence -d(F1)/dT
Temperature (°C)
C
Control
Fluorescence -d(F1)/dT
Temperature (°C)

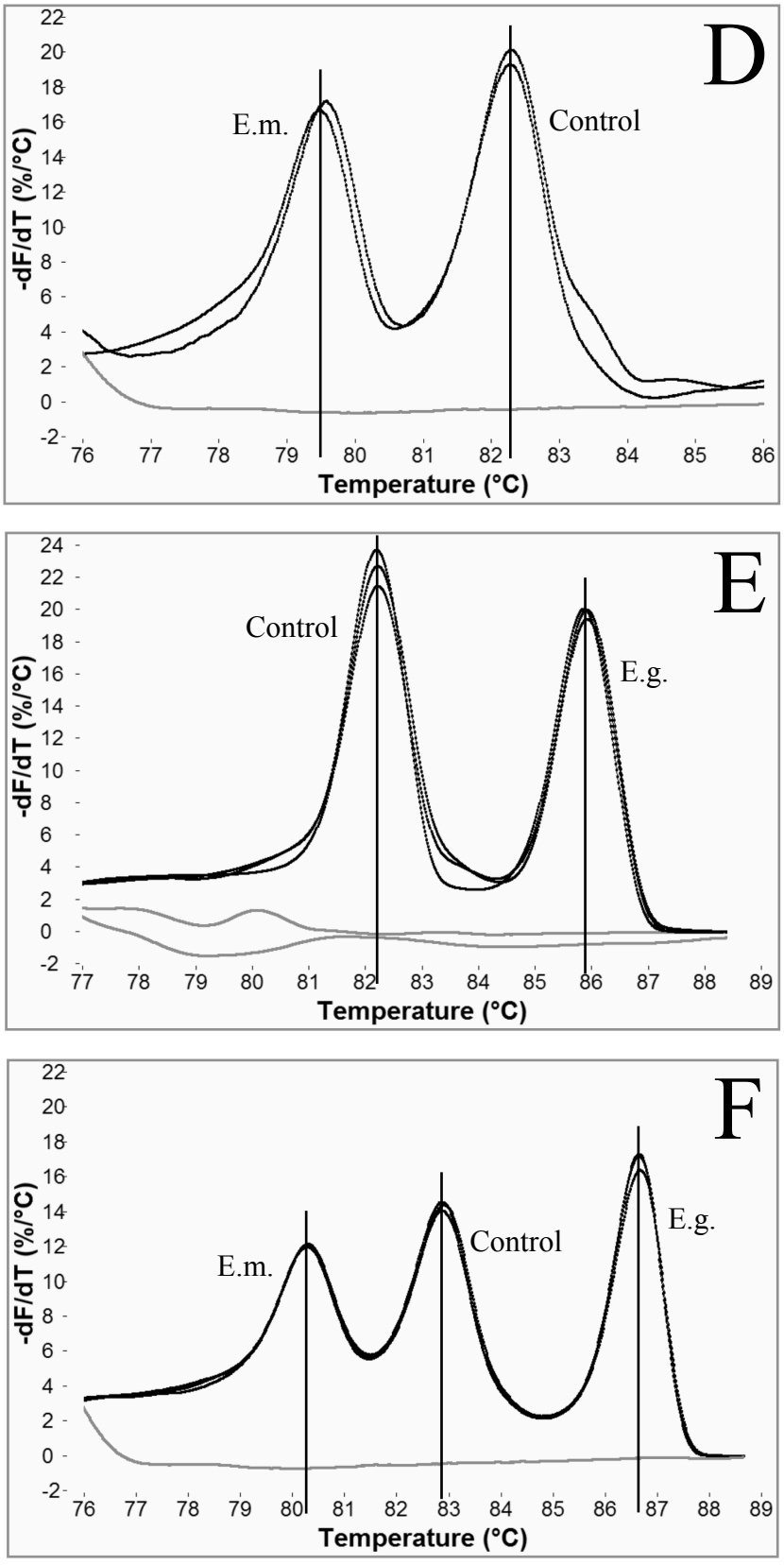


Figure 2. continued.

Dilution Series Results

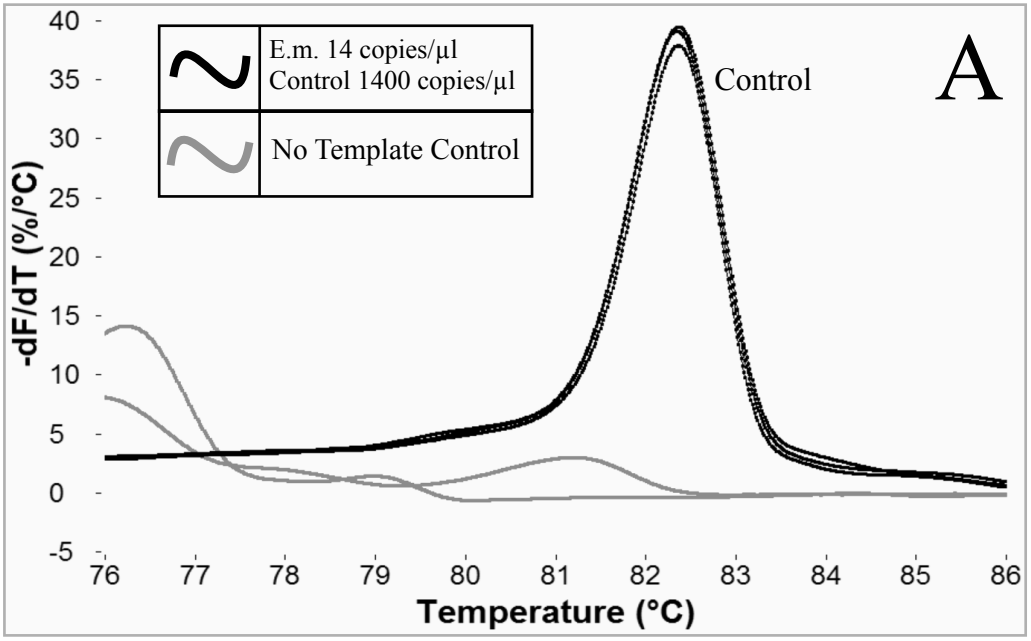
Control DNA at 1400 copies/μl

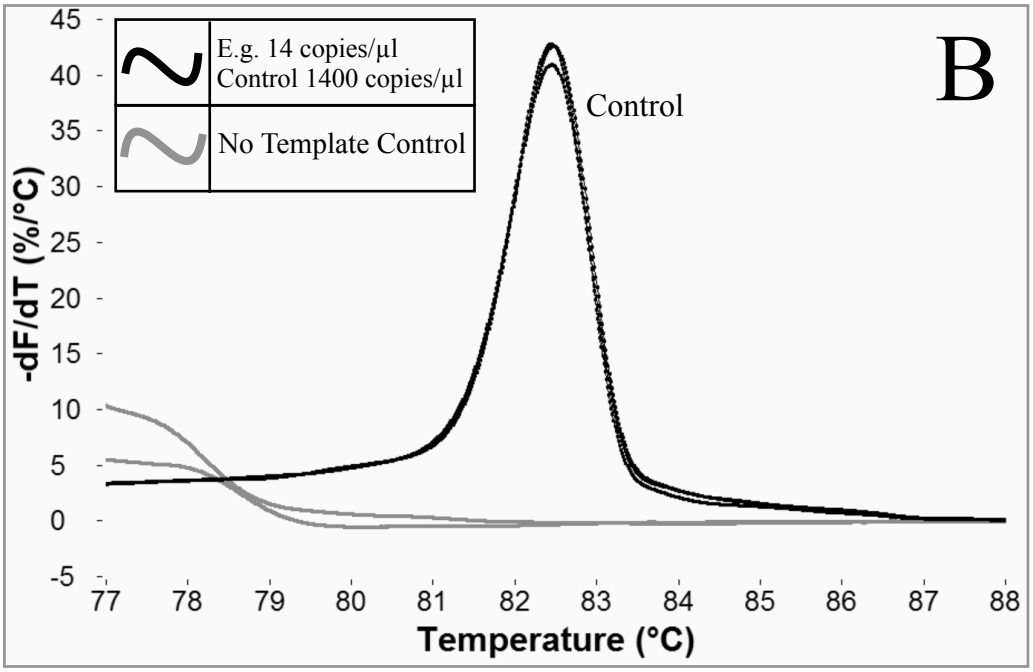
With control DNA concentrations at 1400 copies/μl, there was no detection of *E. multilocularis* or *E. granulosus* at 14 copies/μl. When copy numbers of *E. multilocularis* and *E. granulosus* were increased to 140 copies/μl, there was only very weak detection of both. When copy numbers of *E. multilocularis* and *E. granulosus* were increased to 1400 copies/μl, there was positive detection of both, but the control DNA peak was much higher than that of either. When *E. multilocularis* concentration was increased to 14,000 copies/μl, there was clear detection, but the fluorescence of the control DNA peak was still much higher. With *E. granulosus* at 14,000 copies/μl, its peak and the control DNA peak were nearly equal in height (Figure 3).

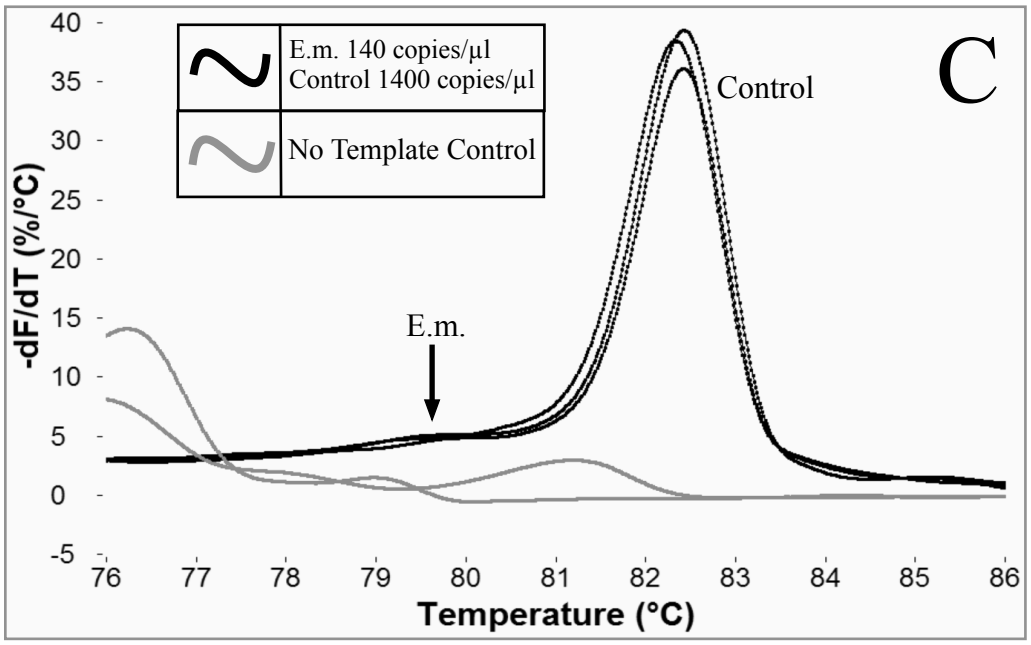
Control DNA at 140 copies/μl

With control DNA concentrations at 140 copies/μl, there was weak positive detection of both *E. multilocularis* and *E. granulosus* at concentrations of 14 copies/μl. There was positive detection of *E. multilocularis* when increased to 140 copies/μl, but with a much lower peak than that of the control. There was positive detection of *E. granulosus* at 140 copies/μl and with only a slightly higher control peak. When copy numbers of *E. multilocularis* were increased to 1400 copies/μl, there was strong positive detection, but with a slightly lower melting peak than the control. There was also strong positive detection of *E. granulosus* at 1400 copies/μl, with its melting peak and that of the control at near equal heights. When copy numbers of *E. multilocularis* were increased to 14,000 copies/μl, there was strong positive detection with its melting peak

Figure 3. High-resolution melting curves from the dilution series with the control DNA template concentration at 1400 copies/µl. (A) *E. multilocularis* at 14 copies/µl. (B) *E. granulosus* at 14 copies/µl. (C) *E. multilocularis* at 140 copies/µl. (D) *E. multilocularis* at 140 copies/µl. (E) *E. multilocularis* at 1400 copies/µl. (F) *E. granulosus* at 1400 copies/µl. (G) *E. multilocularis* at 14,000 copies/µl. (H) *E. granulosus* at 14,000 copies /ul.


A
E.m. 14 copies/µl
Control 1400 copies/µl
No Template Control
Control
-dF/dT (%/°C)
Temperature (°C)
40
35
30
25
20
15
10
5
0
-5
76
77
78
79
80
81
82
83
84
85
86


B
E.g. 14 copies/µl
Control 1400 copies/µl
No Template Control
Control
-dF/dT (%/°C)
Temperature (°C)
45
40
35
30
25
20
15
10
5
0
-5
77
78
79
80
81
82
83
84
85
86
87
88



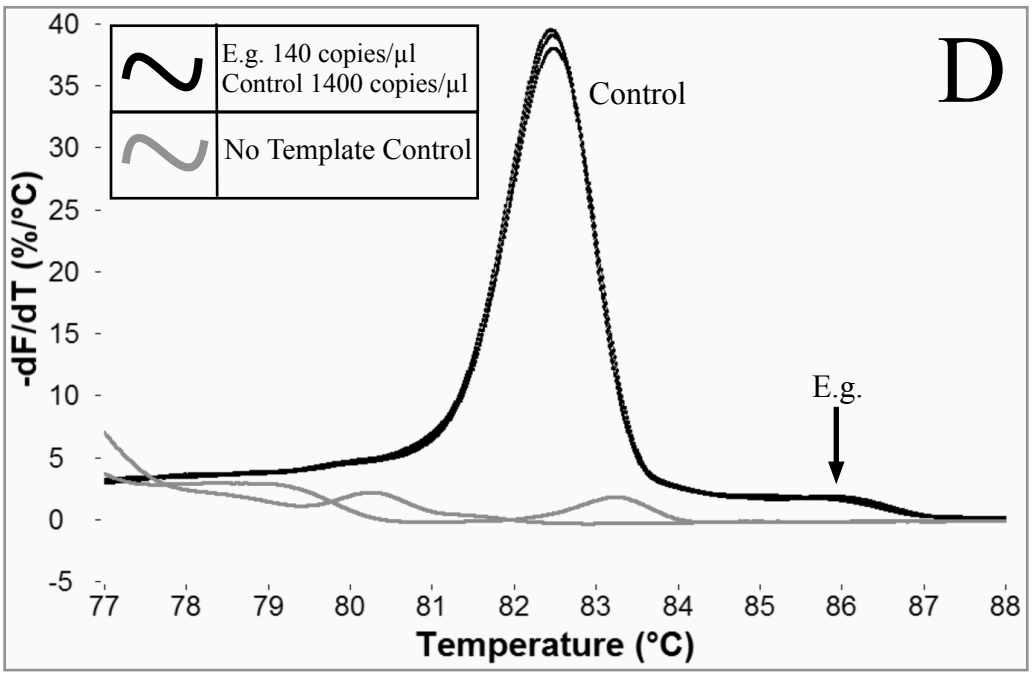
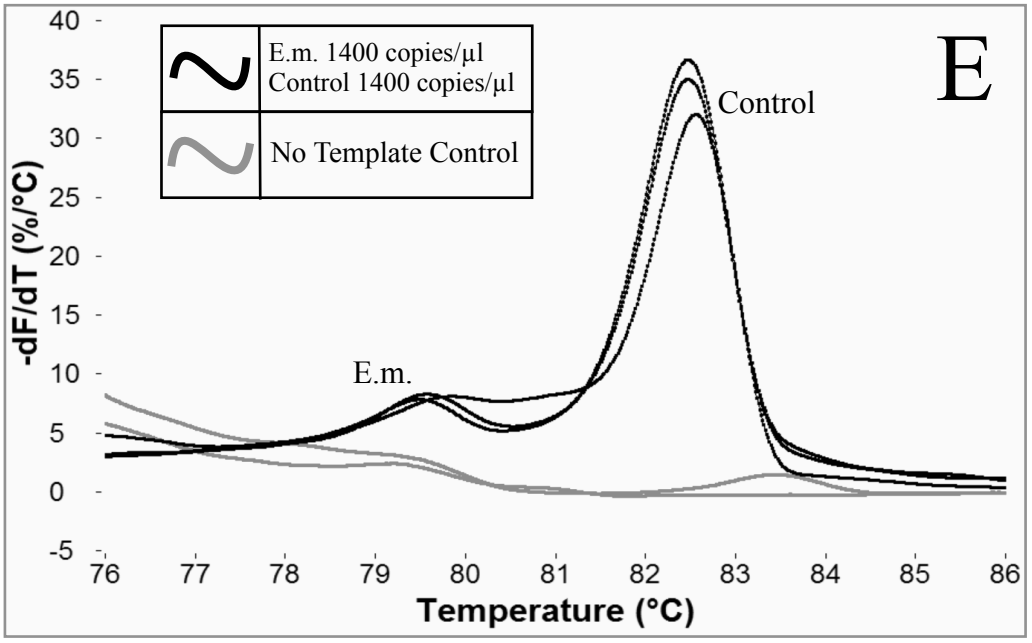


Figure 3. continued.



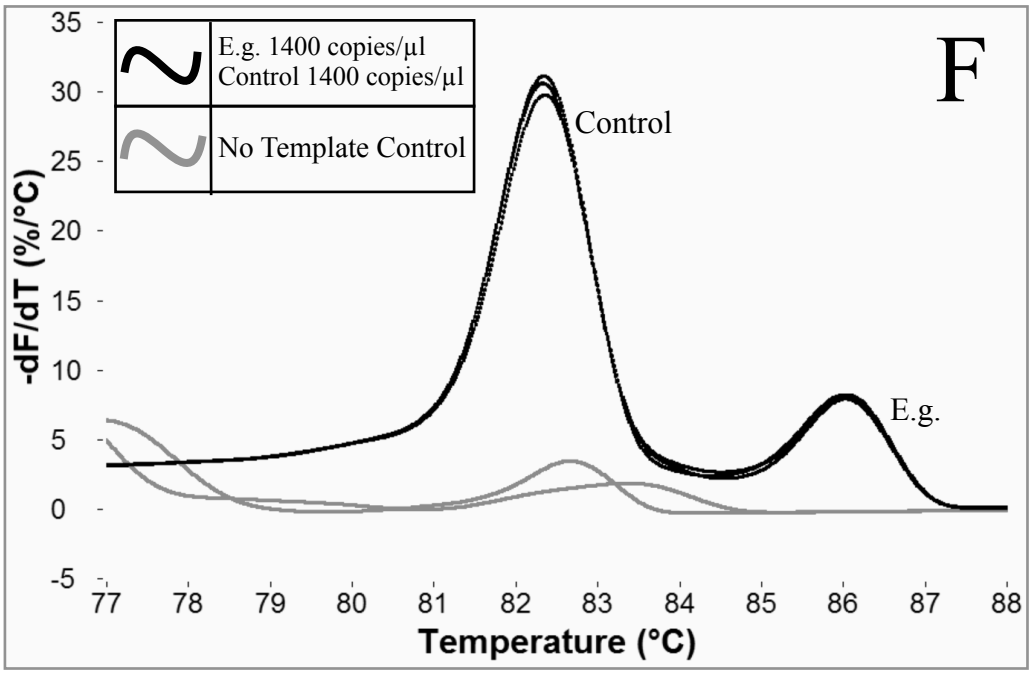
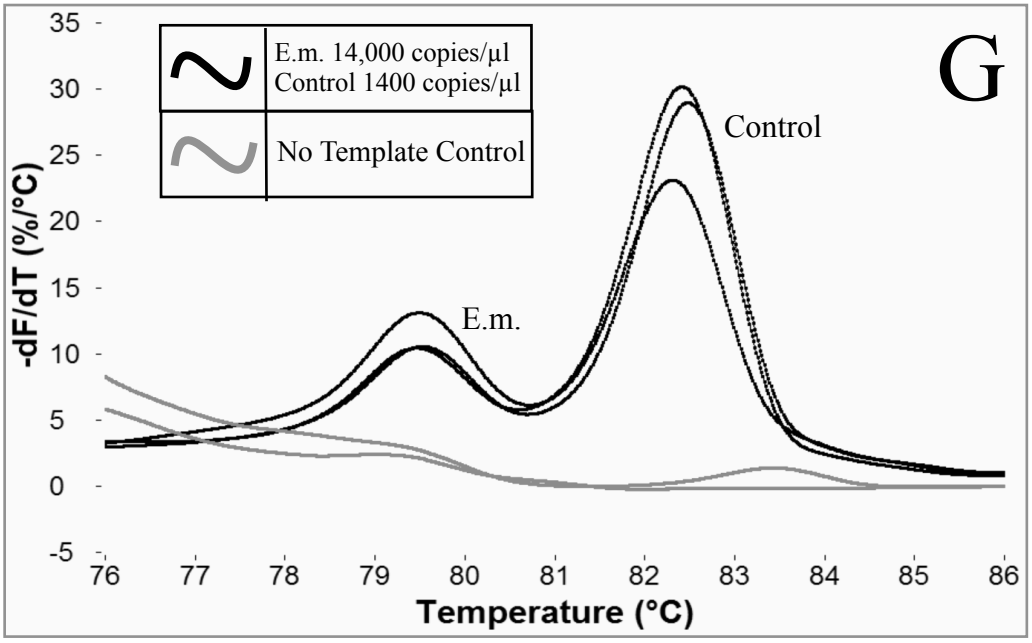


Figure 3. continued.



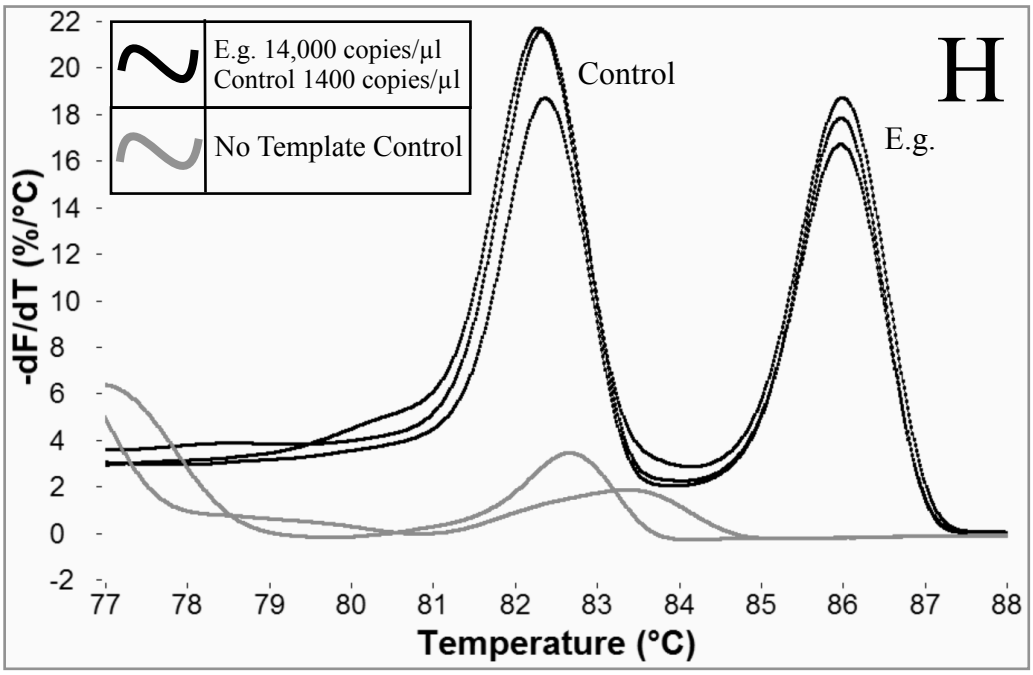


Figure 3. continued.

nearly equal to that of the control. There was also strong positive detection of *E. granulosus* at 14,000 copies/µl and with a significantly higher melting peak than that of the control (Figure 4).

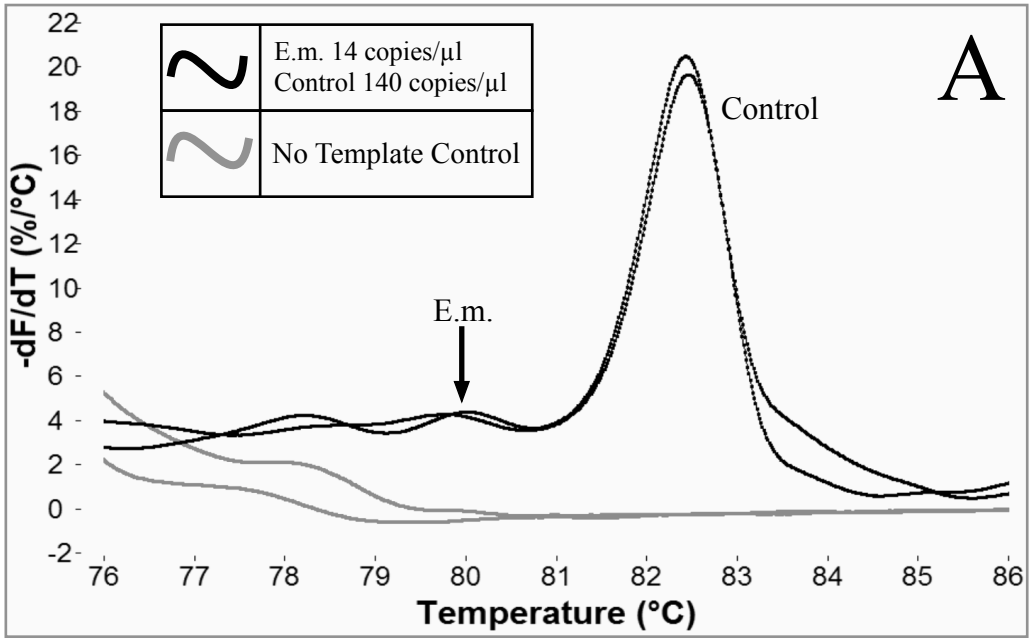
Control DNA at 14 copies/µl

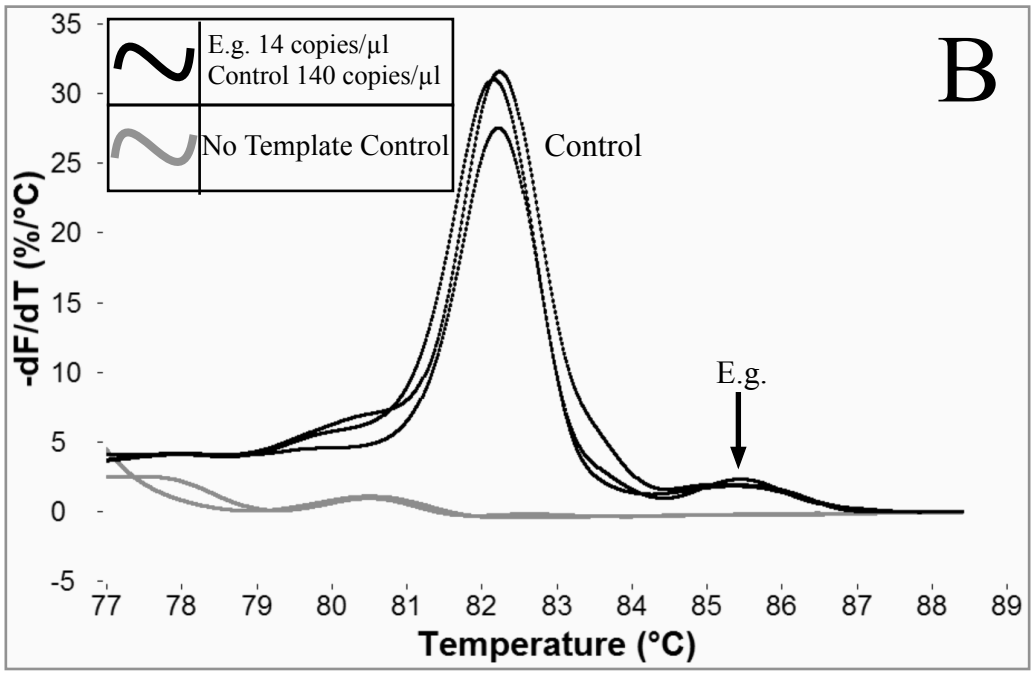
With control DNA concentrations at 14 copies/µl, there was positive detection of both *E. multilocularis* and *E. granulosus* also at 14 copies/µl. Both had melting peaks near equal in height to that of the control. When copy numbers of *E. multilocularis* and *E. granulosus* were increased to 140 copies/µl, both showed strong positive detection with melting peaks exceeding the height of the control. When copy numbers of *E. multilocularis* and *E. granulosus* were increased to 1400 copies/µl, both showed strong positive detection with significantly higher melting peaks than that of the control. When *E. multilocularis* was increased to 14,000 copies/µl, it showed strong positive detection, also with a significantly higher melting peak than that of the control. When *E. granulosus* was increased to 14,000 copies/µl, it showed strong positive detection, but no control DNA amplification is evident (Figure 5).

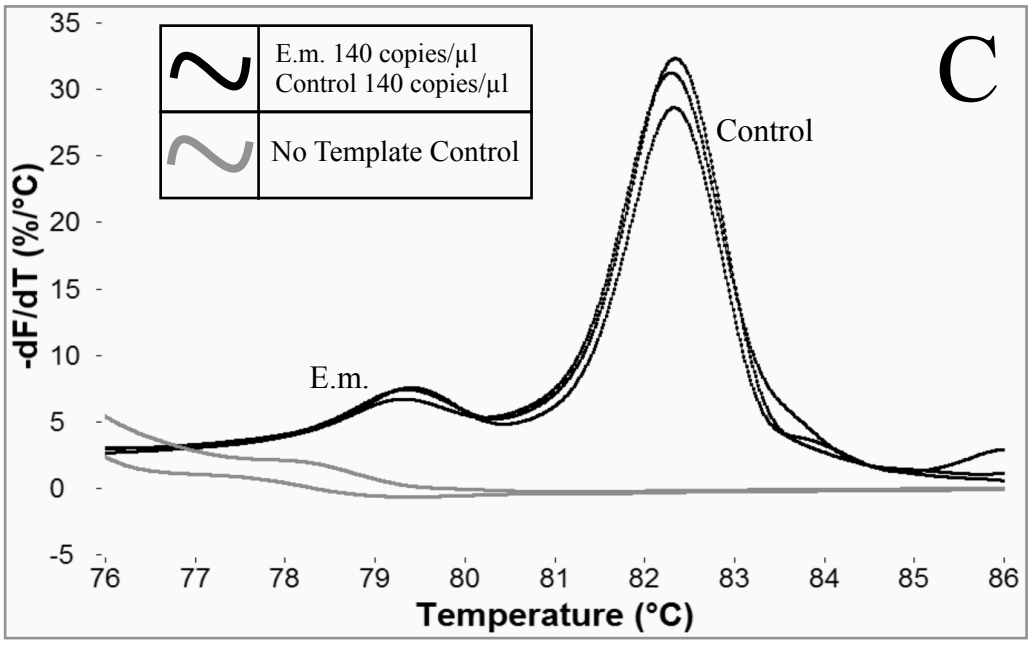
Blind Test Results

Out of the 20 blind samples, 11 were retested with no control or with a reduced concentration of control due to ambiguous results. Even with the retests conducted with reduced control template concentration, blind samples 6 and 18 were called negative, but with a desire to retest due to a possibility of a weak positive for *E. granulosus* or *E. multilocularis*, respectively. Sample 6 was called negative, but was actually positive for *E. granulosus*, at a concentration of 14 copies/µl. Sample 18 was called negative, which

Figure 4. High-resolution melting curves from the dilution series with the control DNA template concentration at 140 copies/µl. (A) *E. multilocularis* at 14 copies/µl. (B) *E. granulosus* at 14 copies/µl. (C) *E. multilocularis* at 140 copies/µl. (D) *E. multilocularis* at 140 copies/µl. (E) *E. multilocularis* at 1400 copies/µl. (F) *E. granulosus* at 1400 copies/µl. (G) *E. multilocularis* at 14,000 copies/µl. (H) *E. granulosus* at 14,000 copies /ul.


A
E.m. 14 copies/µl
Control 140 copies/µl
No Template Control
Control
E.m.
-dF/dT (%/°C)
Temperature (°C)


B
E.g. 14 copies/µl
Control 140 copies/µl
No Template Control
Control
E.g.
-dF/dT (%/°C)
Temperature (°C)



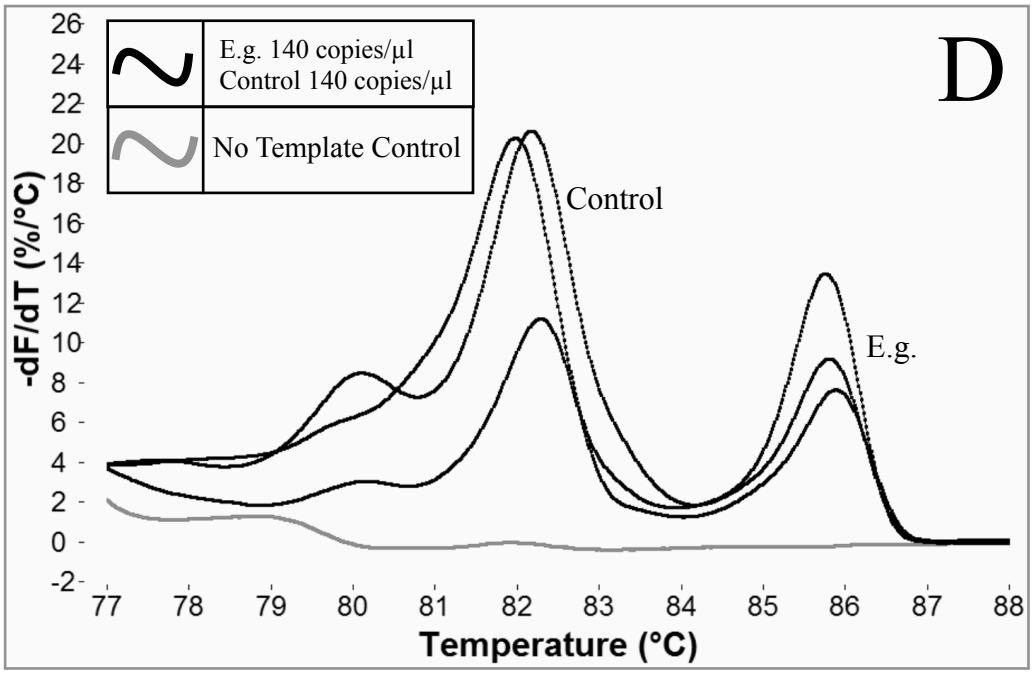
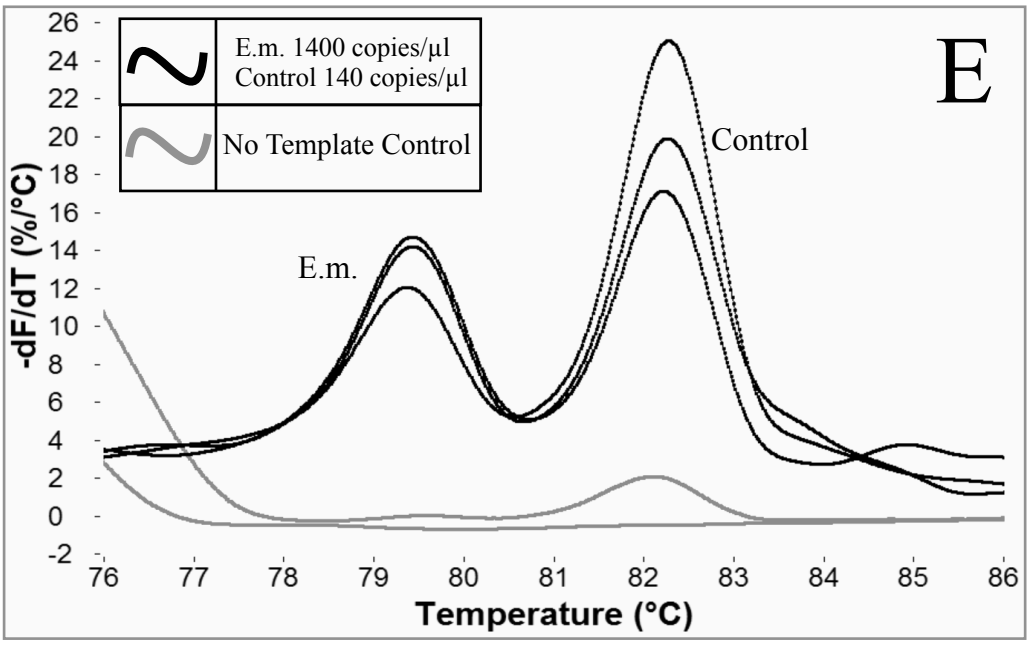


Figure 4. continued.



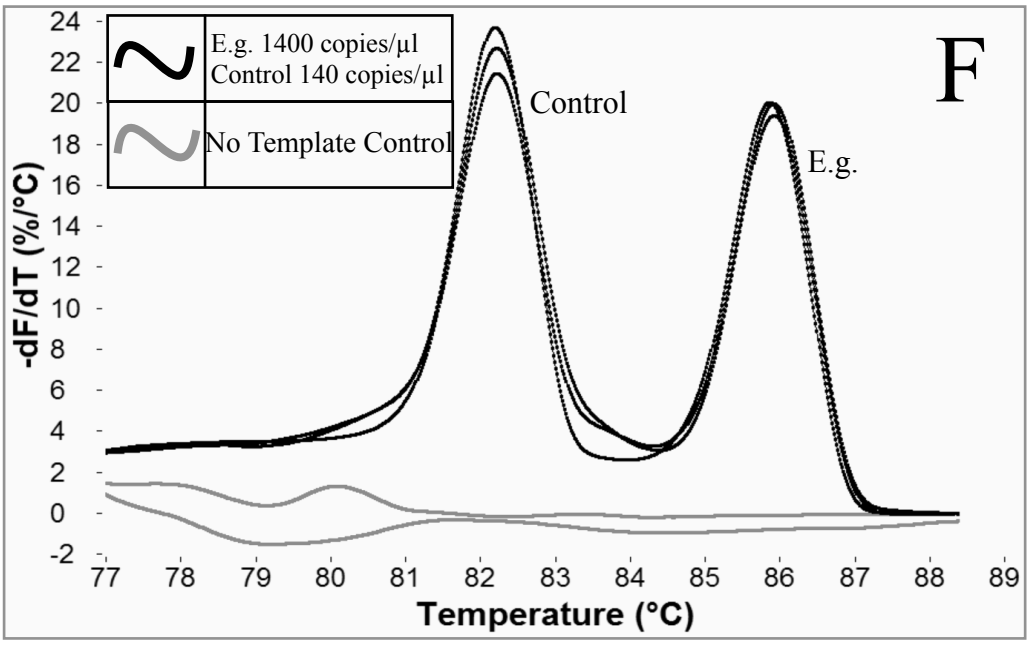
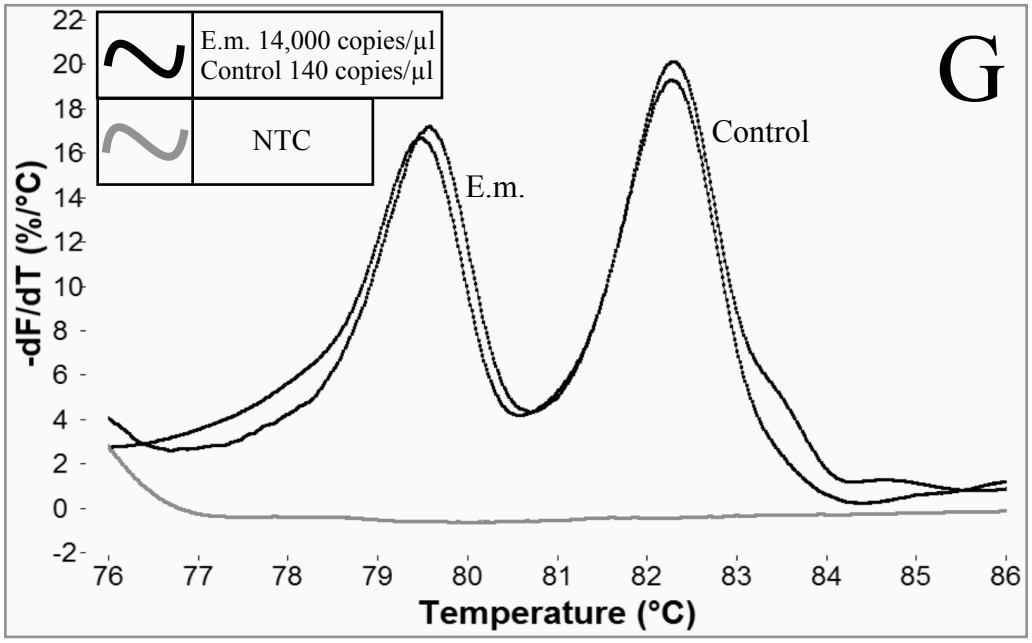


Figure 4. continued.



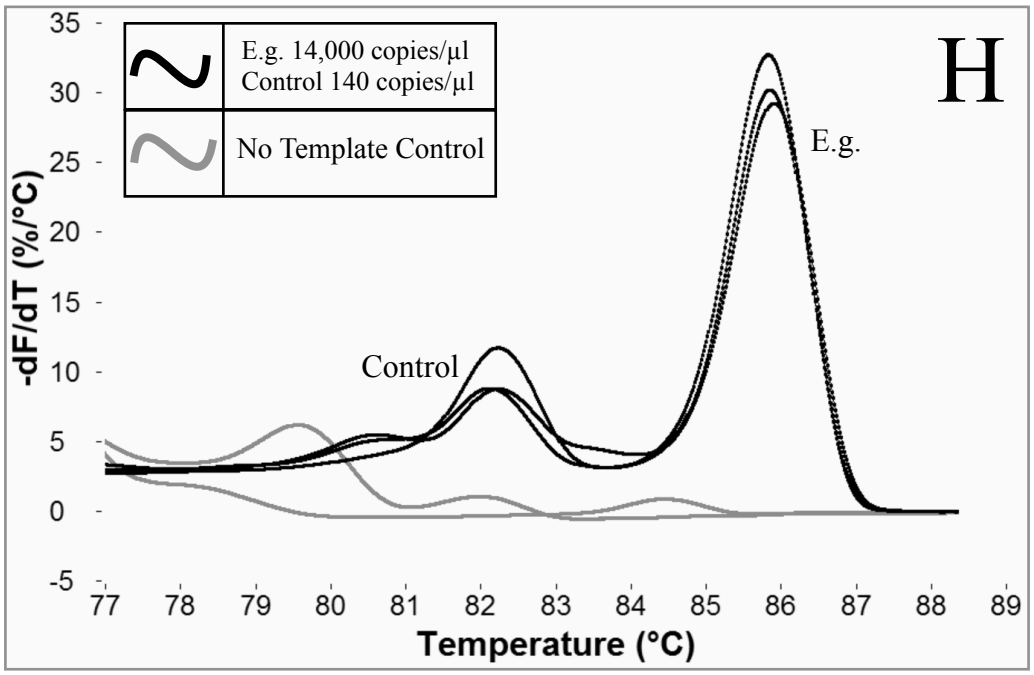
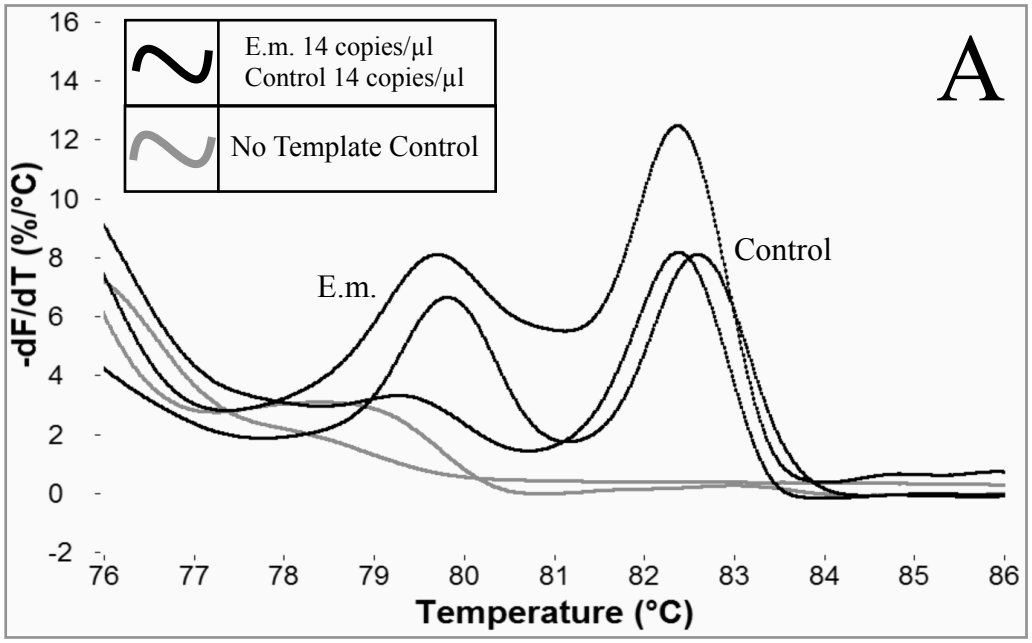
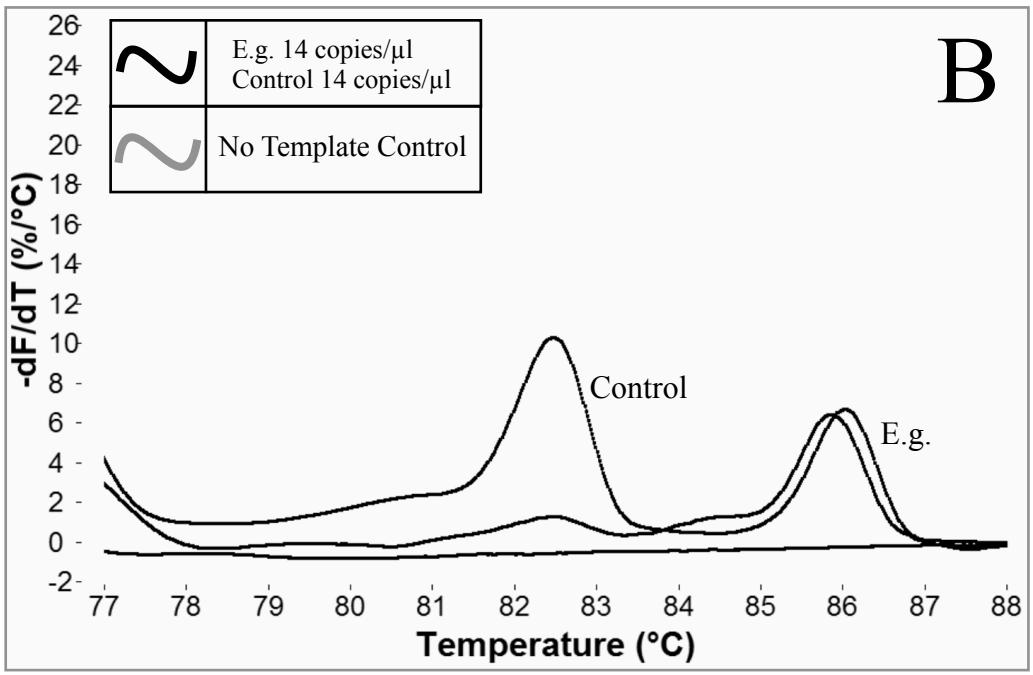


Figure 4. continued.

Figure 5. High-resolution melting curves from the dilution series with the control DNA template concentration at 14 copies/µl. (A) *E. multilocularis* at 14 copies/µl. (B) *E. granulosus* at 14 copies/µl. (C) *E. multilocularis* at 140 copies/µl. (D) *E. multilocularis* at 140 copies/µl. (E) *E. multilocularis* at 1400 copies/µl. (F) *E. granulosus* at 1400 copies/µl. (G) *E. multilocularis* at 14,000 copies/µl. (H) *E. granulosus* at 14,000 copies /ul.


A
E.m. 14 copies/µl
Control 14 copies/µl
No Template Control
E.m.
Control
-dF/dT (%/°C)
Temperature (°C)
16
14
12
10
8
6
4
2
0
-2
76
77
78
79
80
81
82
83
84
85
86


B
E.g. 14 copies/µl
Control 14 copies/µl
No Template Control
Control
E.g.
-dF/dT (%/°C)
Temperature (°C)
26
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
77
78
79
80
81
82
83
84
85
86
87
88

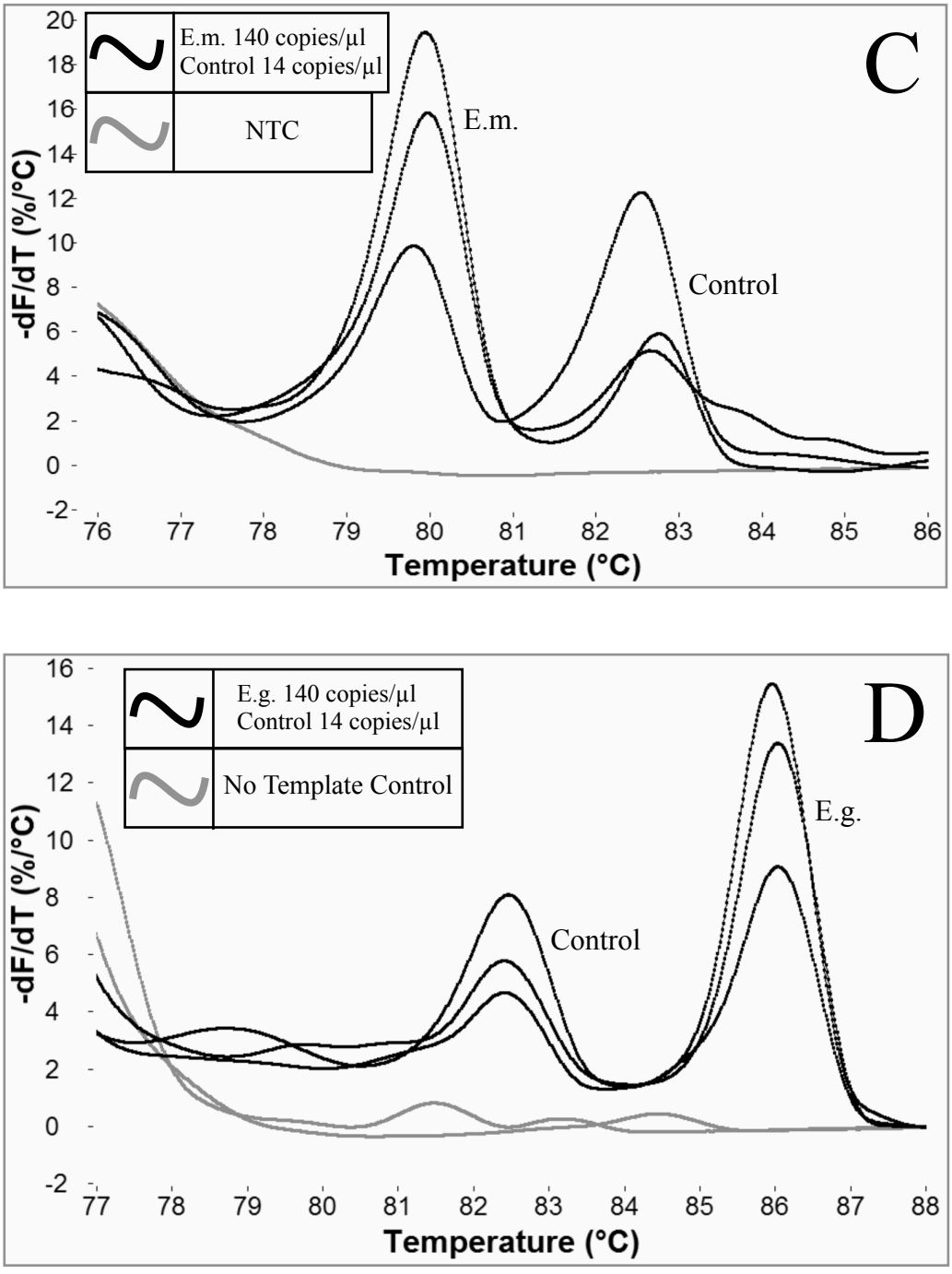
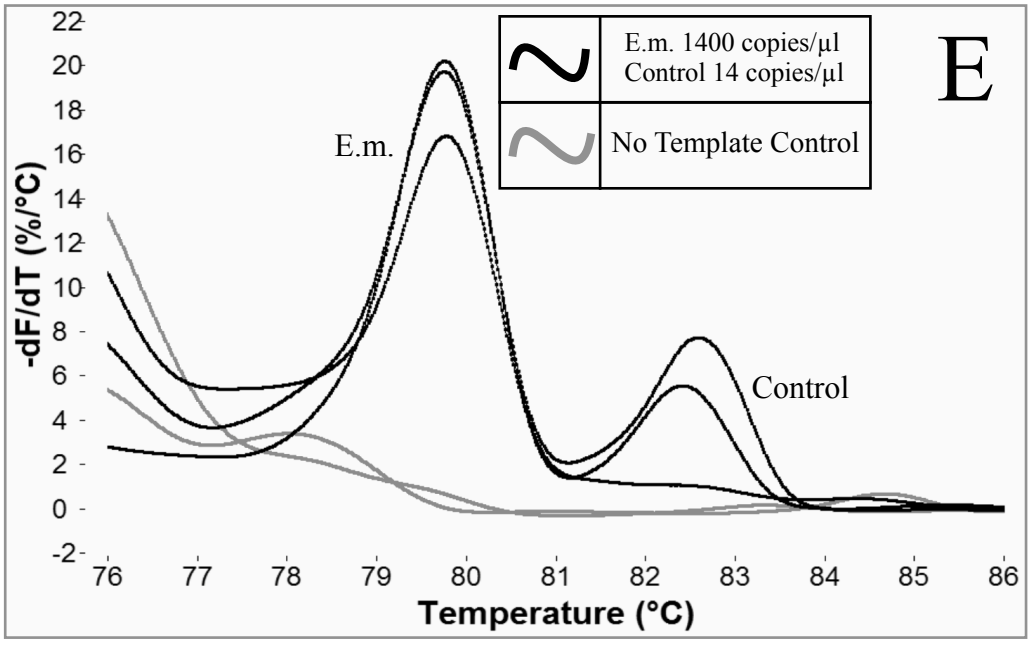


Figure 5. continued.



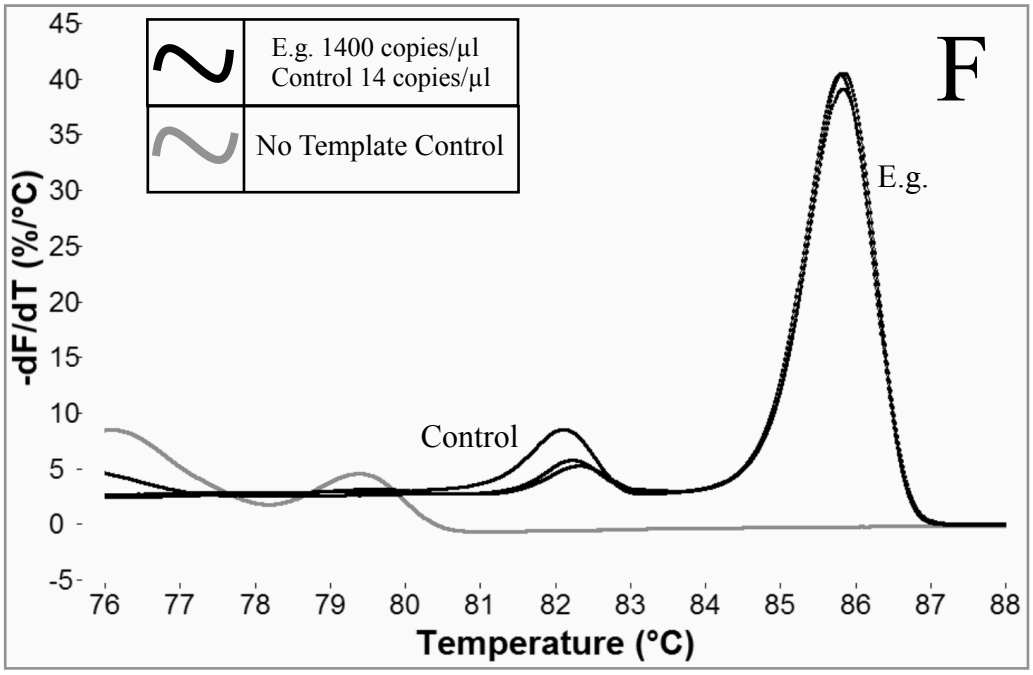
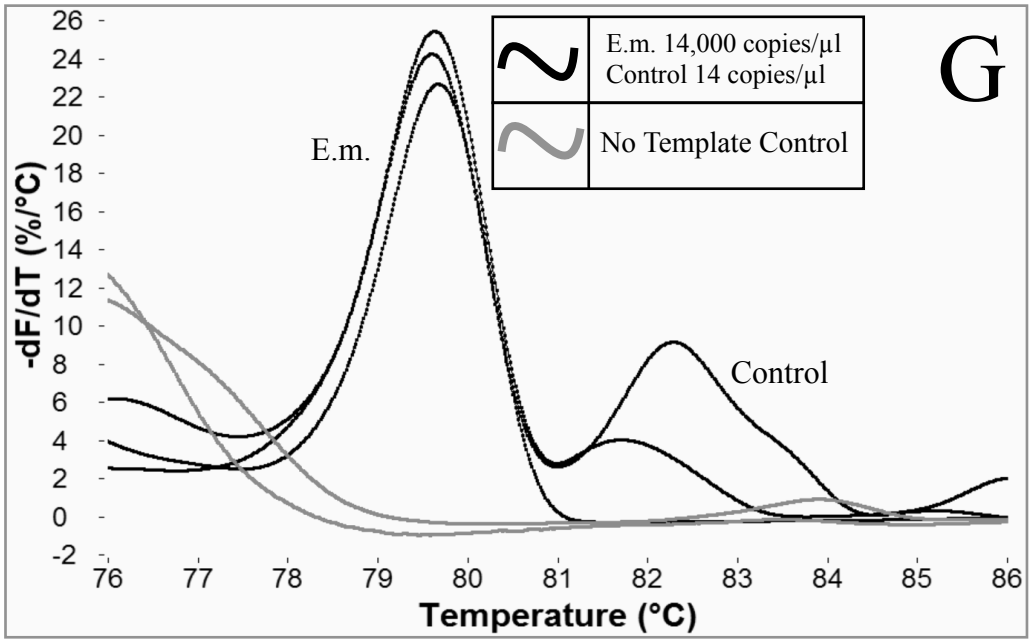


Figure 5. continued.



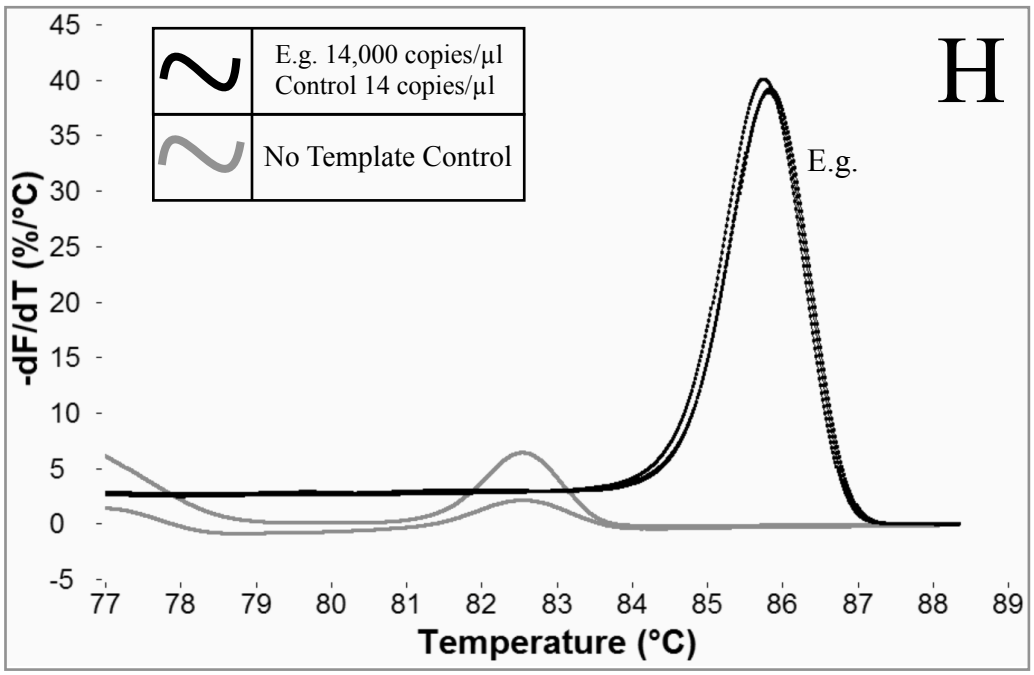


Figure 5. continued.

was correct. Sample 19 was called negative, but was actually positive for *E. multilocularis* at a concentration of 14 copies/µl. All other samples were identified correctly (Table 1, Figure 6).

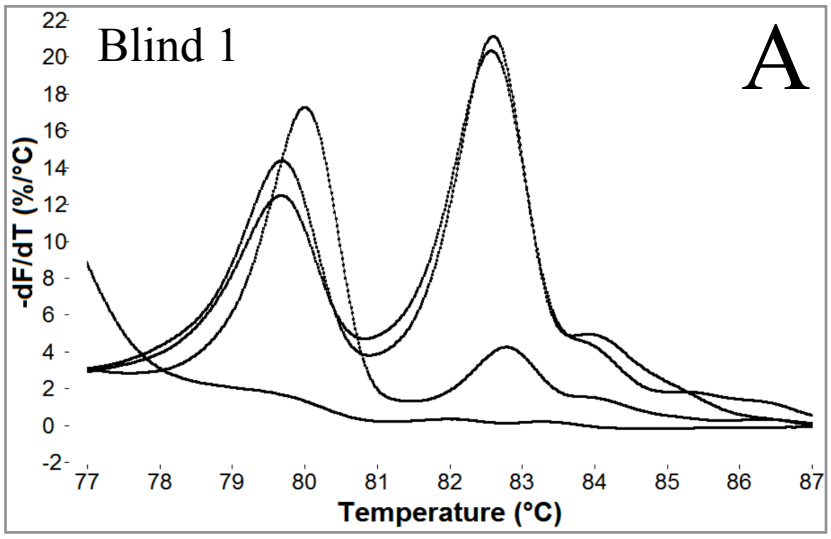
Ambiguous results

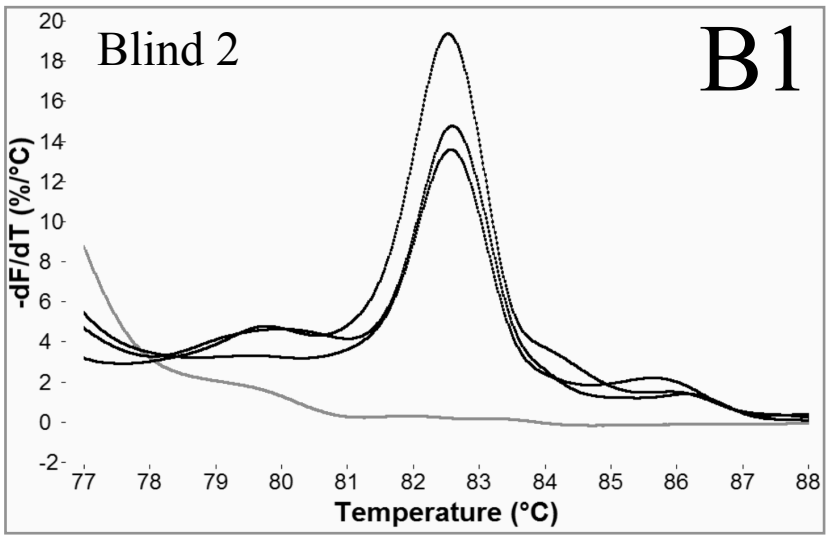
The original HRMA from blind sample 2 produced what looked like a peak at both the *E. multilocularis* and *E. granulosus* positions. *E. multilocularis* positivity was sure, but *E. granulosus* was in question. The sample was repeated with control DNA concentration reduced to 14 copies/µl. The resulting HRMA curve showed a peak at the *E. multilocularis* position and one at the control position (Figure 6, parts B1 and B2). The original HRMA curve from blind sample 3 showed a possible weak *E. granulosus* positive. When repeated with control DNA concentration reduced to 14 copies/µl, the resulting HRMA curve showed a peak at the *E. granulosus* position and one at the control position (Figure 6, parts C1 and C2). The original HRMA curve from blind sample 4 showed possible positives for both *E. multilocularis* and *E. granulosus* with some inconsistency between replicates. When repeated with the control DNA concentration reduced to 14 copies/µl, the resulting HRMA curve showed a peak at the *E. multilocularis* position and one at the control position (Figure 6, parts D1 and D2). The original HRMA curve from blind sample 6 showed a possible weak positive for *E. granulosus*. When repeated with control DNA concentration reduced to 14 copies/µl, the resulting HRMA curve showed a very small peak at the *E. granulosus* position and one at the control position along with an unknown peak at 80.2°C (Figure 6, parts F1 and F2). The original HRMA curve from blind sample 7 showed a possible weak positive for both *E. multilocularis* and *E. granulosus*. When repeated with the control DNA reduced to 14

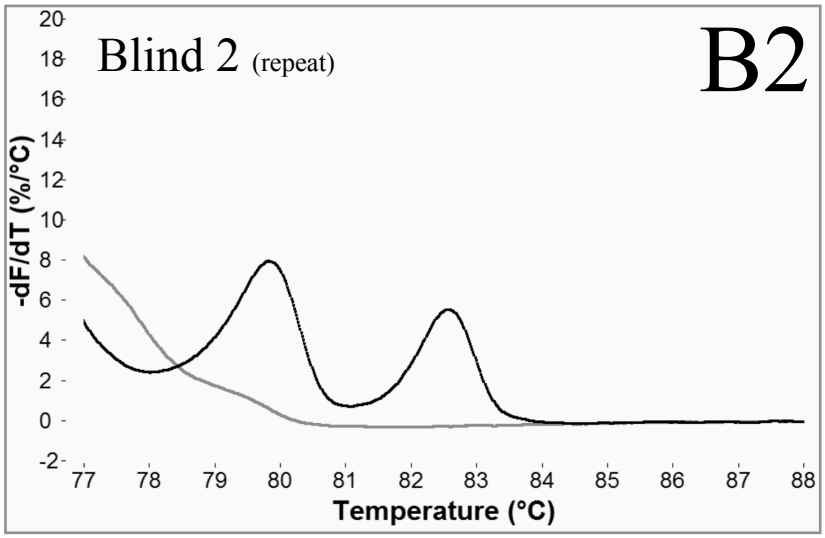
Table 1. Test results of each blind sample are represented by check marks in the E.m. +, E.g. + or Negative columns. E.m.: E. multilocularis, E.g.: E. granulosus; starred results represent the desire to repeat testing due to uncertain results.

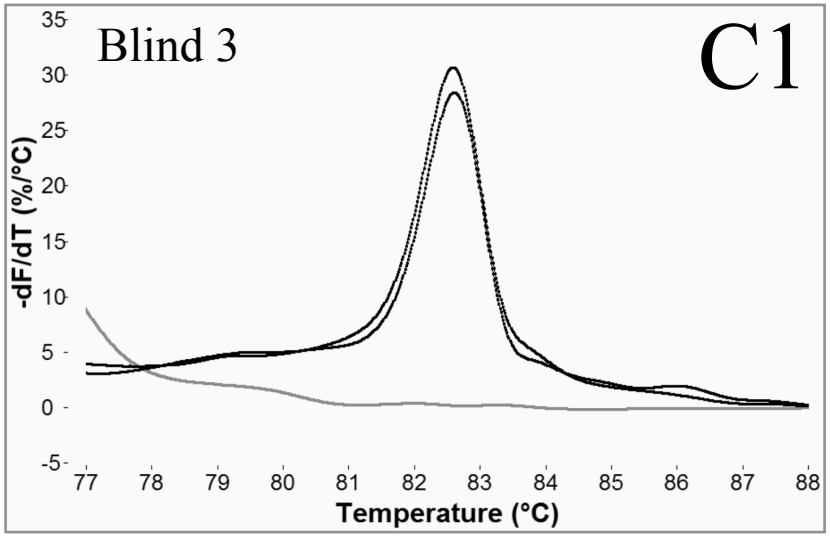
	Result					
	E.m. +	E.g. +	Negative	Retest done	Identity and Concentration (copies/ul)	Correct Identification
Blind 1	✓				E.m. 14,000	Yes
Blind 2	✓			✓	E.m. 140	Yes
Blind 3		✓		✓	E.g. 14	Yes
Blind 4	✓			✓	E.m. 14	Yes
Blind 5		✓			E.g. 14,000	Yes
Blind 6			✓*	✓	E.g. 14	No
Blind 7		✓		✓	E.g. 14	Yes
Blind 8	✓			✓	E.m. 1400	Yes
Blind 9	✓				E.m. 140	Yes
Blind 10		✓			E.g. 140	Yes
Blind 11	✓			✓	E.m. 140	Yes
Blind 12		✓			E.g. 1400	Yes
Blind 13	✓				E.m. 1400	Yes
Blind 14		✓		✓	E.g. 140	Yes
Blind 15			✓		H_2O	Yes
Blind 16		✓			E.g. 140	Yes
Blind 17	✓				E.m. 140	Yes
Blind 18			✓*	✓	H_2O	Yes
Blind 19			✓	✓	E.m. 14	No
Blind 20		✓		✓	E.g. 1400	Yes

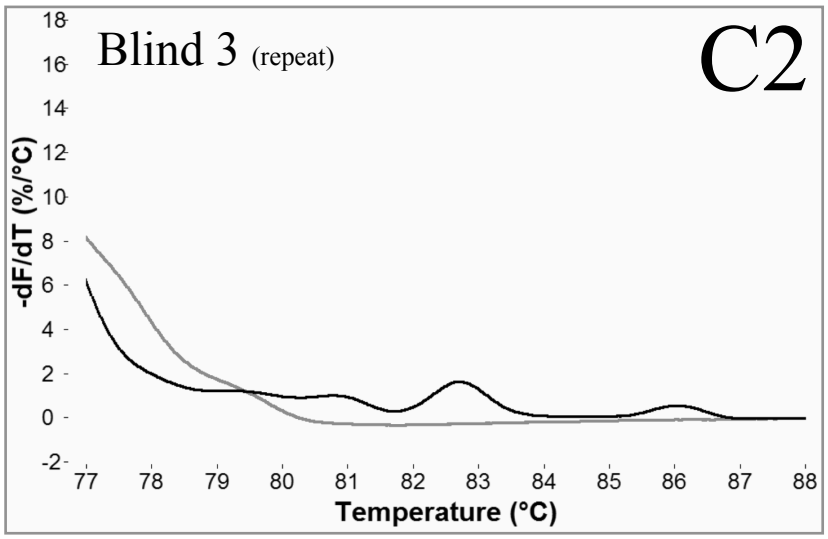
Figure 6. High-resolution melting curves from the blind samples. (A) Blind sample 1. (B1) Blind sample 2. (B2) Blind sample 2 repeat. (C1) Blind sample 3. (C2) Blind sample 3 repeat. (D1) Blind sample 4. (D2) Blind sample 4 repeat. (E) Blind sample 5. (F1) Blind sample 6. (F2) Blind sample 6 repeat. (G1) Blind sample 7. (G2) Blind sample 7 repeat. (H1) Blind sample 8. (H2) Blind sample 8 repeat. (I) Blind sample 9. (J) Blind sample 10. (K1) Blind sample 11. (K2) Blind sample 11 repeat. (L) Blind sample 12. (M) Blind sample 13. (N1) Blind sample 14. (N2) Blind sample 14 repeat. (O) Blind sample 15. (P) Blind sample 16. (Q) Blind sample 17. (R1) Blind sample 18. (R2) Blind sample 18 repeat. (S1) Blind sample 19. (S2) Blind sample repeat. (T1) Blind sample 20. (T2) Blind sample 20 repeat.


Blind 1
A
-dF/dT (%/°C)
Temperature (°C)


Blind 2
B1
-dF/dT (%/°C)
Temperature (°C)


Blind 2 (repeat)
B2
-dF/dT (%/°C)
Temperature (°C)





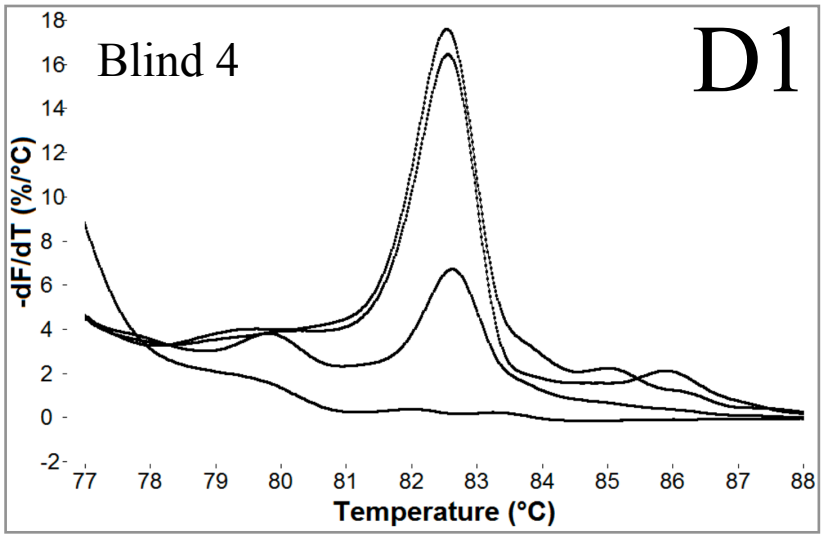


Figure 6. continued.

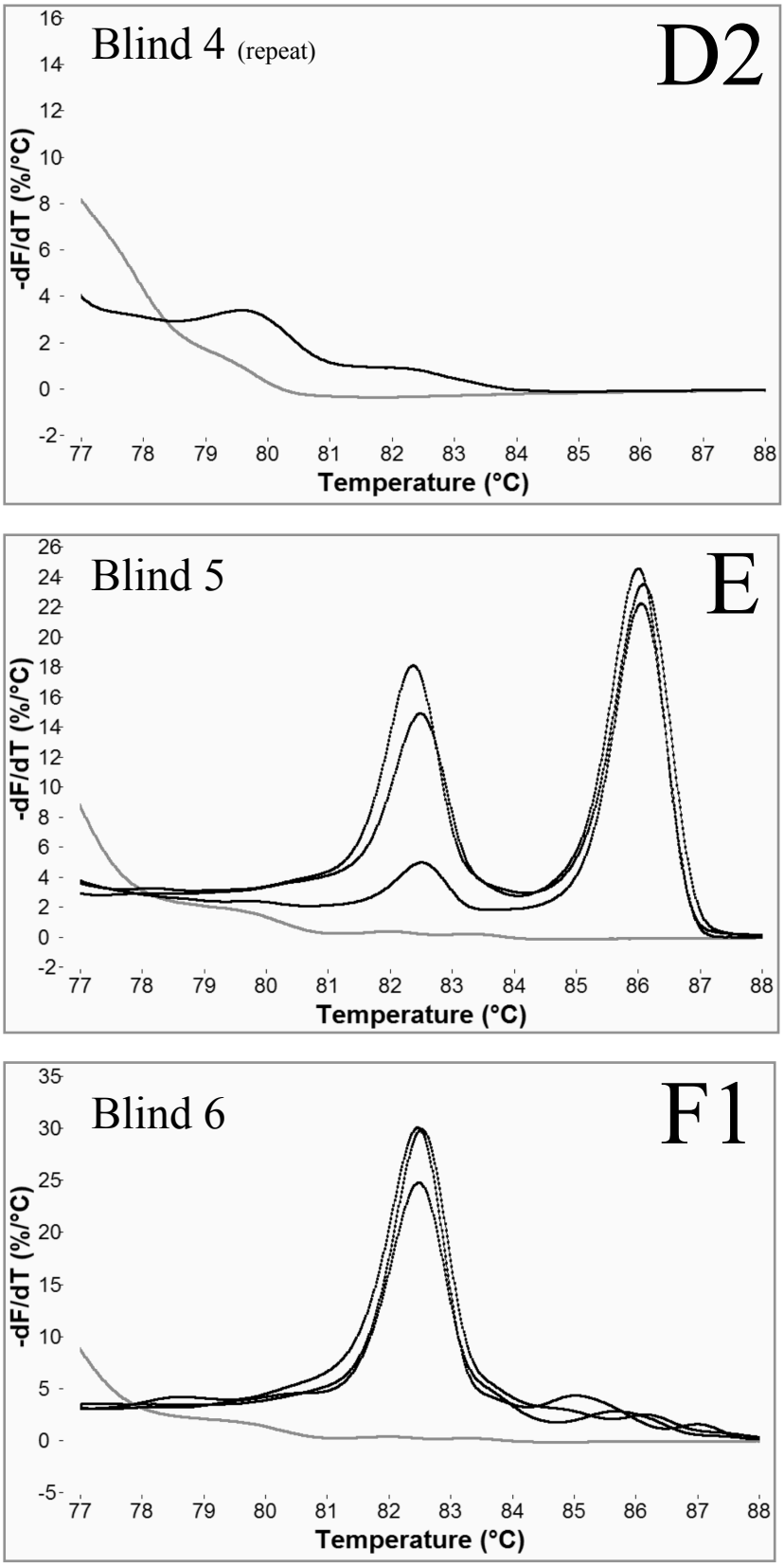
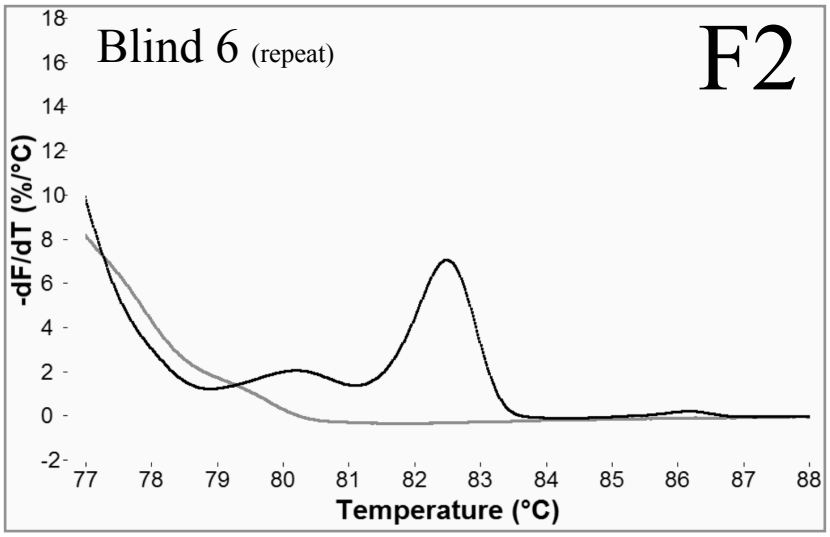
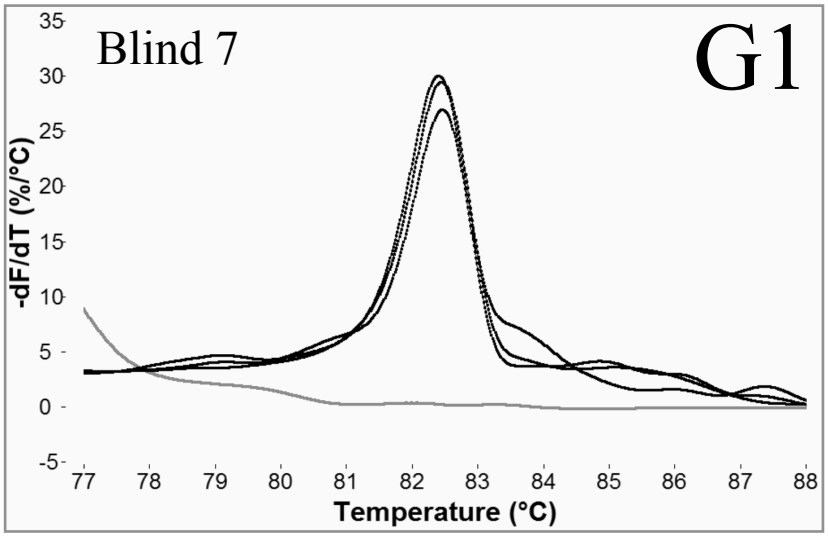


Figure 6. continued.





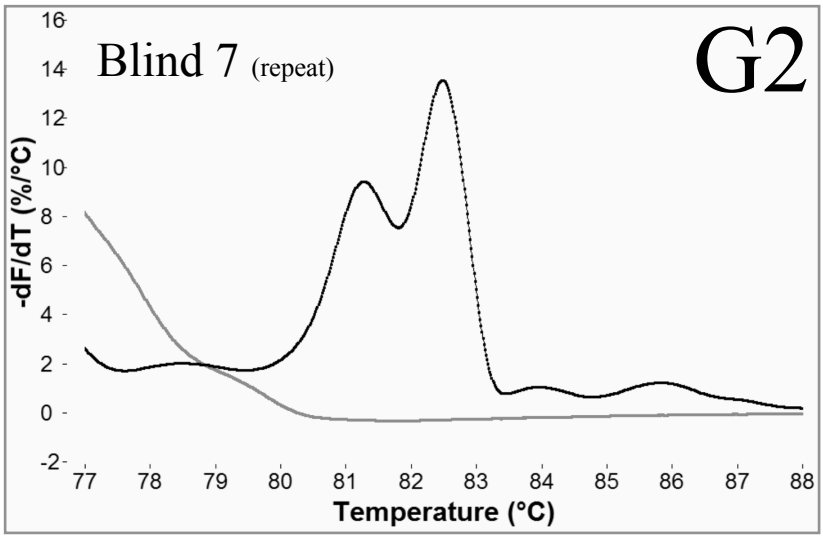


Figure 6. continued.

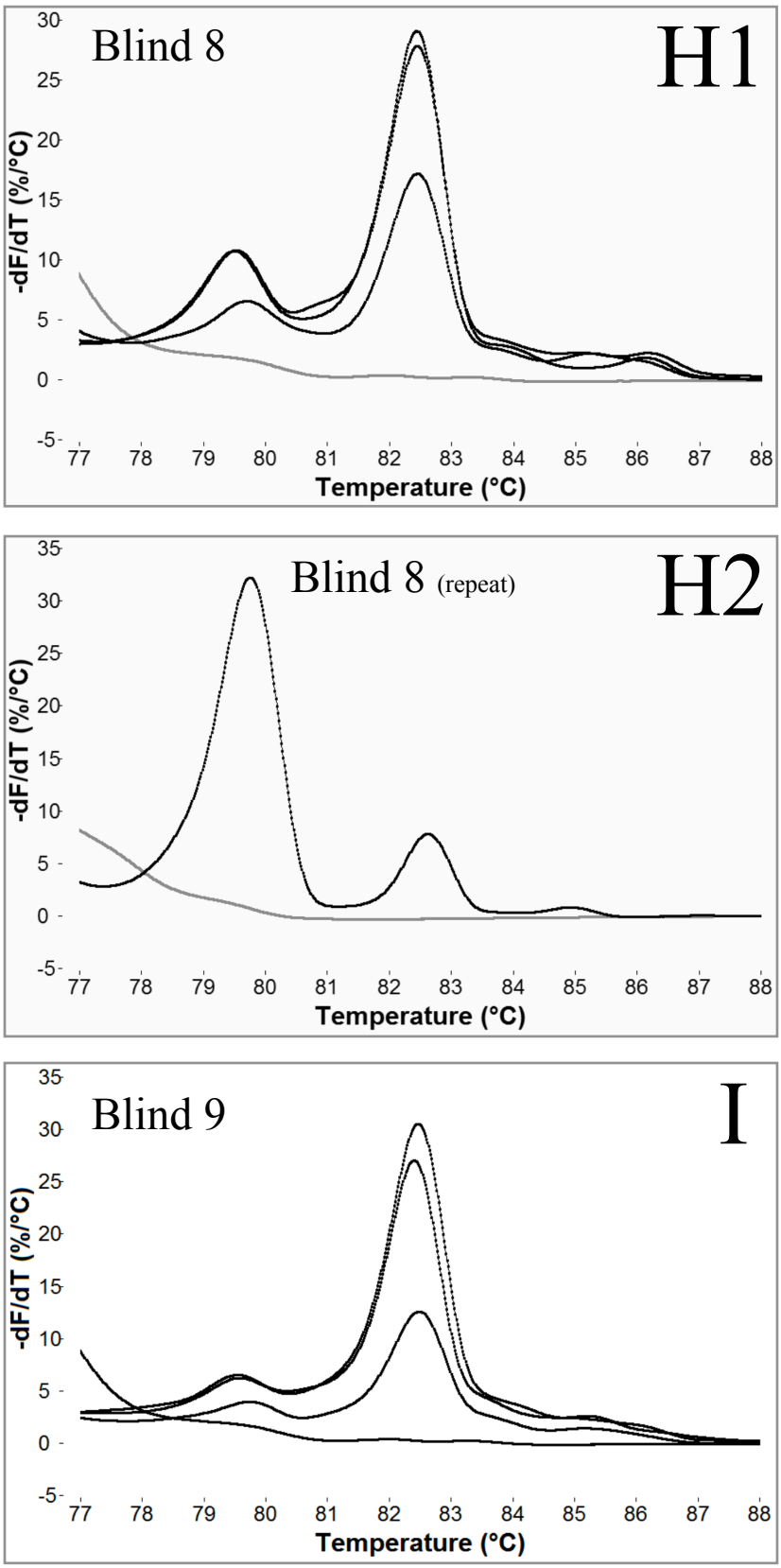


Figure 6. continued.

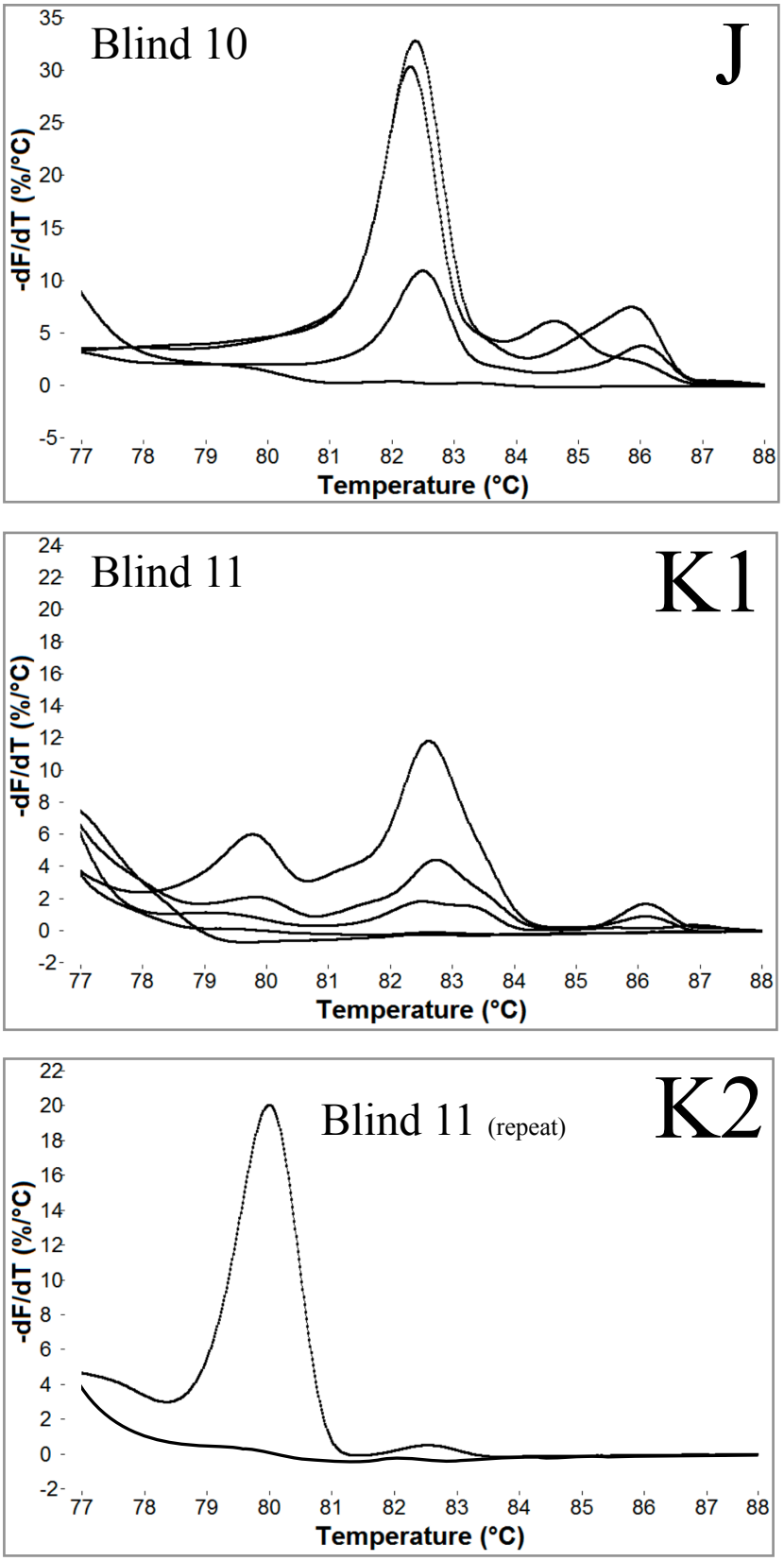


Figure 6. continued.

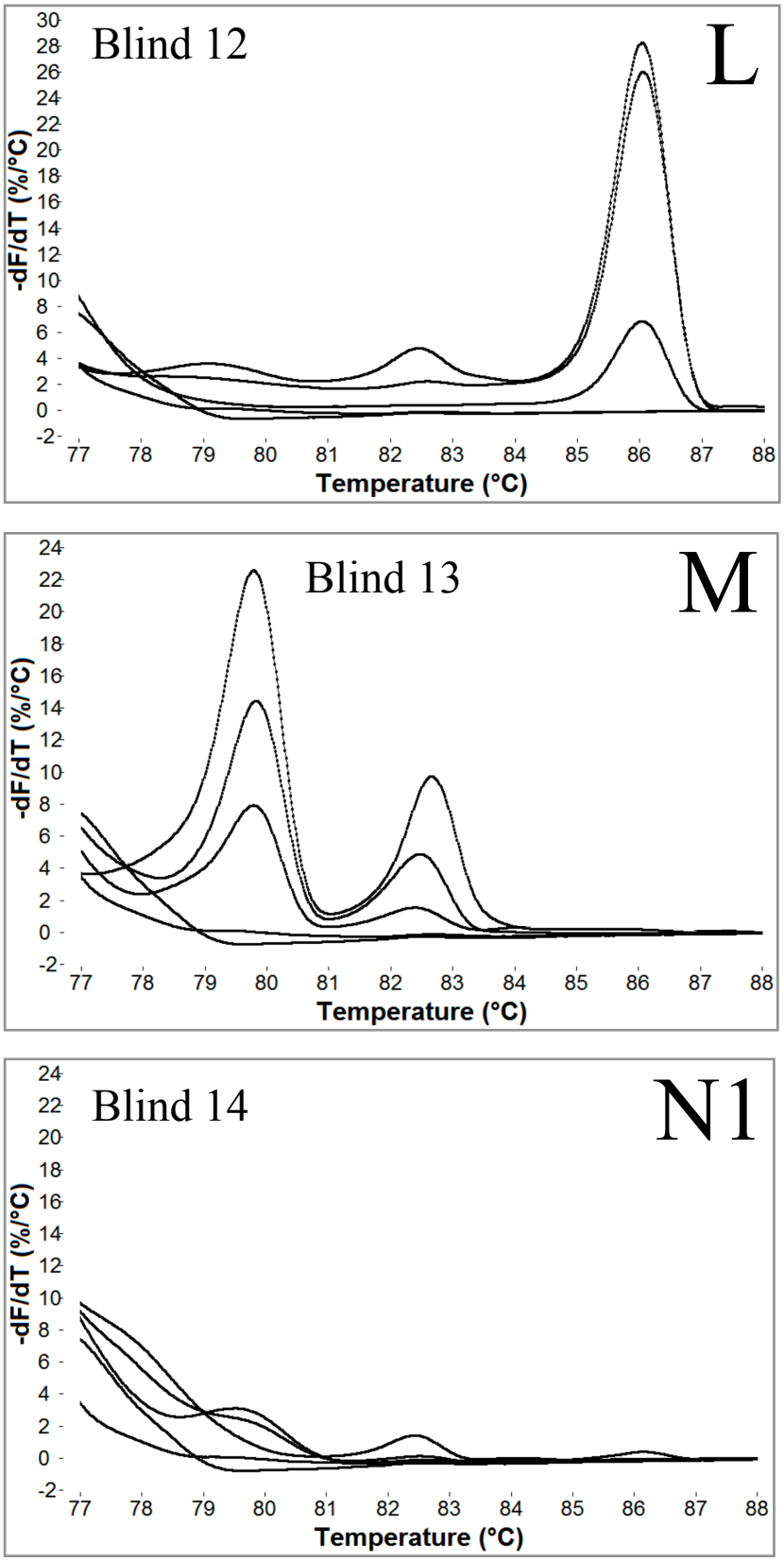


Figure 6. continued.

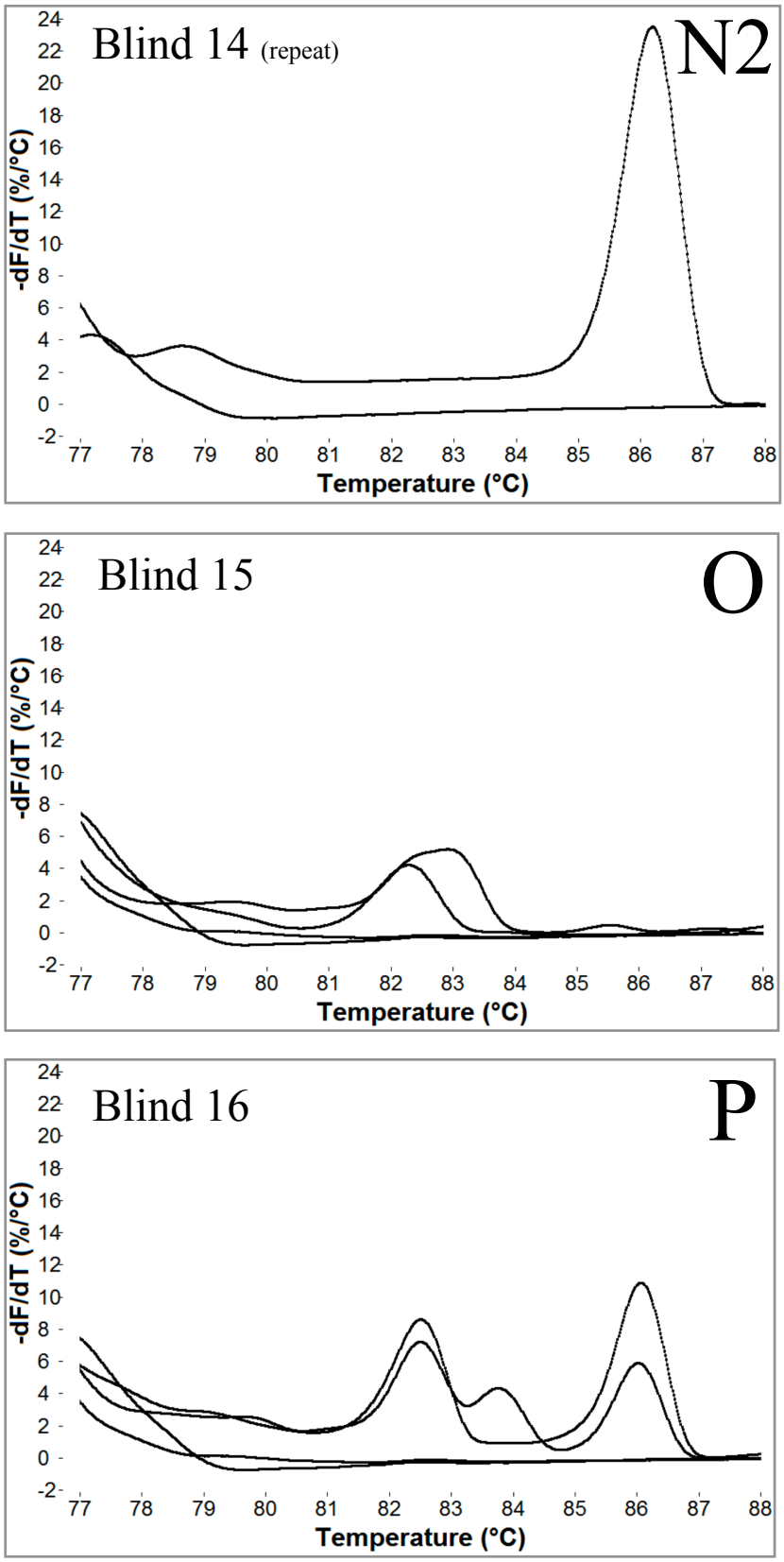


Figure 6. continued.

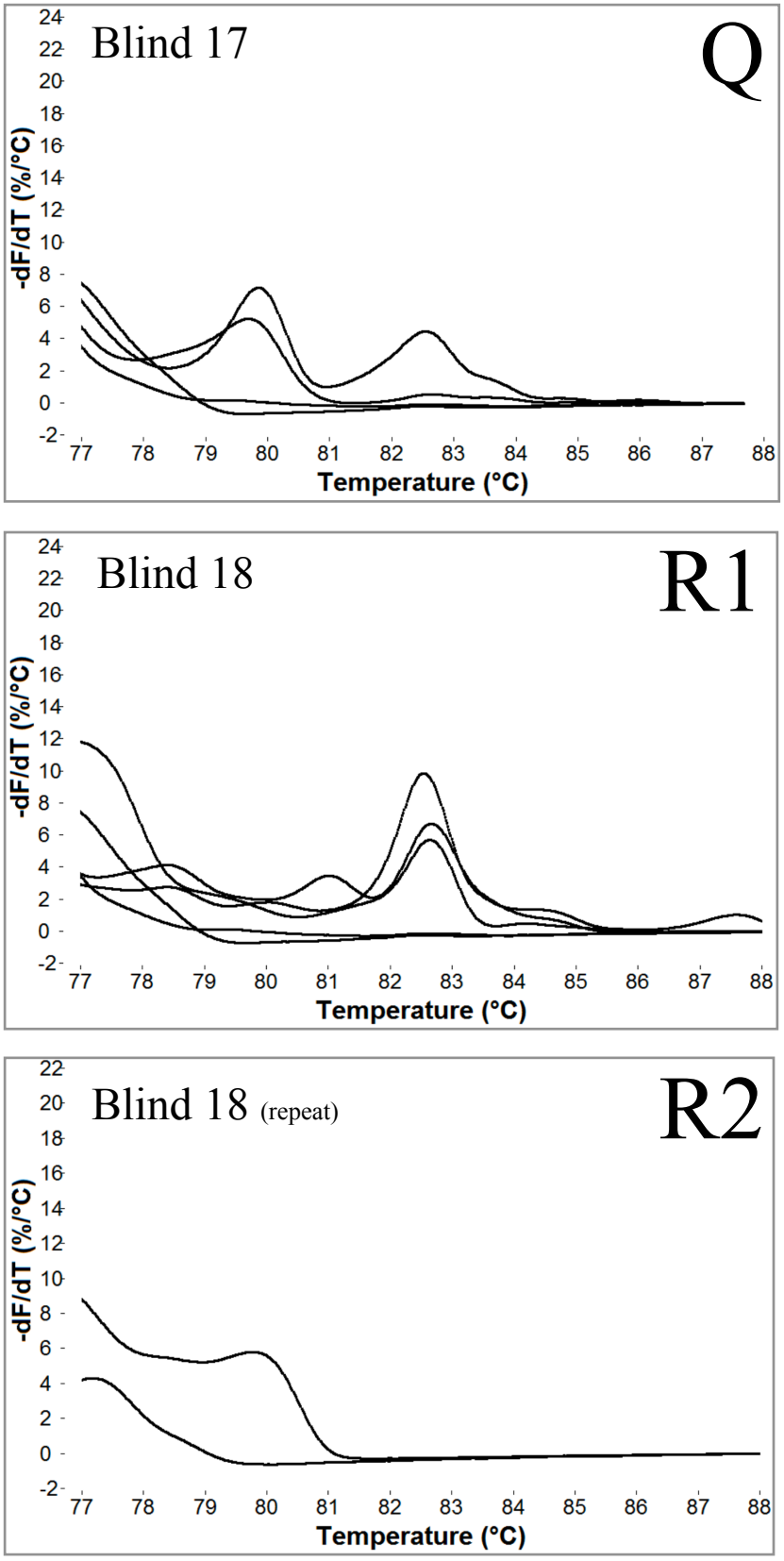
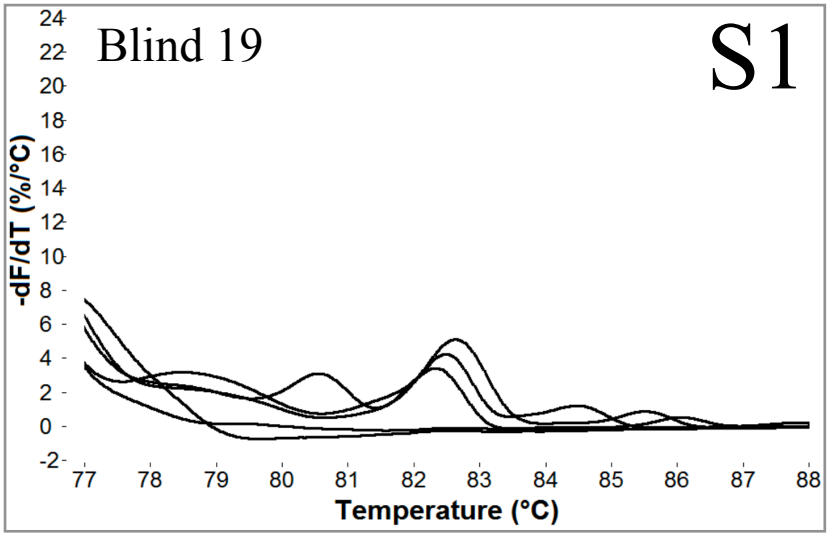
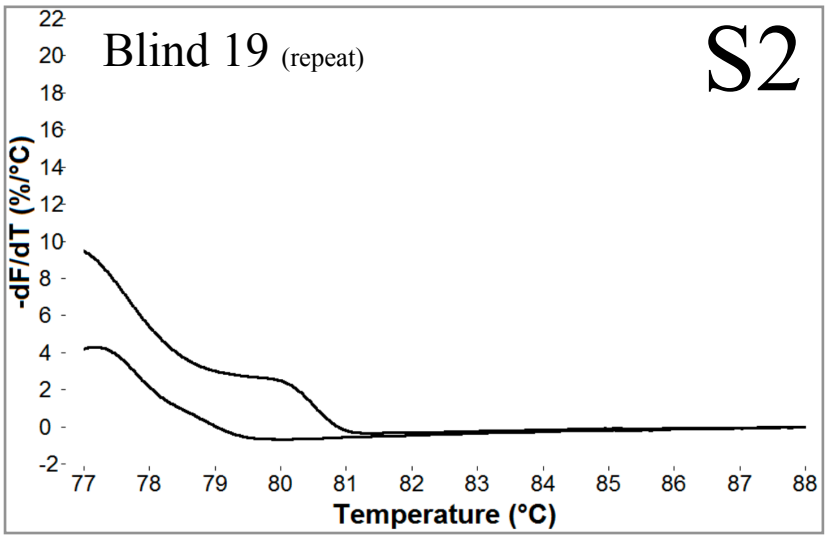


Figure 6. continued.





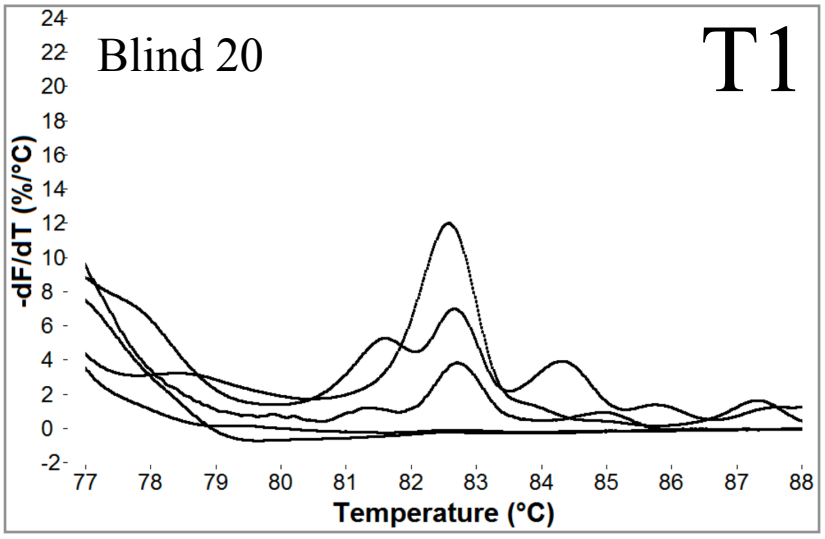


Figure 6. continued.

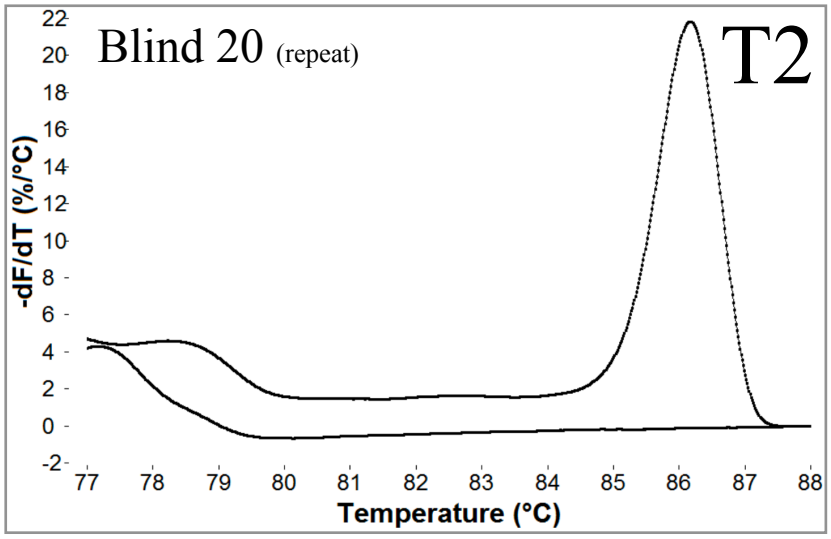


Figure 6. continued.

copies/µl, the resulting HRMA curve showed a peak at the *E. granulosus* position and one at the control position along with an unknown peak at 81.2°C (Figure 6, parts G1 and G2). The original HRMA curve from blind sample 8 showed a strong positive for *E. multilocularis*, but a possible weak positive for *E. granulosus*. When repeated with the control DNA reduced to 14copies/µl, the resulting HRMA curve showed a peak at the *E. multilocularis* position and one at the control position along with a small, unknown peak at 85°C (Figure 6, parts H1 and H2). The original HRMA curve from blind sample 11 showed a strong positive for *E. multilocularis*, but a possible weak positive for *E. granulosus*. When repeated with no control DNA present, the resulting HRMA curve showed a peak at the *E. multilocularis* position and a very small peak at the control position despite the fact that no control DNA template was added (Figure 6, parts K1 and K2). The original HRMA curve from blind sample 14 showed a possible weak positive for *E. granulosus*. When repeated with no control DNA present, the resulting HRMA curve showed a peak at the *E. granulosus* position along with an unknown peak at 78.7°C (Figure 6, parts N1 and N2). The original HRMA curve from blind sample 18 showed a probable negative, but with a possible weak positive of *E. multilocularis*. When repeated with no control DNA present, the resulting HRMA curve showed a peak at 79.8°C, which is a higher melting temperature than expected when *E. multilocularis* is run alone (Figure 6, parts R1 and R2). The original HRMA curve from blind sample 19 showed a probable negative, but possible weak positives for *E. multilocularis* and *E. granulosus*. When repeated with no control DNA present, the resulting HRMA curve showed a peak at 80°C, which a higher melting temperature than expected when *E. multilocularis* is run alone (Figure 6, parts S1 and S2). The original HRMA curve from blind sample 20

showed possible positives for both *E. multilocularis* and *E. granulosus* with some inconsistency between replicates. When repeated with no control DNA present, the resulting HRMA curve showed a peak at the *E. granulosus* position along with an unknown peak at 78.3° (Figure 6, parts T1 and T2).

DISCUSSION

Currently, no other PCR-based assay exists that is designed to test for both *E. multilocularis* and *E. granulosus* concurrently using human serum or plasma. The need for an assay of this nature lies in the current epidemiological trends occurring with both CE and AE. In areas that are co-endemic, a reliable means of differentiation is needed. The limitations of this research lie in the source for genetic materials used. Genetic sequences used for both *E. granulosus* and *E. multilocularis* were synthetic DNA based on the published genetic sequences of these organisms. No actual parasitic material or extracted DNA was available for use in this project. There were also no patient specimens tested in order to validate the assay for possible clinical use. The hope is that further research can be conducted on this method when parasitic DNA and patient samples become available for testing. The goal of this project was to establish a limit of detection for each organism. If there are circulating cells or cell-free DNA present in the blood of infected individuals, it is likely to be present in very small amounts, which makes it essential that any assay attempting to detect it be sensitive.

One challenge that was not foreseen when beginning this research was how much the control DNA and the primer set designed to amplify it might affect the detection levels of the *E. multilocularis* and *E. granulosus* DNA. The reasons behind choosing human DNA as the positive control included the lack of any similar sequences to that of *E. multilocularis* or *E. granulosus* and the easy access to extracted human genomic DNA.

Another reason for its use is the fact that one would expect to find it in human serum or plasma, which would make spiking it into the PCR reaction unnecessary when used on clinical samples. There was a significant difference between the melting temperatures of *E. multilocularis* and *E. granulosus* amplicons, leaving room for a melting peak in between them. Both of the CFTR primer-set candidates were chosen because the melting temperature of the products would fall right in between the melting temperatures of the two parasites. However, when the CFTR3 and CFTR10 primer sets were initially tested by other researchers, the PCR reaction mixture contained $MgCl_2$ at a concentration of 2 mmol/L when all primer sets for *E. multilocularis* and *E. granulosus* had been tested at a $MgCl_2$ concentration of 3 mmol/L.[38,39] Because of this, primer sets were then retested with $MgCl_2$ concentration reduced to 2 mmol/L. Although the primer set for *E. multilocularis* seemed to perform well, there was no amplification of the *E. granulosus* template at the lower $MgCl_2$ concentration. Because the CFTR primer sets also performed well with 3 mmol/L $MgCl_2$, that is the concentration that was used in the PCR mixture for this project despite the fact that it would decrease stringency for the control DNA. It is clear when looking at the HRM melting curves throughout the dilution series and blind samples that there seemed to be preferential amplification of the control DNA over that of the other two templates (Figure 3, Figure 4, Figure 5, Figure 6). The *E. multilocularis* template concentration needed to be at approximately 100 times that of the control DNA before its fluorescence matched that of the control DNA. *E. granulosus* was less dramatic, but still needed a concentration of approximately ten times that of the control DNA in order to reach equal fluorescence. Although the concentration of primers was highest for the control DNA, which could also help explain its preferential

amplification, the multiplex reaction did not have good results when the control DNA primer concentration was decreased. Therefore, it would be worth finding a primer set that performed better at 3 mmol/L $MgCl_2$ and not so well at 2 mmol/L $MgCl_2$. This might help balance the amplification of the templates and eliminate the preferential amplification of the control DNA template.

The reason that half the blind samples were tested with 140 copies/µl and the other half with 14 copies/µl of control DNA template was partially to demonstrate that the control DNA was being preferentially amplified and partially because cleaner HRMA curves were produced when control DNA template concentration was at 140 copies/µl. Despite the higher probability of detecting *E. multilocularis* or *E. granulosus* with control DNA template at 14 copies/µl, there was a possibility that there would be fewer ambiguous results when evaluating cleaner melting curves. This, however, was not the case. Most of the ambiguous results produced for blind samples 1 – 10 were because very small peaks at the positions of *E. multilocularis* and *E. granulosus* were produced that indicated possible weak positives. Most of the ambiguous results produced for blind samples 11 – 20 were because there was more noise present on the HRM curve. This is most attributable to small, aberrant peaks that made positive identification of peaks at the *E. multilocularis* and *E. granulosus* positions more difficult. In the case of all repeated blind samples, results almost always became less ambiguous when control DNA concentrations were decreased or eliminated from the reaction.

The results of the dilution series and blind samples demonstrate a need to look for a different positive control to include in the assay. Using human DNA as a positive control may be inadvisable in this assay for a few reasons. One reason is that given the

abundance of human DNA in the laboratory environment, it makes contamination of the reaction more likely than if a nonhuman source was used. Some of the HRM curves from the dilution series and the blind samples show contamination of the no template controls (Figure 2Results, part H; Figure 3Results, part E; Figure 4Results, part H, Figure 5Results, part K2). It has been shown in this project that an increase in the amount of human DNA can easily wash out low-level detection of either parasite. Therefore, an increase in human DNA template due to contamination could be detrimental to the success of the assay. Another reason to not use human DNA as the control is that it limits the kind of sample that can be used in the assay. If peripheral blood is collected for testing, the only acceptable sample for this assay would be to use plasma, which is the liquid portion of anti-coagulated blood. Serum would be unacceptable as it is derived from coagulated blood, which has a much higher amount of cell-free DNA due to the white blood cell lysis that occurs during the clotting process. Research conducted by Lee et al. compared the amount of cell-free DNA present in plasma vs. serum samples between 18 healthy donors.[40] They found that only two out of the 18 plasma samples were positive for cell-free DNA. The two positive samples each had approximately 40 copies/mL present. However, all 18 serum samples had high concentrations of cell-free DNA present with a mean of 20,000 copies/mL. If serum samples were used to test for *E. multilocularis* and *E. granulosus* with the use of human DNA as a control, there would be so much human DNA present that detection of either parasite would be extremely unlikely. The amount of human DNA present could also pose a problem with improperly collected plasma specimens. If venipuncture was not performed correctly, it could result in cell lysis, which would increase the amount of human DNA in the plasma sample. For

these reasons, it would be advisable to look for a alternative positive control. A possibility would be plant-based DNA, which would be unlikely to have any similarity to the genomes of *E. multilocularis* or *E. granulosus*.

Regardless of the positive control used, one does need to be included and even if it is not preferentially amplified, it could still wash out low-level detection of either organism. This research shows that it is possible to detect both organisms down to 14 copies/µl, although not all of the time. Three of the blind samples contained *E. granulosus* at 14 copies/µl, two of which were called positive. Two of the blind samples contained *E. multilocularis* at 14 copies/µl, one of which was called positive. Trying to detect anything less than 14 copies/µl would likely produce negative results. This raises the question of how many parasitic cells or how much cell-free DNA needs to be present in blood in order to detect it via PCR. The *E. multilocularis* ND5 gene is a mitochondrial gene. This means there will be at least 1000 copies of that gene in one cell. The *E. granulosus* EgG1 Hae III genomic repeat is estimated to occur 6900 times in one cell. According to the American Red Cross, the average adult has approximately 10 pints of blood in his or her body.[41] That equals approximately 4732 mL. If circulating parasitic cells are intact, the genetic contents of one cell would be more than sufficient to detect it via PCR. However, if 5 mL of blood were drawn from a patient, in order to get an average of one cell in that 5 mL, there would need to be 946 cells in circulation. In the case of *E. multilocularis*, this may be possible, but it would be unlikely with *E. granulosus*. If, however, the cells were not intact and had undergone apoptosis, there would be cell-free DNA circulating. In that case, there would need to be approximately 14,000 copies of the *E. multilocularis* ND5 gene or the *E. granulosus* EgG1 Hae III

repeat in circulation for there to be an average of 15 copies in each 5mL of blood. In the case of *E. multilocularis*, this would require the contents of 14 cells to be circulating. For *E. granulosus*, it would only need to be two cells. Given these estimates, it seems probable that *E. multilocularis* could be easily detected in serum or plasma via PCR and at least possible that *E. granulosus* could be detected despite the nature of its lifecycle, especially during the initial phase of infection before any cysts have formed. If there are intact parasitic cells present, it may be slightly more advantageous to use serum rather than plasma. Whole cells would be more likely to rupture and release their contents during the clotting process required for serum collection, making detection easier than if only cell-free DNA were present. It would also be necessary to concentrate the serum or plasma in order to be able to use the small volume of specimen required in each reaction. However, even with concentration, the more serum used in the reaction, the more probable it would be to detect any small amounts of template that may be present. During this project, each 10 µl reaction in the dilution series and blind tests contained 1 µl of forward and reverse primers, 1 µl of control template, 1 µl of either *E. multilocularis* or *E. granulosus* template, 5 µl of water and 2 µl of reaction mixture (see Materials and Methods). Sensitivity could potentially be increased by eliminating the water and using 6 µl of serum instead.

Given the need for a laboratory setting and an analyzer, this method would not be useful for field-testing at this time, especially in rural areas, but innovations in molecular methods could make it possible in the future. Millions of people worldwide are at risk for infection of either or both parasites. Because of the co-endemicity occurring in central Asia and China, there is an increasing need for an assay that can distinguish

between CE and AE more accurately than serological tests. Although more research is needed to find a more suitable positive control, the specificity and sensitivity of this assay would make it possible to diagnose patients in an inexpensive and noninvasive way. It would also make posttreatment monitoring much more accurate as cell-free DNA is eliminated from circulation within hours.[42] Antibody titers may take several years to decrease after successful treatment of AE or CE infection, making the use of serological testing unsuitable for posttreatment monitoring.[1] When parasitic tissue or extracted DNA as well as patient serum samples become available for testing, it could lead to the design of a clinical assay that would be of great value to clinicians and those at risk for AE and CE infection.

REFERENCES

1. Clinical manifestations and diagnosis of echinococcosis. UpToDate Web Site. http://www.uptodate.com/contents/clinical-manifestations-and-diagnosis-of-echinococcosis. Updated December 17, 2015. Accessed February 27, 2016.

2. DPDx - Echinococcosis - Diagnostic Findings. Centers for Disease Control and Prevention Web Site. http://www.cdc.gov/dpdx/echinococcosis/dx.html. Updated November 29, 2013. Accessed February 27, 2016.

3. Echinococcosis. World Health Organization Web Site. http://www.who.int/mediacentre/factsheets/fs377/en/. Updated February 3, 2016. Accessed February 27, 2016.

4. Agudelo Higuita NI, Brunetti E, McCloskey C. Cystic echinococcosis. *J Clin Microbiol.* 2016;54(3):518-523.

5. Edited by Eckert J, Gemmell MA, Meslin F-X, Pawlowski ZS. WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. World Organization for Animal Health and World Health Organization; 2001:265.

6. Ye J, Zhang Q, Ma L, Zheng H. Immunological Characteristics of Recurrent Echinococcosis-Induced Anaphylactic Shock. *Am J Trop Med Hyg.* 2016;94(2):371-377.

7. Zhang W, Zhang Z, Wu W, et al. Epidemiology and control of echinococcosis in central Asia, with particular reference to the People's Republic of China. *Acta Trop.* 2015;141(Pt B):235-243.

8. Tiaoying L, Jiamin Q, Wen Y, et al. Echinococcosis in Tibetan populations, western Sichuan Province, China. *Emerg Infect Dis.* 2005;11(12):1866-1873.

9. Torgerson PR. The emergence of echinococcosis in central Asia. *Parasitology.* 2013;140(13):1667-1673.

10. Yu SH, Wang H, Wu XH, et al. Cystic and alveolar echinococcosis: an epidemiological survey in a Tibetan population in southeast Qinghai, China. *Jpn J Infect Dis.* 2008;61(3):242-246.

11. Wang Q, Huang Y, Huang L, et al. Review of risk factors for human echinococcosis prevalence on the Qinghai-Tibet Plateau, China: a prospective for control options. *Infect Dis Poverty.* 2014;3(1):3.

12. Schantz PM, Wang H, Qiu J, et al. Echinococcosis on the Tibetan Plateau: prevalence and risk factors for cystic and alveolar echinococcosis in Tibetan populations in Qinghai Province, China. *Parasitology.* 2003;127 Suppl:S109-120.

13. Wang Z, Wang X, Liu X. Echinococcosis in China, a review of the epidemiology of Echinococcus spp. *Ecohealth.* 2008;5(2):115-126.

14. Feng X, Qi X, Yang L, et al. Human cystic and alveolar echinococcosis in the Tibet Autonomous Region (TAR), China. *J Helminthol.* 2015;89(6):671-679.

15. Zhang W, Zhang Z, Yimit T, et al. A pilot study for control of hyperendemic cystic hydatid disease in China. *PLoS Negl Trop Dis.* 2009;3(10):e534.

16. Gottstein B, Stojkovic M, Vuitton DA, Millon L, Marcinkute A, Deplazes P. Threat of alveolar echinococcosis to public health--a challenge for Europe. *Trends Parasitol.* 2015;31(9):407-412.

17. Vuitton DA, Zhou H, Bresson-Hadni S, et al. Epidemiology of alveolar echinococcosis with particular reference to China and Europe. *Parasitology.* 2003;127 Suppl:S87-107.

18. Massolo A, Liccioli S, Budke C, Klein C. Echinococcus multilocularis in North America: the great unknown. *Parasite.* 2014;21:73.

19. Miman O, Yazar S. [Alveolar echinococcosis in Turkey: in the light of the literature]. *Turkiye Parazitol Derg.* 2012;36(2):116-120.

20. Farrokh D, Zandi B, Pezeshki Rad M, Tavakoli M. Hepatic alveolar echinococcosis. *Arch Iran Med.* 2015;18(3):199-202.

21. Ziadinov I, Mathis A, Trachsel D, et al. Canine echinococcosis in Kyrgyzstan: using prevalence data adjusted for measurement error to develop transmission dynamics models. *Int J Parasitol.* 2008;38(10):1179-1190.

22. Li T, Chen X, Zhen R, et al. Widespread co-endemicity of human cystic and alveolar echinococcosis on the eastern Tibetan Plateau, northwest Sichuan/southeast Qinghai, China. *Acta Trop.* 2010;113(3):248-256.

23. Sarkari B, Rezaei Z. Immunodiagnosis of human hydatid disease: Where do we stand? *World J Methodol.* 2015;5(4):185-195.

24. Reiter-Owona I, Grüner B, Frosch M, Hoerauf A, Kern P, Tappe D. Serological

confirmatory testing of alveolar and cystic echinococcosis in clinical practice: results of a comparative study with commercialized and in-house assays. *Clin Lab.* 2009;55(1-2):41-48.

25. Orhun A, Müller-Stöver I, Holtfreter MC, Dedelen H, Häussinger D, Richter J. [Epidemiological and clinical characteristics of patients with echinococcosis - management in an infectiological service in Germany]. *Dtsch Med Wochenschr.* 2012;137(20):1039-1044.

26. Mamuti W, Yamasaki H, Sako Y, et al. Molecular cloning, expression, and serological evaluation of an 8-kilodalton subunit of antigen B from Echinococcus multilocularis. *J Clin Microbiol.* 2004;42(3):1082-1088.

27. Tenguria RK, Naik MI. Evaluation of human cystic echinococcosis before and after surgery and chemotherapy by demonstration of antibodies in serum. *Ann Parasitol.* 2014;60(4):297-303.

28. Benabid M, Galai Y, Nouira R, Bouchoucha S, Bouratbine A, Aoun K. Contribution of specific anti-hydatid IgG subclasses in the diagnosis of echinococcosis primary infection and relapses. *Clin Lab.* 2013;59(3-4):293-298.

29. Liu CN, Lou ZZ, Li L, et al. Discrimination between E. granulosus sensu stricto, E. multilocularis and E. shiquicus Using a Multiplex PCR Assay. *PLoS Negl Trop Dis.* 2015;9(9):e0004084.

30. Abbasi I, Branzburg A, Campos-Ponce M, et al. Copro-diagnosis of Echinococcus granulosus infection in dogs by amplification of a newly identified repeated DNA sequence. *Am J Trop Med Hyg.* 2003;69(3):324-330.

31. Xiao N, Nakao M, Qiu J, et al. Dual infection of animal hosts with different Echinococcus species in the eastern Qinghai-Tibet plateau region of China. *Am J Trop Med Hyg.* 2006;75(2):292-294.

32. Abd El Baki MH, El Missiry AM, Abd El Aaty HE, Mohamad AA, Aminou HA. Detection of G1 genotype of human cystic echinococcosis in Egypt. *J Egypt Soc Parasitol.* 2009;39(3):711-721.

33. Safa AH, Harandi MF, Tajaddini M, Rostami-Nejad M, Mohtashami-Pour M, Pestehchian N. Rapid identification of Echinococcus granulosus and E. canadensis using high-resolution melting (HRM) analysis by focusing on single nucleotide polymorphism. *Jpn J Infect Dis.* 2015.

34. Santos GB, Espínola SM, Ferreira HB, Margis R, Zaha A. Rapid detection of Echinococcus species by a high-resolution melting (HRM) approach. *Parasit Vectors.* 2013;6(1):327.

35. Chaya D, Parija SC. Performance of polymerase chain reaction for the diagnosis of cystic echinococcosis using serum, urine, and cyst fluid samples. *Trop Parasitol.* 2014;4(1):43-46.

36. Alberts B, Bray D, Lewis J, et al. *Molecular Biology of the Cell.* 3rd ed. New York (NY): Garland Science; 1995.

37. Hizem A, M'rad S, Oudni-M'rad M, et al. Molecular genotyping of Echinococcus granulosus using formalin-fixed paraffin-embedded preparations from human isolates in unusual tissue sites. *J Helminthol.* 2015:1-5.

38. Montgomery J, Wittwer CT, Kent JO, Zhou L. Scanning the cystic fibrosis transmembrane conductance regulator gene using high-resolution DNA melting analysis. *Clin Chem.* 2007;53(11):1891-1898.

39. Sundberg SO, Wittwer CT, Greer J, Pryor RJ, Elenitoba-Johnson O, Gale BK. Solution-phase DNA mutation scanning and SNP genotyping by nanoliter melting analysis. *Biomed Microdevices.* 2007;9(2):159-166.

40. Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. *Transfusion.* 2001;41(2):276-282.

41. Blood Facts and Statistics. American Red Cross Web Site. http://www.redcrossblood.org/learn-about-blood/blood-facts-and-statistics. Updated 2016. Accessed May 15, 2016.

42. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet.* 1999;64(1):218-224.