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Original Research

Testing Environmental DNA from Wolf Snow Tracks for Species, Sex, and Individual Identification

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Abstract

Monitoring elusive, relatively low-density, large predators, such as the grey wolf (*Canis lupus*), has often been accomplished by live-capture and radiocollaring. Increasingly, non-invasive methods are considered best practice whenever it is possible to use them. Recently, environmental DNA (eDNA) deposited in snow tracks was demonstrated as useful for identifying lynx (*Lynx canadensis*), fisher (*Pekania pennanti*), wolverine (*Gulo gulo*), sika deer (*Cervus nippon*), red fox (*Vulpes vulpes*), and the Japanese marten (*Martes melampus*) to species level using mitochondrial DNA (mtDNA) markers. We tested whether eDNA from fresh wolf snow tracks collected in the Superior National Forest, Minnesota, USA during winter 2019 could be used to identify species, sex, and individual. Seven of the 8 snow track samples were successfully identified to "wolf-dog" species using mtDNA, with alleles amplifying in 5 of the samples at 1 or both of loci u250 and FH2096 in the allele range for wolves for this population. None yielded enough high-quality DNA to obtain genotypes to determine individual or sex. We recommend additional field trials to determine the minimum number of tracks required per individual to obtain sufficient, high-quality eDNA, as well as collecting associated urine or blood (from estrus) when possible. If individual wolves could be identified and sexed by the eDNA in their snow tracks, researchers should be able to determine population and family or group metrics with greater precision and less effort than typically required when conducting winter scat or hair-based genetic field

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studies of low density, elusive carnivores. Furthermore, this method would have applications to other areas of research and management, such as hunting quota determinations, validation of field methods and, in particular regards to wolves and other predators, livestock depredation issues. Overall, this technique holds significant future promise as field and laboratory methods are further refined for greater precision and optimized regarding the varying collection, filtration, and extraction protocols for different species in various environments.

Key Words: *Canis lupus*, Carnivore, eDNA, Genetic Survey, Non-invasive, Population Monitoring.

INTRODUCTION

Monitoring elusive, relatively low-density, large predators, such as the grey wolf (*Canis lupus*), has often been accomplished by live-capture and radiocollaring (Mech *et al.* 1998). Similarly, since the late 1960s, wolves in the Superior National Forest, Minnesota, USA have been radiocollared as part of a long-term U.S. Geological Survey (USGS) wolf-prey study (Mech 2009). Wolves are counted during direct visual observations of radioed wolves and their packmates during winter. Recent advances in genetics and other technology may allow for winter wolf population counts, without relying on live-captures of wolves. Increasingly, such non-invasive methods are considered best practice whenever it is possible to use them (Long *et al.* 2012) for a variety of reasons often including ethical issues.

Wild wolves have been studied using a number of non-invasive methods, such as traditional howling (Harrington and Mech 1982), scent-post (Sargeant *et al.* 1998), and snow track surveys (Kojola *et al.* 2014), and more recently, camera trapping (Galaverni *et al.* 2012) and non-invasive genetics (Lucchini *et al.* 2002). Such non-invasive genetic surveys have most commonly used scat (Stenglein *et al.* 2010; Marucco *et al.* 2012), but also hair (Ausband *et al.* 2011; Stansbury *et al.* 2014), urine in snow (Valiere and Taberlet 2000), and blood in snow from female wolves in estrus or from body injuries (Scandura 2005).

Recently, another technique in genetic surveying has emerged as promising. Environmental DNA (eDNA) is DNA shed into the environment from an organism in the form of sloughed skin cells, metabolic waste, or injured bodily tissue (Taberlet *et al.* 2012; Kelly *et al.* 2014). Environmental DNA has been applied frequently in aquatic systems to determine occurrence of species (e.g., Hunter *et al.* 2018, 2019) and abundance (e.g., Lacoursière-Roussel *et al.* 2016). The technique has also been applied to analyze eDNA in soil and even air (Bohmann *et al.* 2014; Deiner *et al.* 2017; Leempoel *et al.* 2020). A relatively early study of eDNA in mammals was successful in recovering mtDNA (mitochondrial DNA) from an arctic fox (*Vulpes lagopus*) footprint in the snow (Dalén *et al.* 2007). Later, the use of eDNA deposited in snow tracks was demonstrated as also useful for identifying lynx (*Lynx canadensis*), fisher (*Pekania pennanti*), and wolverine (*Gulo gulo*) to species level in North America (Franklin *et al.* 2019). Another study in Japan illustrated its utility in identifying sika deer (*Cervus nippon*), red fox (*V. vulpes*), and the Japanese marten (*Martes melampus*) to species level (Kinoshita *et al.* 2019).

In the context of the long-term USGS wolf-prey study, we were interested in testing whether eDNA from numerous fresh snow tracks from a single wolf could be used to identify the wolf tracks to species level, sex and individual. If individual identification was possible, it would mean that winter wolf counts could be accomplished using this non-invasive method and population estimates with error (rather than just a minimum count) could be generated (e.g., Pennell *et al.* 2013), along with many other parameters of interest, if the data were analyzed in a capture-mark-recapture framework (see Williams *et al.* 2002). Current snow track survey methods (that do not rely on radiotelemetry for individual wolf identification) can sometimes result in double-counting because pack wolves do not always travel together and tracks in different locations are sometimes counted as from different individuals when they could be from the same wolves (Barber-Meyer, unpublished data). Furthermore, this new technique would have applications to other areas of research and management, such as validation of field techniques (Kinoshita *et al.* 2019), hunting quota determinations (Hellström *et al.* 2019), and, in particular regards to wolves and other predators, livestock depredation issues. Thus, we tested whether eDNA from fresh wolf snow tracks collected in the Superior National Forest, Minnesota, USA during winter 2019 could be used to identify species, sex, and individual.

STUDY AREA

Field crews collected wolf snow tracks as part of a long-term wolf-prey research project (Mech 2009). The USGS Minnesota Wolf and Deer Project study area included 2,060 km^2, comprised of both wilderness (Boundary Waters Canoe Area Wilderness, BWCAW) and non-wilderness, in the Superior National Forest, Minnesota, USA (48° N, 92° W - see Nelson and Mech 1981 for a detailed description). From

mid-November through mid-April snowfall averages 150 cm (Nelson and Mech 2006). Average monthly temperatures range from approximately 4 to 18°C during May – October and approximately -18 to 2°C during November – April (Heinselman 1996). The area is a transition zone between the hardwood forests typical of areas farther south in Minnesota and the southern boreal forest of neighboring Ontario, Canada (Pastor and Mladenoff 1992). Forest overstory is predominately conifers, e.g., jack pine (*Pinus banksiana*), black spruce (*Picea mariana*), white spruce (*P. glauca*), white pine (*P. strobus*), red pine (*P. resinosa*), balsam fir (*Abies balsamea*), white cedar (*Thuja occidentalis*), and tamarack (*Larix laricina*), interspersed with quaking aspen (*Populus tremuloides*) and white birch (*Betula papyrifera*) (Heinselman 1996). Topography includes rocky ridges, swamps, numerous lakes, and uneven upland ranging from 325 to 700 m above sea level (Heinselmann 1996). Generally, in the southwestern portion of our study area, white-tailed deer (*Odocoileus virginianus*) are the wolf's main prey, and in the northeastern, the primary large ungulate (wolf prey) is moose (*Alces americanus*) (Frenzel 1974; Mech 2009). During winter 2019, mean resident-pack wolf (not including transients or lone wolves) density was 13/1,000 km^2 (Barber-Meyer, unpublished data). The sex ratio of wolves in our study area approximates parity (Mech 2009). Due to motor restrictions in the BWCAW, field crews were only able to access a subset of the broader USGS study area (mainly the southern and western portions) by truck or snowmobile during winter (Figure 1). It was in this subset area that wolf snow tracks were collected.

MATERIAL & METHODS

Track collection, storage, and transfer

During late February through mid-late March 2019 snow track samples from individual wolves were opportunistically collected by crews conducting other field work. Because samples were collected over ~1 month, some of them could have been from the same wolves. During the sample collection period, the daily (24-h) average maximum temperature was -2.70ºC, the daily average temperature was -7.94ºC, and the daily average minimum temperature was -14.75ºC (KELO automated weather station at Ely, MN, USA, http://www.wunderground.com/history/monthly/us/mn/ely/KELO/date/2109-5, accessed on 16 April 2020). Daily (24-h) maximum, average, and minimum wind speeds during this period were 24.29 kmph, 10.30 kmph, and 1.02

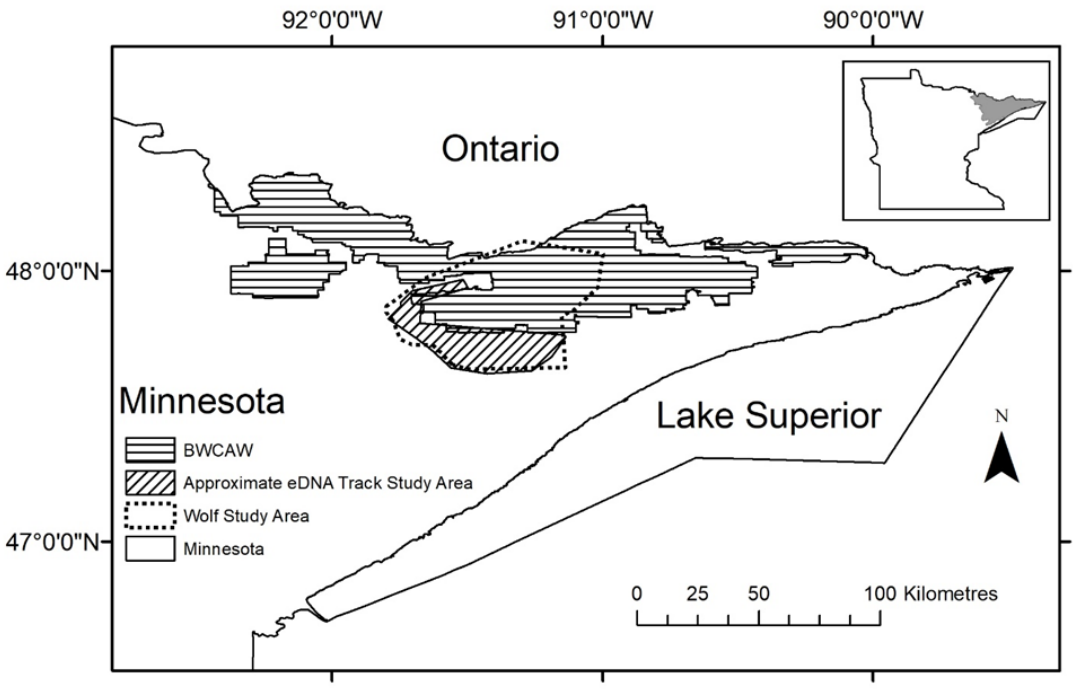

Figure 1. Map showing the area where wolf snow tracks were collected during winter 2019 in northeastern Minnesota, USA. The larger U.S. Geological Survey Wolf Study Area and the Boundary Waters Canoe Area Wilderness (BWCAW) are also delineated.

kmph, respectively (KELO automated weather station at Ely, MN, USA, http://www.wunderground.com/history/monthly/us/mn/ely/KELO/date/2109-5, accessed on 16 April 2020). While snowmobiling or skiing / snowshoeing on trails, crews scouted for trails of tracks made by individual wolves (i.e., no other wolves stepped in the line of tracks). When a crew member located a fresh (i.e., pad and claw details still well-defined, crisp edges, track not windblown or obviously melted, generally <48 h old) trail of wild wolf tracks that were confirmed to be from a single individual, they collected 10 individual snow tracks from the single wolf's trail (Hellström *et al.* 2019; Kinoshita *et al.* 2019). New latex gloves were worn for each sample and the edge of a sterile 120-ml specimen cup was used to scoop 5 tracks into the cup. Care was taken to ensure only the surface layer of snow was collected under the track itself to minimize debris and the volume of water relative to potential eDNA (Franklin *et al.* 2019). Two specimen cups were filled (5 tracks per cup) for each trail of tracks for a total of 10 tracks per sample. We used ArcMap v. 10.6.1 and geospatial layers from the Minnesota Geographic Data Clearinghouse (http://www.mngeo.state.mn.us/chouse/data.html) to generate the polygon from where tracks were collected (Figure 1).

Samples from 8 trails of wolf snow tracks (plus 1 large snow sample, associated with a wolf track sample, included only wolf urine in snow and was collected in a new, large gallon Ziploc bag) were stored in a cooler or packed in snow until arrival at field headquarters later that day. Once at headquarters, tracks were stored in a -20ºC freezer until shipping. Samples were shipped in a cooler on dry ice overnight during late May 2019 to the National Genomics Center for Wildlife and Fish Conservation (U.S. Forest Service, Missoula, Montana, USA) for genetic analysis.

Genetic analysis

Snow in the containers from the 9 snow samples (8 wolf track trails and also 1 bag of urine in snow) were processed following methods described in Franklin *et al.* (2019). Generally, each sample (2 specimen cups containing 10 tracks total per sample and the snow urine sample) was melted at room temperature and filtered through a Whatman® 1827-047 Microfiber Filter with a 1.5-micron mesh size. Paired samples were run through the same filter. DNA was extracted from the filter using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following methods in Franklin *et al.* (2019). The 9 samples were tested using a panel of 9 variable microsatellite loci: Cph5, u250, FH2088, FH2096, FH2054, Pez17, c20.253, FH2001, and FH2079 (Ostrander *et al.* 1993; Fredholm and Winteroe 1995; Francisco *et al.* 1996), plus a sexing locus (K9-SRY; Kun *et al.* 2013). Samples were tested using the multi-tube approach in order to determine concordance of alleles (McKelvey and Schwartz 2004). Quantitative-polymerase chain reaction (qPCR) was conducted for the presence of *Canis lupus* mtDNA using the respective assay described in Knapp *et al.* (2016). Each snow-track eDNA extract was analyzed using a QuantStudio 3 Real-time PCR Instrument (Life Technologies) in 15 µl reactions containing 7.5 µl Environmental Master Mix 2.0 (Life Technologies), 900 nM each forward and reverse primer, 250 nM of probe, 4 µl eDNA extract, and PCR-grade water for the remaining volume. Thermocycler conditions consisted of initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. The assay used was designed for domestic dog (*Canis lupus familiaris*) and detects the presence of wolf or dog mtDNA (but does not distinguish between the species). Each snow-track eDNA extract was analyzed in triplicate and DNA from 2 wolf scats from the study area, previously confirmed by genetic analysis to be *Canis lupus* sp., were used as positive controls in the qPCR assays. A no-template control that used molecular grade distilled water in place of a DNA template was also included in the analysis.

RESULTS

Field crews collected snow-track samples during late February – March 2019 from up to 9 wolves consisting of 10 single tracks each (plus 1 bag of snow urine associated with nearby tracks) (Table 1). Some of the samples could have been from the same individuals, and snow-track sample USGS_B and snow-urine sample USGS_BU likely were (Table 1). Positive detections for *Canis lupus* sp. mtDNA were obtained from 8 of the 9 samples, (positive in all 3 replicates, Table 1). Mean Ct value (cycle threshold) was lowest (indicating higher amounts of target mtDNA) in the snow sample that contained only wolf urine (no tracks) and was similar to Ct values obtained from the positive control, wolf-scat samples. Mean Ct values from the other 7 samples (all snow-tracks) that yielded mtDNA ranged from 32.43–35.52. These values are similar to those obtained by the same genetics lab for mtDNA for another canid species, coyote (*C. latrans*), with Ct values ranging from 25.71– 36.35 (Table 2).

None of our samples yielded enough high-quality nuclear DNA (nDNA) to obtain concordant alleles from the replicates, nor a full genotype to determine individual. Also, none of the samples yielded PCR products at the sexing locus. However, because we did not obtain quality nDNA for genotyping, we did not have confidence in the sexing locus results. The sexing locus test assesses the sex determining region on the Y-chromosome and, therefore, only males

Table 1. Results of eDNA analysis of wild wolf snow track samples collected in the Superior National Forest, Minnesota, USA during winter 2019. Snow track samples were evaluated for the presence of *Canis lupus* DNA using quantitative-polymerase chain reaction (qPCR) and PCR amplification at 2 of the microsatellite loci.

		qPCR assay *Canis lupus* sp.			Microsatellite amplification	
Sample ID	**Sample type**	***Canis lupus* DNA detected?**	**Wells amplified**	**Mean Ct[1]**	**u250**	**FH2096**
USGS_S45	Wolf scat – control	Y	3	22.93	y	y
USGS_S176	Wolf scat – control	Y	3	27.7	y	y
USGS_A	Wolf snow tracks	Y	3	35.52	n	n
USGS_B	Wolf snow tracks	Y	3	32.43	y	n
USGS_C	Wolf snow tracks	Y	3	35.27	n	n
USGS_D	Wolf snow tracks	Y	3	35.37	y	n
USGS_E	Wolf snow tracks	Y	3	33.4	y	y
USGS_F	Wolf snow tracks	N			n	n
USGS_G	Wolf snow tracks	Y	3	32.8	y	y
USGS_H	Wolf snow tracks	y	3	35.38	y	y
USGS_BU[2]	Wolf urine in snow	y	3	25.74	y	y

[1]Lower Mean Ct values (cycle threshold) indicate higher amounts of target mtDNA.
[2]Collected at the same location and on the same date as USGS_B snow track sample. Snow sample USGS_BU contained only wolf urine (no tracks). These are likely from the same animal.

Table 2. Table 2. Coyote (*Canis latrans*) mtDNA detected in saliva swabs (Wengert et al. 2013) collected from bighorn sheep (*Ovis canadensis*) carcasses in 4 predation cases. Analyses were conducted by the U.S. Forest Service, National Genomics Center for Wildlife and Fish Conservation, Missoula, Montana, using quantitative-polymerase chain reaction (qPCR) methods. These values are presented for comparison with our wolf (*C. lupus*) Ct values (Table 1).

Predation case	**Saliva swab location**	**Coyote Ct value**
Case#1	Leg puncture	36.3510
Case#1	Rib A	33.2330
Case#1	Rib B	30.9255
Case#2	Rib A	36.1109
Case#2	Rib B	32.3905
Case#2	Shoulder A	27.8479
Case#2	Shoulder B	29.9969
Case#3	Bite mark A	25.7060
Case#3	Bite mark B	26.9355
Case#3	Bite mark C	26.7613
Case#3	Bite mark D	26.7167
Case#4	Skin puncture A	30.4694
Case#4	Skin puncture B	31.3965

yield a PCR product. Thus, females and samples from males with poor DNA are indistinguishable. Nevertheless, 6 of the 9 paired snow samples (including the urine sample) [5 of the 8 paired snow track samples] amplified alleles at 1 or both of loci u250 and FH2096 in the allele range for wolves for this population, though allele amplifications were not consistent between replicates for any sample, and in many cases, only 1 of the replicates had an allele (Table 1).

DISCUSSION

Non-invasive sampling methods are increasing in diversity, along with advances in technology and statistical analysis (Long *et al.* 2012). Non-invasive genetic surveys represent a rapidly evolving area as the technology available changes so quickly (e.g., Hellström *et al.* 2019). In particular, eDNA has opened new possibilities of quickly and accurately screening for occurrence of species important in conservation (Hunter *et al.* 2018, 2019) and estimating abundance of aquatic species (Lacoursière-Roussel *et al.* 2016). Outside of the traditional aquatic environment, mammalian eDNA from snow tracks have been amplified with conventional PCR methods and species-level identifications accomplished via DNA metabarcoding and Sanger sequencing analyses (Kinoshita *et al.* 2019). Further, eDNA coupled with qPCR has been successfully used to confirm species from mammalian snow tracks using mtDNA, to reduce camera trap species misidentification from snow column analysis, and to obtain sufficient genetic material for species identification from hair samples that overwintered and did not have DNA suitable for conventional PCR (Franklin *et al.* 2019).

Because of the relatively higher abundance and encounter rate of tracks versus scat or hair (i.e., increased sample size and recapture rate), if individual wolves (or other large carnivores) could be identified and sexed by the eDNA in their snow tracks, researchers should be able to determine metrics such as family (pack) counts, survival, reproduction, dispersal, predation, and population abundance and trends with greater precision and less field effort than is typically required when conducting traditional winter (periods of snow cover) scat or hair-based genetic surveys (Hellström *et al.* 2019; Kinoshita *et al.* 2019). This kind of information should also lead to better management information for setting hunting quotas for large carnivores (Hellström *et al.* 2019). Similarly, other field techniques that relate to species identification of footprints in snow (such as occupancy, quantitative track measurements combined with snow conditions to identify species) could be validated using this approach (Kinoshita *et al.* 2019).

The ability to identify individuals and sex should also lead to greater specificity in dealing with large carnivore livestock depredation conflicts. Although most depredations in our wider region occur during the snow-free period (e.g., as in Michigan per Edge *et al.* 2011), recent work indicates that eDNA from soil samples is also useful in identifying terrestrial mammals (e.g., Leempoel *et al.* 2020). The ability to identify individuals present at the depredation through tracks at the scene and match that eDNA with the individual(s) targeted for negative conditioning or removal, should increase the public's tolerance for sometimes controversial management actions such as lethal removal. Already, swabs of carnivore saliva deposited on livestock carcasses during feeding have been successfully analyzed to obtain sufficient DNA for genotype, sex, and individual identification of the wild animal (particularly for mountain lion, *Puma concolor*) (Pilgrim, unpublished data). Feeding trials of captive predators have demonstrated similar success (Piaggio *et al.* 2019).

Unfortunately, none of the wolf track samples in this study yielded adequate eDNA to identify sex or individual. Yet, some of the wolf track samples yielded some amplification in the wolf-allele range indicating the presence of wolf nDNA. Of course, a primary challenge in using nDNA is the significantly fewer copies of nDNA relative to mtDNA per eukaryotic cell (Hellström *et al.* 2019). Therefore, improving field collection and laboratory methods to recover as many cells as possible from snowtracking should produce better results (for additional recommendations see also Franklin *et al.* 2019, Hellström *et al.* 2019, and Kinoshita *et al.* 2019). Use of taxon-specific microsatellite primers that generate smaller products (i.e., <100bp) may also help improve genotyping success due to greater specificity (Hellström *et al.* 2019; Kinoshita *et al.* 2019). Because one of the primary limitations is the amount of high-quality DNA recovered, we recommended collecting and comparing eDNA from samples of 20-30 tracks and also 40-50 tracks per individual (when possible) to begin to determine the minimum number of snow tracks required (as the 10 tracks per individual that field crews collected in this study did not yield sufficient, high-quality eDNA). Additional research should also be conducted to determine how fresh tracks must be and how environmental conditions influence eDNA (e.g., freeze / thaw cycles, temperature thresholds, exposure to sunlight) (Hellström *et al.* 2019). Also, because the snow sample with urine yielded the most DNA, whenever possible, researchers should collect urine (and blood from female wolves in estrus) when in association with snow tracks to maximize their ability to determine individual and sex (Valiere and Taberlet 2000; Scandura 2005). Encouragingly, others have reported obtaining nDNA from lynx snow tracks but, as of yet, obtaining a full genotype for individual ID has not been demonstrated (Hellström *et al.* 2019). Thus, overall, this

technique holds significant future promise as field and laboratory methods are further refined for greater precision and optimized regarding the varying collection, filtration, and extraction protocols for different species in different environments (Hellström *et al.* 2019).

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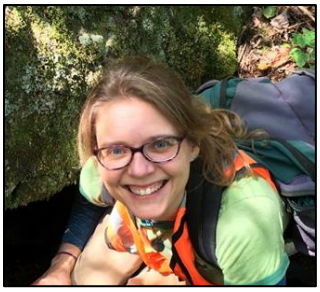

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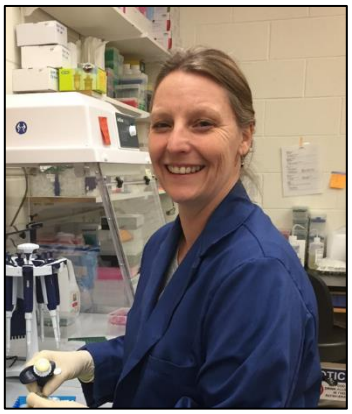

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