

Detection of *Myocoptes musculus* in Fur Swab and Fecal Samples by Using PCR Analysis

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Current methods for detecting mites in mouse colonies have limitations in terms of cost, accuracy, and throughput. To address these limitations, we developed PCR assays to detect *Myocoptes musculus* in fecal samples. Using a newly generated ribosomal RNA sequence of *M. musculus* (MC28S), we developed PCR and qPCR assays capable of detecting *M. musculus* mites or eggs ingested during grooming. To determine our ability to detect mites, we tested fur swabs and feces from mouse colonies experimentally infested with *M. musculus* and *Demodex musculi*, 2) *Myobia musculi* and *Radfordia affinis*, 3) *M. musculus* and *M. musculi*, and 4) no mites (negative control). The MC28S PCR and qPCR assays positively identified *M. musculus* in groups 1 and 3. The MC28S PCR assay detected *M. musculus* in 9 of 10 fecal samples from known-positive animals, whereas the qPCR assay correctly identified *M. musculus* in all 10 fecal samples. To our knowledge, this report is the first description of PCR-based detection of murine mites in feces. By eliminating the need for pelt examinations, mite detection from fecal samples can facilitate mite detection in sentinel or quarantine programs.

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Successful diagnosis of the common mites *Myocoptes musculus*, *Myobia musculi*, *Radfordia affinis*, and *Demodex musculi* is important for the management of research rodent colonies due to the organisms' ability to persistently infect mice, leading to potential clinical disease and research complications. These adverse clinical signs and research-affecting effects include—but are not limited to—ulcerative dermatitis, self-excoriation, erythema, alopecia, reduced weight gain, pruritus, decreased life span, altered IgE levels, and changes in inflammatory cytokines.^{5,12,14,17,23} *M. musculus* is a parasitic fur mite that populates the inguinal region, abdomen, and dorsum, as well as the head and neck.^{4,20} Other common fur mites, such as *M. musculi* and *R. affinis*, infest the skin and hair shafts of the intrascapular and dorsal cervical regions, whereas *D. musculi* primarily resides in the pilosebaceous unit of mice.^{19,26}

Various methods of detecting murine mites are well established in the literature; however, each methodology presents particular advantages and disadvantages in terms of accuracy, efficiency, ease of use, cost, time, and labor. Some of the most common diagnostic methods for detecting murine mites include fur plucks, deep skin scrapings, superficial skin scrapings, tape impressions, pelt exams, IgE detection, and PCR-based assays on samples from fur, cages, and air exhaust plenums.^{11,13,18,22,23} Observation-based methods, such as fur plucks, skin scrapings, and pelt exams, involve simple collection techniques that are easily performed by a trained technician. In a recent study, deep skin scrapings and a 18S rRNA PCR assay performed equally well in detecting *Demodex musculi* on laboratory mice.¹⁸ However, disadvantages of these techniques include 1) the necessity to sample multiple sites per mouse,¹⁸ 2) the need for special-

ized training for microscopic detection, and 3) low throughput. Variability between users in terms of collection or detection can affect the accuracy of diagnosis. In recent years, murine mite detection has shifted toward PCR-based assays.^{2,8,11,13,18,22} PCR testing can increase throughput and may reduce concerns regarding sampling error, because swabbing techniques cover a greater surface area than a single pluck or scraping. In addition, the sensitivity of PCR assays have enabled scientists to detect mites at the cage and ventilated-rack levels, allowing more flexibility in sampling.^{6,11,22} However, inhouse PCR testing requires specialized equipment, whereas sending samples to outside commercial diagnostic labs may be cost-prohibitive for some laboratories. To simplify mite detection, our goal was to develop PCR-based assays for *M. musculus* that could detect mite DNA in fecal samples, given the assumption that mites or mite eggs (or both) are ingested during grooming. To accomplish this goal, we generated ribosomal RNA PCR products from *M. musculus* for next-generation sequencing using generic mite primers. Using the newly generated DNA sequences, we developed *M. musculus*-specific PCR assays. After validating these primers on fur swabs, we demonstrated the detection of *M. musculus* in fecal samples from mice with known mite infestations.

Materials and Methods

Collection of mite samples from mouse colonies. Samples used in this study originated from mice infested during naturally occurring outbreaks at Memorial Sloan Kettering. Sentinel Tac:SW and NOD.Cg-Prkd^{scid} Il2rg^{tm1Wjl}/SzJ mice were cohoused with known-infested NSG mice to expand each group. Mice in each group were confirmed as mite-positive by using microscopic methods (that is, superficial skin scrapes) and pelt swabs sent to a commercial laboratory for confirmation by PCR analysis. For negative controls, culled aged breeders free of known murine viruses, *Helicobacter* spp., mites, and other parasites were sampled postmortem at the Massachusetts Institute of Technology.

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Table 1. Primers used in PCR amplifications for *M. musculus*

Target	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe	Program
rRNA (sequencing)	MC_18S_seq F1 5' AGG TGA AAC CGC GAA TGG CTC A 3'	MC_28S_seq R1 5' AAC GCC GCA TCT CTT GAA CGC 3'		MCrRNA
rRNA (sequencing)	MC_18S_seq F1 5' AGG TGA AAC CGC GAA TGG CTC A 3'	MC_28s_seq_R2 5' AGT ACA TCA CCC CGG CAG GT 3'		MCrRNA
rRNA (sequencing)	MC_18S_seq F2 5' CGC ACG CGC GCT ACA CTG AAA A 3'	MC_28s_seq_R2 5' AGT ACA TCA CCC CGG CAG GT 3'		MCrRNA
28S rRNA (PCR or qPCR)	MC28s F1 5' GAG ACC GAT AGC AAA CAA GTA C 3'	MC28s R1 5' CAT AGC CTC CGA AAA GAC CCA CTG 3'	5' Cy5-GCC CTT CGG TCG ATT CAA TC-IBRQ 3'	MITE58 or default qPCR

Table 2. Pairwise identity of mite sequences available in NCBI compared with sequenced *M. musculus* (MC) contig

	18S rRNA sequence used	18S rRNA % pairwise identity with MC contig	28S rRNA sequence used	28S rRNA % pairwise identity with MC contig
<i>M. musculus</i>	JF834893 (partial, 935 bp)	98.4% ^a	not applicable	not applicable
<i>M. japonensis</i>	JQ000333	97.2%	JQ000641	95.7%
<i>D. pteronyssus</i>	JQ000249	93.7%	JQ000557	89.9%
<i>D. farinae</i>	JQ000247	92.9%	JQ000555	89.4%
<i>M. musculi</i>	JF834895	83.0%	JF934703 (partial, 444 bp)	68.7%

^a14 mismatches in first 28 bases of JF834893

Mice at Memorial Sloan Kettering were housed on autoclaved aspen chip bedding (PWI Industries Canada, Quebec, Canada) in solid-bottom polysulfone 'shoebox' containment cages maintained in an IVC system (Thoren Caging Systems, Hazelton, PA). γ -irradiated feed (LabDiet 5053, PMI, St Louis, MO) and acidified water (pH 2.5 to 2.8 in a polysulfone bottle [Techniplast, West Chester, PA]) were provided without restriction. Mice at the Massachusetts Institute of Technology were maintained in static microisolation cages and were fed a commercial chow (Prolab RMH3000, PMI Nutrition International, Richmond, IN) without restriction. At both institutions, 12:12-h light:dark photoperiods were maintained. Room temperature was maintained at 72 ± 2 °F (22.2 ± 1.1 °C) and relative humidity at 30% to 70%. The animal care and use programs at Memorial Sloan Kettering and the Massachusetts Institute of Technology are accredited by AAALAC, and all animals were maintained in accordance with the recommendations provided in the *Guide for the Use and Care of Laboratory Animals*.¹⁰ The use of these animals was approved by the respective IACUC.

The experiments described were completed in 2 phases requiring separate sample collections. First, fur plucks were collected and analyzed through microscopy to identify samples containing *M. musculus* mites and eggs. DNA from *M. musculus*-positive samples was used to generate PCR templates for sequencing *M. musculus* rRNA genes to develop *Myocoptes*-specific PCR primers. The second phase of experiments consisted of validating the *M. musculus*-specific primers using fecal and fur swab samples. For the validation studies, mouse samples were collected from negative control mice and those infested with different species of mites. Group 1 mice were infested with *M. musculus* and *D. musculi*; group 2 mice were infested with *M. musculi* and *R. affinis*; and group 3 mice were infested with *M. musculus* and *M. musculi*. For groups 1 through 3, a Sticky Head Cotton Bud swab (Daiso, Hiroshima, Japan) was used to sample the entire mouse body by rubbing against the direction of fur growth. The swab was

rotated during collection to maximize sampling of mites, mite eggs, and mite debris on the fur. Given the preferred niches of murine mites, we heavily sampled the regions around the ears, back of the neck, periscapular area, head (especially under the chin), inguinal area, and base of the tail. In addition, paired fecal samples were collected from swabbed mice to determine whether mite DNA was present in their feces. A total of 5 mice were sampled for each group.

DNA extraction. For the DNA extraction from fur plucks in the sequencing experiments, *M. musculus* samples were processed by using the *Isolation of Genomic DNA from Tissues* protocol provided with the QIAmp DNA Micro Kit (Qiagen, Hilden, Germany). Samples were incubated for 5 h to complete the lysis process, and carrier RNA was added to buffer AL to maximize yield. For the validation studies using fur swabs, DNA was extracted by using the High Pure PCR Template Preparation Kit (version 20, Roche Molecular Systems, Mannheim, Germany) according to the manufacturer's instructions. Fecal DNA was extracted with the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. DNA from all samples was stored at -20 °C until tested.

Sequencing the 18S and 28S rRNA genes of *M. musculus*. To generate PCR products from *M. musculus*, ribosomal (18S and 28S rRNA) genes from the suborder *Psoroptidia* were aligned by using Geneious software (Biomatters, Auckland, New Zealand). 18S rRNA sequences from this suborder included *Myocoptes japonensis* (NCBI accession nos. EU152597.1 and JQ000333.1), *Sturnophagoides bakeri* (JQ000246.1), *Dermatophagoides farinae* (JQ000247.1), and *Dermatophagoides pteronyssinus* (JQ000249.1). 28S rRNA sequences included *M. japonensis* (EU152723.1 and JQ000641.1), *Sturnophagoides bakeri* (JQ000554.1), *D. farinae* (JQ000555.1), and *D. pteronyssinus* (JQ000557.1). Based on consensus regions, forward and reverse primers spanning the 18S and 28S rRNA region were designed using Primer3.²⁸ These generic *Psoroptidia* primers (Table 1) were used to generate 18S and 28S rRNA PCR products from *M. musculus* DNA by us-

ing Phusion High-Fidelity DNA Polymerase (New England Bio Labs, Ipswich, MA). The MCrRNA thermocycling program used to generate PCR products for MiSeq sequencing consisted of: 30 s at 98 °C for initial denaturation; 36 cycles of denaturation at 98 °C for 10 s, annealing at 59 °C for 30 s, and extension at 72 °C for 1 min; final extension at 72 °C for 7 min; and a hold at 6 °C thereafter.

PCR products were pooled for sequencing at the BioMicro Center at the Massachusetts Institute of Technology. Briefly, the sequencing library was prepared by using NexteraXT and sequenced on an MiSeq sequencer (Illumina, San Diego, CA) to generate two 150-bp paired reads by using the 300-cycle MiSeq Reagent Kit (version 2; MS-102-2002, Illumina, San Diego). FASTQC was used to check read quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Paired reads ($n = 3,118,749$) were generated but were down-sampled to 3000 reads for assembly by using the Trinity Assembler⁷ for rRNA gene assembly.

Primer design and PCR reactions. Multiple primer pairs were designed to target the newly sequenced 28S rRNA gene of *M. musculus* (Table 1). PCR reactions were run in a thermocycler (MJ Research PTC-200, Gradient Thermal Cycler, Marshall Scientific, Hampton, NH) using the Phusion Hot-Start Flex 2× master mix (New England Bio Labs, Ipswich, MA), water, 250 nM each of forward and reverse primers, and 3 μL DNA. The following conditions were used for the PCR reactions (MITE58): initial denaturation for 3 min at 94 °C; 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min and a hold at 6 °C thereafter. Select PCR products from these reactions were confirmed by Sanger sequencing at a commercial laboratory (Quintara Biosciences, Boston, MA).

Cloning PCR products to generate standards. A positive control was developed by cloning *M. musculus* PCR products generated by using MC28S into the pCR 2.1 TOPO plasmid vector by using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, plasmids were transformed into Transform One TOP10 cells (Invitrogen) by using the manufacturer's chemical transformation protocol. Eight *M. musculus*-transformed colonies were selected and cultured in medium containing ampicillin and X-gal. The PureLink Plasmid Miniprep Kit (Invitrogen) was then used to isolate the plasmid from the bacteria. All colonies underwent PCR verification to confirm the presence of *M. musculus*-specific inserts in the plasmids. Three colonies yielding *M. musculus*-specific bands at 178 bases were selected. To generate standard curves, we used the known product sizes and the vector size of 3900 bases and estimated the number of plasmid copies of *M. musculus* as $(\text{mass of plasmids [in ng]} \times 6.0221 \times 10^{23} \text{ molecules/mole}) / (4078 \text{ bases} \times 650 \text{ g/mole} \times 10^9 \text{ ng/g})$. DNA concentration was quantified (ThermoFisher Scientific, Waltham, MA), and standards for qPCR reactions were generated by diluting *M. musculus* plasmid stocks.

qPCR reaction. qPCR reactions included 2× PrimeTime Gene Master Mix (Integrated DNA Technologies, Coralville, IA), Primetime qPCR Primer/Probe Mix for MC_28S (*M. musculus*), water, and 5 μL DNA. Technical duplicates or triplicates were placed in the wells of a MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (Applied Biosystems, Foster City, CA) and sealed (MicroAmp Optical Adhesive Film, Applied Biosystems) prior to running on a Fast Real-Time thermocycler (model 7500, Applied Biosystems); the default qPCR program (20 s at 95 °C; 40 cycles at 95 °C for 20 s and 30 s at 60 °C). To

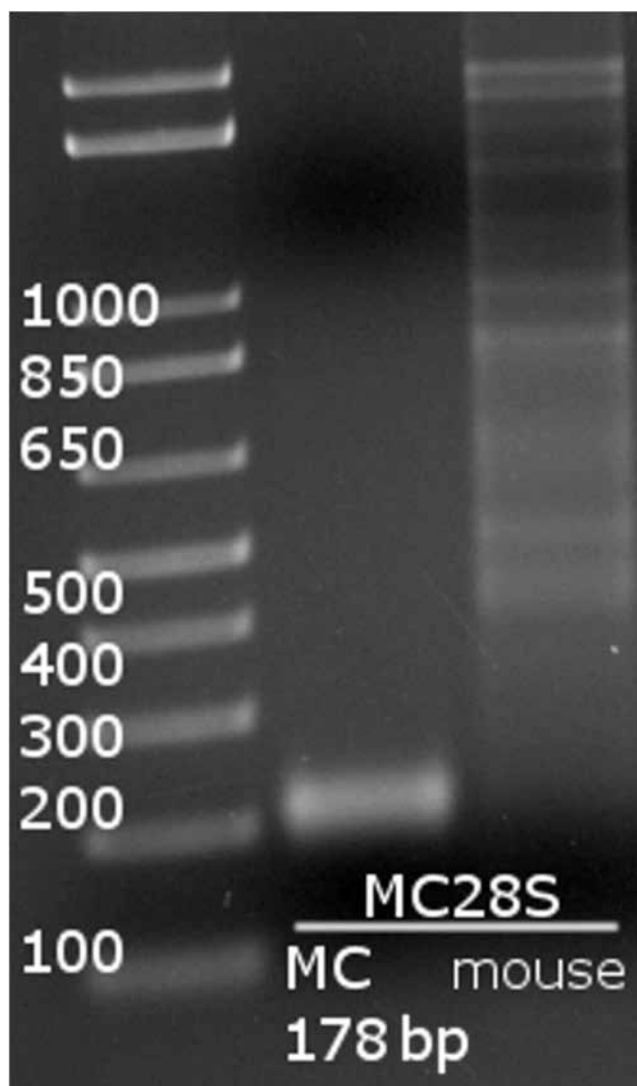


Figure 1. PCR detection using MC28S primers. MC28S primers generate a 178-bp product in the presence of *M. musculus* DNA. Lane 1, 1-kb ladder; lane 2, MC28S primers with *M. musculus* DNA; lane 3, MC28S primers with mouse DNA.

determine the limit of detection for the MC28S qPCR assay, 10-fold dilutions ranging from 10^6 to 0 copies of *M. musculus* plasmid were replicated 8 times over 4 separate runs. To establish the limit of detection, the lowest dilution level at which all 8 replicates were positive was determined.^{9,15,16} Linear regression analysis was performed by using Excel 2016 (Microsoft, Redmond, WA); the correlation coefficient was calculated by using the CORREL function, and the amplification efficiency was computed as $10^{(-1/\text{slope})} - 1$.

Results

***M. musculus* ribosomal RNA sequencing.** Prior to this study, the only published sequence of *M. musculus* was a 935-bp partial sequence from 18S rRNA. Here we obtained sequences for the 18S and 28S rRNA genes within the sub-order *Psoroptidia* to which *Myocoptes* belong, for the closely related *Myocoptes japonensis* and several dust mite species (*Dermatophagoides* spp. and *Sturnophagoides bakeri*). Alignment of both 18S and 28S rRNA genes revealed conserved regions. The compared regions among these species were 93.8% and 86.1% identical for the 18S and 28S rRNA genes, respectively.

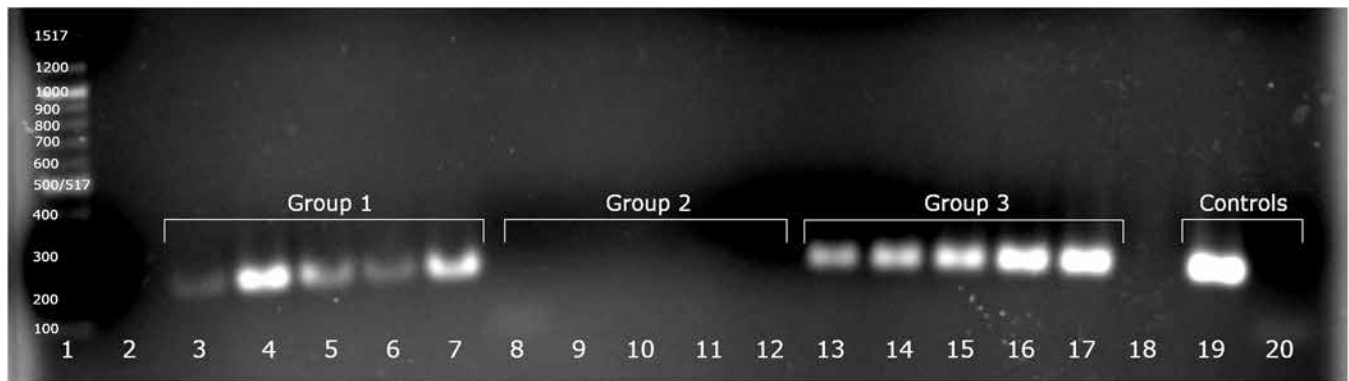


Figure 2. PCR detection of *M. musculus* by using MC28S primers against fur swab samples. Lane 1, 100-bp ladder; lanes 3 through 7, samples containing *M. musculus* and *D. musculi* (group 1); lanes 8 through 12, samples containing *M. musculi* and *R. affinis* (group 2); lanes 13 through 17, samples containing *M. musculus* and *M. musculi* (group 3); lane 19, positive control; and lane 20, negative control.

Table 3. Results of *M. musculus* PCR and qPCR assays

Group	Infested mites	PCR		qPCR	
		Swab	Feces	Swab	Feces
1	<i>M. musculus</i> and <i>D. musculi</i>	5	5	5	5
2	<i>M. musculi</i> and <i>R. affinis</i>	0	0	0	0
3	<i>M. musculus</i> and <i>M. musculi</i>	5	4	5	5

Data in each column are given as the number of mite-positive samples among a total of 5 samples.

Using the alignment of related species, we designed forward primers within the 18S rRNA gene and reverse primers within the 28S rRNA gene that would target the *Psoroptidia* mite species. Using these primers, we generated 3 PCR products from the *M. musculus* DNA, which we sent for MiSeq sequencing. A 6351-base contig was generated that spanned both the 18S and 28S rRNA gene of *M. musculus*. Homology analysis of assembled contigs with published mite sequences confirmed the correct assembly of the short reads (Table 2). Sequences for the 18S (NCBI accession no., KT384411.1) and 28S (KT384412.1) rRNA genes of *M. musculus* were deposited at NCBI. These sequences were used to generate species-specific PCR primers to identify *M. musculus*.

Development of PCR assay for the detection of *M. musculus*.

After multiple rounds of testing, we selected the MC28S primer pair (Table 1) because it specifically targeted *M. musculus* DNA and did not yield similarly sized bands when tested against mouse DNA (Figure 1). Furthermore, BLAST and Primer-BLAST analysis of the MC28S primer pair demonstrates specificity for the *Myocoptes* genus, and the only predicted PCR products with one or fewer mismatches are from *M. musculus* and *M. japonensis*.¹ To validate the MC28S primers, swab samples were collected from experimentally maintained mouse colonies with known mite infestations. Specifically, we used samples from mice infested with *M. musculus* and *D. musculi* ($n = 5$), *M. musculi* and *R. affinis* ($n = 5$), or *M. musculus* and *M. musculi* ($n = 5$). MC28S primers positively identified all 10 samples containing *M. musculus* but none of the 5 samples devoid of *M. musculus* (Figure 2, Table 3). To confirm the accuracy of our results, we sequenced the PCR product generated from sample 1E by using MC28S primers, which verified the positive identification of *M. musculus* (99.4% sequence identity over 154 bases).

Quantification of detection by using a qPCR assay for *M. musculus*. Because the MC28S PCR assay showed specificity for *M. musculus*, we developed a probe-based qPCR assay was developed as qPCR analysis is faster than conventional PCR amplification and may show increased assay sensitivity and specificity. Relying on the specificity of the PCR primers,

we designed a probe within the target region of the MC28S primers. Using 8 replicates of the *M. musculus* standard curve, we determined that the limit of detection for the MC28S qPCR assay was 100 copies of *M. musculus*, because this dilution was the lowest at which 100% of the replicates were positive. At 10 copies, *M. musculus* was detected in 75% of the replicates, whereas at 100 copies *M. musculus* was detected in 100% of the samples. (Figure 3 A).^{9,15,16} Using samples from fur swabs, the MC28S qPCR assay correctly identified all 10 mice in groups 1 and 3 as positive for *M. musculus* and all 5 group 2 samples as negative (Figure 3 B, Table 3).

Detection of murine mites in fecal DNA by using PCR-based assays. Because swabbing for mites may increase the workload within surveillance programs, MC28S assays were tested against DNA extracted from feces, which are commonly collected for colony health surveillance. The MC28S assay correctly detected *M. musculus* in all 5 fecal samples from group 1 mice and 4 of the 5 samples from group 3; in addition, sample 3B yielded a very faint band (Figure 4). Furthermore, we obtained a weak band from sample 2C, which lacked *M. musculus* (Figure 4). Leveraging the increased sensitivity of qPCR analysis, we tested the 15 fecal samples with the MC28S qPCR assay and confirmed that all group 1 and 3 samples were indeed positive, but all group 2 samples were considered negative (Figure 3 C, Table 3).

Discussion

PCR-based assays provide advantages in terms of the ability to 1) detect few copies of DNA in complex samples, 2) comprehensively sample whole animals, cages, racks, and so forth by using swabs, and 3) test a large number of samples without linearly increasing time and labor.^{6,11,22} PCR testing has been used to detect mites in a variety of animal species, including humans, dogs, bats, and marmots.^{21,25,27} In addition, many publications have shown the successful detection of murine mites in laboratory settings using external diagnostic labs.^{6,11,22} However, the costs associated with external testing may limit clinicians' ability to test mice with suspected acariasis. Inhouse PCR testing can provide laboratories the ability to cost-efficiently and rapidly

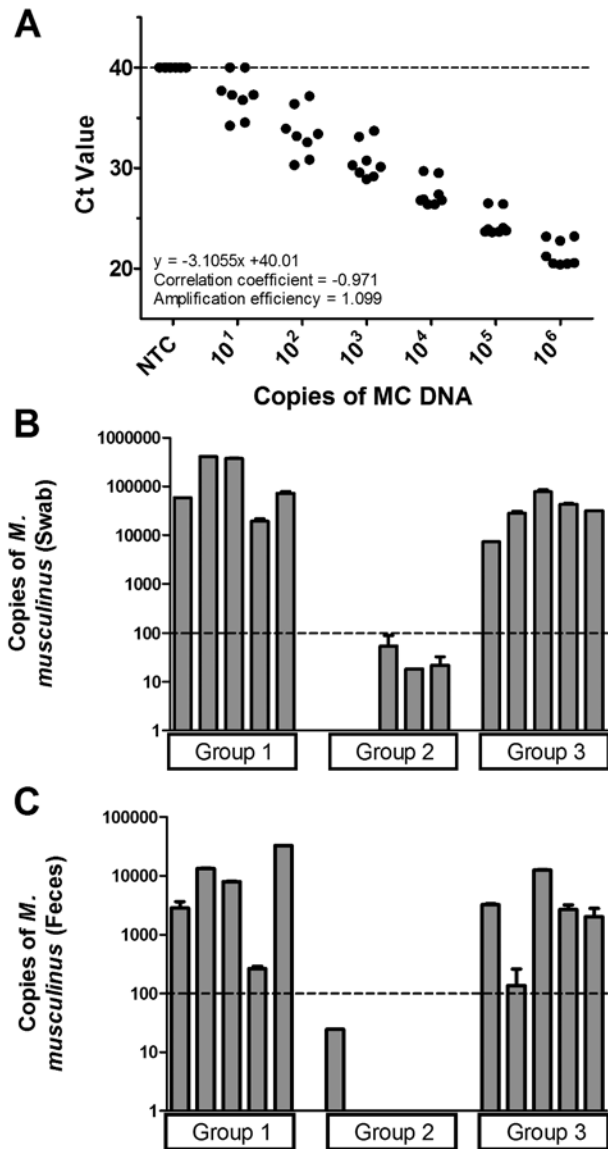


Figure 3. (A) Replicates of the standard curve analysis show that the limit of detection of the *M. musculus* PCR assay is 100 copies. (B) Detection of *M. musculus* in swab samples by using MC28S qPCR assay for groups 1 (infested with *M. musculus* and *D. muscui*), 2 (*M. muscui* and *R. affinis*), and 3 (*M. musculus* and *M. muscui*). (C) Detection of *M. musculus* in fecal samples by using the MC28S qPCR assay for groups 1, 2, and 3.

screen for mites. To date, we have found only 2 publications that describe primer sequences to detect murine mites in laboratory mice; these studies focused on pelt and skin samples.^{8,24}

To further simplify the process of inhouse testing for mites in laboratory mice, we developed PCR assays that could detect *M. musculus* DNA in fecal samples. Because constant grooming suggests that mites and mite eggs might be ingested, veterinarians have speculated about the ability of PCR tests to detect mites in feces. Anecdotally, we understand that some technicians have reported observing mites in fecal exams. To our knowledge, this report is the first to describe *M. musculus* detection in feces using PCR. We here present robust *M. musculus*-specific PCR and qPCR assays capable of detecting *M. musculus* DNA in complex mixtures including fecal and fur swab samples. In silico analysis of both the PCR and qPCR binding sites predicted efficient binding to *Myocoptes* species

only. While the qPCR probe shares sequence homology with other mite species within the suborder *Psoroptidia* to which *Myocoptes* belongs, none of these mites naturally infest mice. In addition, no homology was observed to *M. muscui*,³ *R. affinis* or *D. muscui*, which are taxonomically distinct from *Myocoptes*. Because specific binding of both primers and probes is necessary for efficient 5' nuclease qPCR assays, our *in silico* analysis only predicts the efficient binding of all 3 components to *Myocoptes* DNA sequences. Validation by using both types of samples with known combinations of mites and Sanger sequencing of select PCR products demonstrated that the PCR assay was indeed specific and detected *M. musculus*. The developed probe-based qPCR assay can be used by laboratories with real-time PCR capabilities that are interested in reducing the duration of the assay and hands-on time. In summary, our results demonstrate that fecal samples can be used in the PCR assays presented to screen for *M. musculus* infestations in laboratory mice, thus giving laboratory animal veterinarians additional options when designing health surveillance programs.

Acknowledgments

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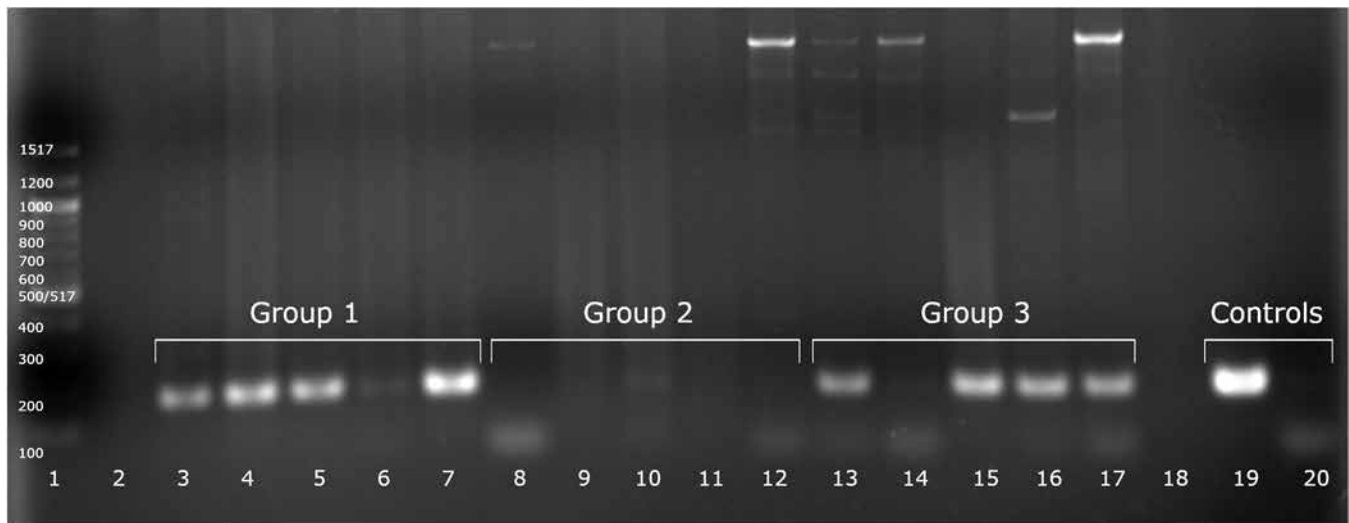


Figure 4. PCR detection of *M. musculus* by using the MC28S primer set with fecal samples. Lane 1, 100-bp ladder; lanes 3 through 7, samples containing *M. musculus* and *D. musculi* (group 1); lanes 8 through 12, samples containing *M. musculi* and *R. affinis* (group 2); lanes 13 through 17, samples containing *M. musculus* and *M. musculi* (group 3); lane 19, positive control; and lane 20, negative control.

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