國立臺灣大學獸醫專業學院分子暨比較病理生物學研究所

碩士論文

Graduate Institute of Molecular and Comparative Pathobiology
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National Taiwan University

Master Thesis

建立分子生物診斷法檢測實驗動物嚙齒類毛蝨並與傳統診斷法比較

Development of a Molecular Assay for Laboratory Rodent Fur Mite Diagnosis and Comparison with the Traditional Diagnostic Methods

周知林

Chih-Lin Chou

指導教授：萬灼華 博士

Advisor: Cho-Hua Wan, DVM, Ph.D.

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建立分子生物診斷法檢測實驗動物嚙齒類毛蝨並與傳統診斷法比較

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本論文係周知林君（R06644005）在國立臺灣大學獸醫專業學院分子暨比較病理生物學研究所完成之碩士學位論文，於民國 108 年 7 月 31 日承下列考試委員審查通過及口試及格，特此證明

口試委員：萬灼芽（簽名）

（指導教授）

系主任、所長 萬灼芽（簽名）

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中文摘要

實驗鼠感染外寄生蟲毛蟎(fur mite)是實驗動物中心常見且難徹底解決的問題之一，乃主要受限於外寄生蟲診斷方法敏感度較差、檢測樣本不易具有代表性及常用的衛兵鼠健康監測系統並不適用於監控實驗鼠的外寄生蟲感染情形。為了提升毛蟎檢測的敏感性與效率，本研究建立了一個多重引子聚合酶鏈鎖反應檢測法（multiplex PCR），此檢測法能同時偵測並區別不同種類毛蟎，包括 *Myocoptes musculinus* (COP)、*Myobia musculi* (MOB)/*Radfordia* spp. (RAD)以及於臺灣發現的新種毛蟎(species A；SPA)，更可藉由同時檢測囓齒動物管家基因（housekeeping gene）以確定檢體的品質。當多種毛蟎等量感染時，此檢測法能同時偵測出僅 10 copies 的各種毛蟎。而當同時有多種不等量的毛蟎感染時，此檢測法仍能偵測到感染量相差 10 至 100 倍的不同毛蟎感染。為了比較此多重引子聚合酶鏈鎖反應與多種傳統診斷法（拔毛測試、膠帶測試、毛皮檢查），本研究分別以不同診斷法來檢測 48 隻囓齒動物與 25 個鼠籠的毛蟎汙染情形。於診斷個體動物毛蟎感染情形，此多重引子聚合酶鏈鎖反應檢測法的敏感度與準確度（86 %與 95.1 %）明顯高於所有傳統診斷方法（敏感度：6 % - 46 %，準確度： 67.4 % - 81.3 %）。更值得一提的是，當應用於檢測鼠籠擦拭樣本（環境樣本）時，此毛蟎多重引子聚合酶鏈鎖反應法可完全正確地區別檢測出每個鼠籠的不同毛蟎的汙染情形，其敏感度與準確度均達 100 %。本研究所建立的毛蟎多重引子聚合酶鏈鎖反應檢測法（COP/MOB-RAD/SPA/Actin multiplex PCR assay）是一可靠的替代診斷方法，能應用於實驗動物中心的例行性病原監測（不論動物或環境樣本），更可用於實驗動物中心疑似毛蟎感染的追蹤調查。

關鍵字：囓齒類毛蟎、*Myocoptes musculinus*、*Myobia musculi*、*Radfordia* spp.、臺灣新種囓齒類毛蟎、多重引子聚合酶鏈鎖反應、環境監測

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ABSTRACT

Rodent fur mite infestation is a persistent and intractable problem in laboratory rodent colonies, due to insensitive diagnostics, unrepresentative samples for testing, and improper sentinel system. To improve the sensitivity and efficiency of fur mite detection, a multiplex PCR assay was developed to simultaneously detect and differentiate different species of fur mites, including *Myocoptes musculinus* (COP), *Myobia musculi* (MOB) and/or *Radfordia* spp. (RAD), and species A (SPA; a novel rodent fur mite identified in Taiwan), with the existence of a rodent housekeeping gene. This multiplex PCR could specifically detect as low as 10 copies of each species in equal-amount triple infestation. Super-infestation with 10 to 100-fold differences in mite burdens could be also detected. In comparison of the multiple PCR and traditional methods (pluck test, tape test, and pelt exam) for fur mite diagnosis, 48 rodents and 25 cage environment samples were evaluated for the fur mite infestation. In screening the status of various fur mites on individual animals, the multiplex PCR assay showed distinctly higher in sensitivity and accuracy (86 % and 95.1 %) than that of traditional methods (sensitivity: 6 % - 46 %, accuracy: 67.4 % - 81.3 %). Interestingly, by using cage wipe environmental samples, the multiplex PCR assay exhibited 100 % in both sensitivity and accuracy on the fur mite detection and differentiation. The COP/MOB-RAD/SPA/Actin multiplex PCR assay developed in this study could be a reliable alternative method for routine pathogen monitoring (animal or environment) or for tracing the suspect fur mite outbreak in rodent colonies.

Keywords: rodent fur mite, *Myocoptes musculinus*, *Myobia musculi*, *Radfordia* spp., novel rodent fur mite in Taiwan, multiplex PCR, environmental monitoring

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CONTENTS

doi:10.6342/NTU201903951

doi:10.6342/NTU201903951

LIST OF FIGURES

doi:10.6342/NTU201903951

LIST OF TABLES

doi:10.6342/NTU201903951

Introduction

Fur mite infestation caused by *Myocoptes musculinus, Myobia musculi, Radfordia affinis* or *Radfordia ensifera* is among the challenging problems in contemporary laboratory rodent colonies (Baker, 2007). Even the prevalence of fur mite infestation in mice is relatively low in North America and Europe (0.11% and 0.43%, respectively) (Pritchett-Corning *et al.*, 2009), fur mite problems of rodent colonies are still reported in many research institutions (Carty *et al*., 2008). All these indicate the fur mite infestation is a consistent and difficult problem that need to be solved in laboratory rodent colonies.

The fur mite infestation is usually subclinical in rodents; however, it has been reported to be associated with pruritus, erythema, alopecia, ulcerative dermatitis and even weight loss in susceptible strains or under heavy infestation. (Baker, 2007; Iijima *et al.*, 2000; Jungmann *et al.*, 1996; Sahinduran *et al*., 2010). In addition, the immunological modulations, including stimulation in T-helper-2 (Th2) type immune response, increase in inflammatory cytokines, and elevation in serum immunoglobulin E (IgE), which would confound research data, have been reported in mice, under current infestation and after elimination of fur mites (Iijima *et al.*, 2000; Johnston *et al.*, 2009; Jungmann *et al.*, 1996; Morita *et al.*, 1999; Pochanke *et al.*, 2006). Thus, detection and eradication of fur mite infestations are necessary for laboratory rodent colonies.

To sensitively and accurately diagnose the fur mite infestation in laboratory rodent

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colonies is a persistent challenge due to the tiny size of fur mites (about 0.16-0.5 x 0.13-0.2 mm in size) and the external characteristics to distinguish each species. The adult *Myocoptes* spp. are circular (male) or oval (female) in shape, with ambulacral suckers on the first and second pairs of legs. The genera of *Myobia* and *Radfordia* are very similar in size and morphology. Both of them are oval to elongate in shape and the first pair of legs are short and compressed, which are highly adapted for hair clasping. Differentiation of these two genera mainly depends on the tarsus ends on the second pair of legs. *Myobia musculi* has a single empodial claw, while *Radfordia affinis* has two tarsal claws of uneven length, and *R. ensifera* has paired, equal-in-length claws. These fur mites generally stay on skin surface; however, their habitat locations are different, *Myocoptes* spp. typically at the dorsum, abdomen and inguinal regions, while *Myobia* spp. and *Radfordia* spp. mainly at the head and cervical areas (Baker, 2007). All these make the accurate diagnosis of fur mite infestation more difficult.

Traditional diagnostic methods applied for the fur mite detection include the tape test, the pluck test, and the pelt exam. The tape test and the pluck test, the two common antemortem methods, microscopically examine for the existence of eggs and adults of fur mites on the fur pluck samples and the tape-impressed samples, respectively, collected from the specific regions of live mice and rats (Bauer *et al.*, 2016; Bornstein *et al.*, 2006; Gerwin *et al.*, 2017; Jensen *et al.*, 2013; Karlsson *et al.*, 2014; Macy *et al.*, 2009; Metcalf Pate *et al.*, 2011; Miller *et al.*, 2018; Rice *et al.*, 2013; Weiss *et al*., 2012).

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In the postmortem pelt exam, the “gold standard” for fur mite diagnosis in laboratory rodents, the pelt of a dead animal is directly examined under a dissecting microscope for the eggs and adults of fur mites (Karlsson *et al.*, 2014; Pritchett-Corning *et al.*, 2009). Unfortunately, these traditional diagnostic methods have to rely on microscopic examination and pose difficulties in terms of low load of mite infestations, incorrect sampling sites and personnel skills in mite identification. With these difficulties, the traditional diagnostic methods could not efficiently and accurately demonstrate the ectoparasite infestation status, and the animals need to be sacrificed for “gold standard” diagnostics. Moreover, many institutions use the soiled-bedding sentinel system for health monitoring of laboratory rodent colonies; however, the transmission of fur mite is mainly by direct contact, not easily through the fecal–oral route, and thus the results of fur mite detection would be unreliable by testing the soiled bedding sentinels (Baker, 2007; Clifford, 2014; Lindstrom *et al*., 2011). Therefore, it is still a challenge to sensitively and accurately detect rodent fur mites due to poor diagnostic methods, low prevalence of fur mite infestation, improper sentinel system, and incorrect sampling strategies. A more reliable diagnostics is stringently needed for the fur mite detection.

In recent years, molecular diagnostic assays for various pathogens detection have been developed and applied to monitor the pathogen infections in laboratory animals (Grove *et al*., 2012; Henderson *et al*., 2014; Jensen *et al*., 2013). Many recent studies have reported that PCR assay is a sensitive and reliable diagnostic method to detect fur

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mites (Jensen *et al*., 2013; Karlsson *et al*., 2014; Weiss *et al*., 2012). However, these PCR assays cannot simultaneously detect and differentiate the species of fur mites.

Fur mite infestation is still a problem in conventional laboratory rodent colonies in Taiwan. A novel unclassified fur-mite-like ectoparasite, species A (Figure 2C), was first found by the Laboratory Animal Medicine Diagnostic Laboratory at National Taiwan University during a routine health monitoring of a research mouse colony. In this study, a multiplex PCR assay was developed to detect and differentiate different fur mites, including *Myocoptes musculinus* (COP), *Myobia musculi* (MOB) and/or *Radfordia* spp. (RAD), and species A (SPA) in co-infested laboratory rodents. This assay is more sensitive and reliable than traditional methods and could be applied to monitor both live animals and the environment samples, regardless of any housing systems.

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Methods and Materials

Animals and Sample Collection

All animals were handled according to protocols approved by the Institutional Animal Care and Use Committee in National Taiwan University and the procedures were conducted in compliance with the Animal Protection Act (2018) and the Guidebook for the Care and Use of Laboratory Animals (Council of Agriculture, 2010) in Taiwan. A total of 31 mice and 3 rats, housed in 19 cages, were obtained from 11 different laboratory rodent colonies in Taiwan (Table 1). These animals varied in age, microbial status, and genetic backgrounds, including BALB/cAnNCrlBltw, BALB/cByJNarl, Bltw:CD1(ICR), C57BL/6JNarl, C57BL/6NCrlBltw, B6CBA genetically-engineered, and ICR genetically-engineered mice, and Bltw:SD rats. Another 14 mice (*Mus musculus*) in 6 cages were obtained from 4 pet stores, with higher potential of ectoparasite infestation. These 48 animals and 25 cages were evaluated by PCR for fur mite status. For PCR, fur swab samples and cage wipe samples were collected by swabbing the surface of each animal with a sterile flocked swab (Puritan Medical Products, Guilford, ME, USA) and by wiping the walls of each cage with Kimwipes™ (Kimtech® Science™, GA, USA). These samples were frozen at -80℃ until use. For the traditional diagnostic tests, antemortem samples were collected

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from multiple area, including head, neck, base of tail and inguinal regions for either the tape test or the pluck test. After carbon dioxide euthanasia, pelage samples were excised from head, ears, scruff and posterior dorsum for the pelt exam.

DNA Extraction

DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of manufacturer, with some adjustments suggested by Grove (2012). Briefly, all samples were incubated at 56℃ for 14 to 18 h in 180 μL Buffer ATL (Qiagen) and 20 μL Proteinase K (Qiagen). Mixtures were added to 200 μL Buffer AL (Qiagen) and incubated at 70℃ for 10 min, followed by adding 200 μL 99 % ethanol. Total mixtures were transferred to QIAamp Mini spin column and centrifuged at 6000 x *g* for 1 min. The columns were washed with 500 μL Buffer AW1 (Qiagen) and centrifuged at 6000 x *g* for 1 min. Then, the columns were washed with 500 μL Buffer AW2 (Qiagen) and centrifuged at 20000 x *g* for 3 min, followed by centrifuging in a new collecting tube at full speed for 1 min. DNA was eluted in 75 μL Buffer AE (Qiagen) and stored at -20℃ until use.

PCR Analysis

Multiple oligonucleotide primers to target the 18S ribosomal RNA genes of fur mites and the beta actin gene (a housekeeping gene) of rodent were offered by Laboratory Animal Medicine Diagnostic Laboratory at National Taiwan University.

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These primers were selected for the COP/MOB-RAD/SPA/Actin multiplex PCR assay. PCR mixture was in a total volume of 50 μL, containing 5 μL of extracted DNA sample, 0.2 mM of each dNTP (Promega, Madison, WI, USA), 1X Green GoTaq® Flexi Buffer (Promega), 1.75 mM $MgCl_2$ (Promega), 0.5 μg/μL bovine serum albumin (BSA; Sigma-Aldrich®, St. Louis, USA), 0.25 to 0.4 μM of each forward and reverse primers, and 1.25U of GoTaq® Flexi DNA Polymerase (Promega). PCR was performed in a thermocycler (Labcycler, SensoQuest, Gottingen, Germany), with the following profile: an initial denaturation for 2 min at 95℃, 40 cycles of amplification (denaturation for 30 s at 95℃, annealing for 30 s at 54℃, and extension for 35 s at 72℃), and a final extension for 5 min at 72℃. PCR products (10 μL) were analyzed by electrophoresis in 3 % agarose gels, stained with ethidium bromide, and visualized with UV light. In PCR assay, plasmids containing the 18S ribosomal RNA genes of COP, RAD and SPA (10^2 copies) were served as the positive controls. To mimic the background of clinical PCR samples, the cage wipe DNA extracts of the ectoparasite-free colony (no-parasite cage wipe, NP) were also added in plasmid positive controls. The NP and water (no-template control, NT) were used as negative controls.

To assess the specificity of the PCR assay, DNA of target fur mite(s) (10^2 copies) and genes (10^5 copies) of other ectoparasites, including louse, tropical rat mite and mold mite, were amplified with background DNA extracted from no-parasite cage wipe samples. Both NP and NT were included as negative controls.

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To assess the sensitivity of the PCR assay, serial dilutions of each fur mite DNA in equal amounts (10^3, 10^2, 10^1, and 10^0 copies) were amplified to determine the detection limit of the fur mite PCR assays. To evaluate the efficiency of detecting dual and triple co-infestations with different amounts of rodent fur mites, serial dilutions (10^4, 10^3, 10^2, and 10^1 copies) of one fur mite were co-amplified with a fixed copy number (10^4 copies) of DNA samples of one or the other two kinds of fur mites. No-parasite cage wipe samples were added to every reactions as background DNA except the no-template control.

DNA Sequencing

Amplified DNA fragments were purified with the QIAquick® PCR Purification Kit (Qiagen) or the QIAquick® Gel Extraction Kit (Qiagen), following the instructions recommended by the manufacturer. The purified amplicons were sequenced in the Center for Biotechnology and Department of Medical Research in National Taiwan University Hospital. Sequence data were analyzed with EditSeq™ and MegAlign™ of Lasergene® (DNASTAR). These data were also compared with the sequences of the 18S ribosomal RNA genes of *Myocoptes musculinus* (GenBank accession number KT384411), *Myobia musculi* (GenBank accession number JF834895), *Radfordia affinis* (GenBank accession number MN153812) and species A (unpublished).

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Traditional Diagnostic Methods

Tape test sample was collected by pressing on fur surface of several regions with a clear cellophane adhesive tape (approximately 4 x 2 cm) and adhered to a glass microscope slide. The slides were microscopically examined to identify both adults and eggs and to differentiate fur mite species. To perform the pluck test, fur samples collected from several regions were microscopically examined for the presence of fur mites or eggs and species differentiation. For the pelt exam, the pelage samples were examined under a dissecting microscope for the existence of adults and eggs of fur mite, and the species of adult mites were differentiated under a light microscope. During the examination, furs were separately viewed to confirm whether fur mites or eggs were presented in the region of hair roots and the surface of skins. Each pelt exam lasted approximately 20 min.

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Results

Ectoparasite Identification

Myocoptes musculinus (COP) (Figure 2A), *Radfordia* spp. (RAD) (Figure 2B), species A (SPA) (Figure 2C), louse, tropical rat mite and mold mite were identified based on external characteristics by microscopic examination. The parasite species were confirmed by the amplification of the partial 18S ribosomal RNA genes and DNA sequencing, and stored at -80℃ for further use as parasite controls. Due to the 18S ribosomal RNA genes of *Myobia musculi* (MOB) and *Radfordia affinis* are almost identical (99.75 % in 1601 bp), the *Radfordia* spp. is used as positive controls for both MOB and RAD.

Specificity and Sensitivity of Single Specific PCR

The COP-specific, MOB/RAD-specific, and SPA-specific Single PCR assays successfully amplified the expected fragments of COP (294 bp in length), MOB/RAD (472 bp in length) and SPA (522 bp in length), respectively and confirmed by DNA sequencing. To evaluate the specificity of each Single PCR assay, the target fur mite gene (10^2 copies) and other ectoparasite genes (10^5 copies) were amplified by the COP, MOB/RAD, or SPA Single PCR assay, with the no-parasite cage wipe samples as background DNA. The COP-specific PCR assay amplified the target gene only form

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COP sample and no DNA fragments were detected in other ectoparasite samples, the no-parasite cage wipe control (NP) and the no-template control (NT) (Figure 3). In the MOB/RAD single PCR assay, only the target gene was amplified from MOB/RAD sample and no bands in other ectoparasites or negative controls (NP and NT) (Figure 4). Similarly, by the SPA-specific PCR assay, the amplicon in correct size was only detected in SPA and no fragments were amplified from other samples (Figure 5).

To evaluate the sensitivity of each Single PCR assay (COP-specific, MOB/RAD-specific or SPA-specific), serial dilutions (10^3, 10^2, 10^1, 10^0 copies) of each fur mite DNA were amplified by the specific PCR assays, with the no-parasite cage wipe background DNA to mimic the clinical samples. All three specific Single PCR assays could independently detect as low as 10 copies of target gene each (Figure 6). The β-actin (a housekeeping gene) fragment (134 bp in size) was also amplified from all fur mite samples and the NP control except the NT control.

Specificity and Sensitivity of the COP/MOB-RAD/SPA/Actin Multiplex PCR Assay

All specific primers were applied in the COP/MOB-RAD/SPA/Actin multiplex PCR assay and simultaneously amplified the expected fragments from three different fur mites and the housekeeping gene independently. To evaluate the specificity of the multiplex PCR assay, COP, MOB/RAD and SPA (10^2 copies of each sample) and other

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ectoparasites (10^5 copies each) including louse, tropical rat mite, and mold mite were screened by the multiplex PCR. The correct-size product (294 bp, 472 bp or 522 bp in size) was amplified from the target fur mite (COP, MOB/RAD or SPA) by the multiplex PCR, respectively. The β-actin DNA fragment (134 bp in size) was also amplified in every sample except the no-template control (Figure 7). Occasionally, few weak non-specific bands were amplified in ectoparasite samples and NP control.

To evaluate the sensitivity of the multiplex PCR assay for single or equal-amount triple infestation, serial dilutions (10^3, 10^2, 10^1, 10^0 copies) of each fur mite DNA (single or triple infestation) were amplified by the multiplex PCR assay. In single infestation, the multiplex PCR assay detected as low as 10 copies of each target fur mite DNA (COP, MOB/RAD or SPA) with the present of housekeeping gene (Figure 8). The detection limit of the multiplex PCR assay in COP/MOB-RAD/SPA co-infestation remained the same as in single infestation, 10 copies for each fur mite. The β-actin gene was detected in every sample except the NT control (Figure 9). Occasionally, few weak non-specific bands were amplified.

Detection of Different Amounts of Fur Mite Super-infestations by the Multiplex PCR Assay

To evaluate the efficiency of the multiplex PCR assay to detect multiple fur mites in super-infestations, serial dilutions (10^4, 10^3, 10^2, 10^1 copies) of one fur mite DNA

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mixed with high amounts (10^4 copies) of one or two other fur mite(s) were screened by the multiplex PCR. In COP & MOB/RAD dual infestation, the multiplex PCR assay detected 10^2 copies of COP DNA in the presence of high amounts (10^4 copies) of MOB/RAD DNA (Figure 10A); vice versa, similar amplification efficiency was observed in the reverse case (Figure 10B). Similarly, in COP & SPA dual infestation, 10^2 copies of COP DNA were also amplified, in the presence of high amounts (10^4 copies) of SPA (Figure 10C); vice versa, similar amplification efficiency was detected (10^2 copies of SPA with 10^4 copies of COP co-infestation) (Figure 10D). In MOB/RAD & SPA dual infestation, the multiplex PCR could also detect 10^2 copies of SPA with high amounts of MOB/RAD (Figure 10F); however, ten-fold decrease in amplification efficiency for MOB/RAD (10^3 copies) was noted in combined with high amount of SPA (10^4 copies) (Figure 10E). While co-infected with heavy loads of two other fur mites (10^4 copies of each), COP (10^2 copies) and SPA (10^2 copies) could still be detected by the multiplex PCR (Figures 11A and 11C); however, the detection limit of MOB/RAD was only 10^3 copies (Figure 11B). A fragment (134 bp in size) of the housekeeping gene was amplified in every reaction except the NT control. Non-specific bands were occasionally observed.

Comparison of Fur mite Diagnostic Methods in Animals

To compare the sensitivity and accuracy of different methods for fur mite diagnosis, all 48 animals were screened by the multiple PCR assay (fur swab samples) and three

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traditional methods (pluck test, tape test, and pelt exam) (Tables 1 and 2). By the multiplex PCR assay, 17 animals were detected to be fur-mite positive, including 9 animals with triple infestations of COP, MOB/RAD, and SPA and 8 animals with dual infestations (6 with COP & MOB/RAD, 2 with COP & SPA) (Tables 1 and 2, Figure 12). In traditional methods, 14 animals were detected to be fur-mite positive by pluck testing, including 3 COP-positive and 11 with eggs only, whereas only 12 animals were positive by tape testing, including 9 single infestations (8 with COP, 1 with non-COP fur mite), 2 dual infestations (COP & non-COP fur mite), and 1 animal with eggs only (Tables 1 and 2). By the pelt exam, 17 animals were diagnosed as fur-mite positive, including 4 single infestations (COP), 12 dual infestations (3 with COP & RAD, 4 with COP & SPA, 5 with COP & non-COP fur mite), and 1 animal with eggs only (Tables 1 and 2). The real infection conditions of these animals, confirmed by parasite morphology, single PCRs and DNA sequencing, were 16 with triple infestation of COP, MOB/RAD and SPA, 1 with COP & SPA dual infestation, and 31 fur-mite negative (Tables 1 and 2). In each PCR testing, a fragment of the housekeeping gene was amplified in every clinical sample, positive control (10^2 copies of each fur mite), and NP control, except the NT control. For fur mite-specific diagnosis, the results exhibited that the multiplex PCR testing had significantly higher sensitivity and accuracy (86 % and 95.1 %) than the pluck test (6 % and 67.4 %), the tape test (20 % and 72.2 %), and the pelt exam (46 % and 81.3 %), respectively (Table 2). Due to the indistinguishable morphology of the

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eggs and nymphs of different fur mites, the identification of eggs, nymphs and unclarified fur mites was not included in data analysis in this study.

Environment (cage wipe) Samples by the Multiplex PCR Assay for Fur Mite Detection

To evaluate the reliability of environment sample to reflect the animal infection status, cage wipe samples collected from 25 cages, housing those 48 animals, were tested by the multiplex PCR assay. By the multiplex PCR assay, ten cages, housing the 17 fur mite-infested mice, were detected to be fur-mite positive, including 9 cages with triple contamination of COP, MOB/RAD and SPA, and 1 cage with COP & SPA dual contamination, and the other 15 cages were fur-mite negative (Tables 1 and 3, Figure 12). In screening the cage wipe samples (environment samples), the COP/MOB-RAD/SPA/Actin multiplex PCR results were all consistent with the pathogen contamination status, confirmed by three single PCRs and DNA sequencing, with 100% in both sensitivity and accuracy (Tables 1 and 3). In each PCR testing, a fragment of the housekeeping gene was amplified in every clinical sample, positive control, and NP control except the NT control.

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Discussion

Detection of fur mites continues to be challenging in health monitoring of laboratory rodents. Typically, the pelt will be examined under a stereoscopic microscope for fur mite infestation after rodents are euthanized (Pritchett-Corning *et al.*, 2009). However, the genetically modified animals are extremely valuable and seldom sacrificed only for health monitoring purpose. Unfortunately, the traditional antemortem tests are relatively insensitive in fur mite diagnosis and may produce false negative results (Karlsson *et al.*, 2014). In this study, the COP/MOB-RAD/SPA/Actin multiplex PCR assay was developed to detect and differentiate two groups of common fur mites (COP & MOB/RAD) and one new species of fur mite (SPA) without animal sacrifice.

To the best of our knowledge, this multiplex PCR assay is the first molecular diagnostic method to simultaneously detect and differentiate different fur mites (COP, MOB/RAD, and SPA), with a housekeeping gene to monitor the existence of DNA extracts and the interference of the inhibition factors. In equal-amount triple infestation, the detection limit of the multiplex PCR assay could reach as low as 10 copies of each fur mite. Mixed infestation of fur mites in varying degrees of burden has been reported in naturally-infested rodents (Baker, 2007; Weiss *et al.*, 2012; Whary *et al.*, 2015). Distinct difference in amounts of various pathogens might cause an obstacle in detecting pathogen in light infection (Wang *et al.*, 2013). Similar findings were also observed in

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this study. The multiplex PCR assay was demonstrated with the ability to diagnose super-infestation with 10 or 100-fold differences in fur mite concentration. A slight decrease in sensitivity (10^2 copies of COP and/or SPA) was found in super-infestation (10^4 copies) of other fur mite(s), compared to the sensitivity (10 copies each) in equal-amount co-infestation. Distinct decrease in sensitivity of MOB/RAD detection (10^3 copies) was noted in super-infestations of either SPA only or SPA/COP both. This decline in sensitivity might be related to the competition of a shared primer applied in both MOB/RAD and SPA amplification under super-infestation.

Recently, molecular methods have been developed and applied to detect multiple pathogens, including fur mites in laboratory rodents (Gerwin *et al*., 2017; Grove *et al*., 2012; Henderson *et al*., 2013; Jensen *et al*., 2013; Karlsson *et al.*, 2014; Miller *et al*., 2017; Weiss *et al.*, 2012). Similar to previous reports, the multiplex PCR assay developed in this study was very reliable and sensitive in identifying the fur mite-infested animals without fur mite differentiation, same as the pelt exam (0/17 false-negative result, FNR), whereas two traditional antemortem tests had higher FNRs (3/17; 5/17) (Karlsson *et al.*, 2014; Metcalf Pate *et al.*, 2011; Rice *et al.*, 2013; Weiss *et al*., 2012). In addition to differentiate both COP and MOB/RAD as the specific PCRs published before (Grove *et al*., 2012), the multiplex PCR assay can also detect and differentiate a novel fur mite (SPA), simultaneously. The comparison of traditional methods and molecular diagnostics for fur-mite specific diagnosis was also evaluated in

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this study. For the fur-mite specific diagnosis in individual animal level, the COP/MOB-RAD/SPA/Actin multiplex PCR testing on fur swabs had least FNR (7/50) than that of traditional diagnostics (47/50 in pluck test, 40/50 in tape test and 27/50 in pelt exam) (Table 2). In traditional testings, the higher false-negative results in the fur-mite specific diagnosis might be due to randomly-collected samples, which were unrepresentative of the entire animal, low load(s) of certain species in super-infestation, light or early infestation of fur mites, and personnel biases, as previously reported (Karlsson *et al.*, 2014; Metcalf Pate *et al.*, 2011; Ricart Arbona *et al*., 2010; Rice *et al.*, 2013; Weiss *et al*., 2012). In addition, compared to *Myocoptes*, the genera of *Myobia*, *Radfordia* and species A are difficult to detect and differentiate by traditional tests due to (1) parasite behavior: large numbers of *Myobia*, *Radfordia* and species A still remain in feeding positions after the death of host (Karlsson *et al.*, 2014; Wan, personal communication), (2) morphological similarity: the differentiation of *Myobia*, *Radfordia* and species A is mainly based on the tarsal terminus of the second pair of legs (Figure 2) (Baker, 2007; Wan, personal communication). In screening the animal fur swabs by the multiplex PCR, more FNRs were detected in SPA (6/17) than MOB/RAD (1/16), inconsistent with the efficiency results of super-infestation (Table 2, Figures 11B and 12). The reason for this remained unclear, but it might be due to inconsistence in fur swab sampling and large difference in parasite loads of MOB/RAD and SPA on these animals. Interestingly, the infection status of resident animals could be completely

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reflected by screening cage wipes (environment sample) (Table 3), even for the cages housing the super-infested animals (Figure 12). It is possible that cage wipes could easily collect eggs or parts of dead fur mites, which are more likely evenly-distributed on cages with no distribution difference among various fur mite species. In this study, all fur-mite positive samples were collected from colonies with heavy endemic infestation. Additional studies should be performed in light infestation or early outbreak colonies to assess the efficacy of this multiplex PCR in diagnosis of various fur mites in low prevalence.

Environmental monitoring system has become a trend to ensure the microbial status of laboratory rodent colonies (Gerwin *et al.*, 2017; Jensen *et al.*, 2013; Kapoor *et al.*, 2017; Macy *et al.*, 2009; Miller *et al.*, 2016; Miller *et al.*, 2018). The environment samples, including exhaust air particles and filter samples of individually-ventilated cage system (IVC) have been evaluated and applied as subjects in routine hygienic monitoring of rodent colonies (Gerwin *et al.*, 2017; Jensen *et al.*, 2013; Kapoor *et al.*, 2017; Macy *et al.*, 2009; Miller *et al.*, 2018); however, it might be difficult to apply in some facilities. In some research facilities, not only many different rodent housing systems were in use, simultaneously, including open cage systems, microisolator cages, and IVCs, but also the IVCs might be in multiple models of various brands. Thus, for each housing system, the location and special devices should be evaluated to ensure the reliability and efficacy for environment sample collection (Bauer *et al.*, 2016; Gerwin *et*

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al., 2017; Jensen *et al.*, 2013; Kapoor *et al.*, 2017; Macy *et al.*, 2009; Miller *et al.*, 2016; Miller *et al.*, 2018). In this study, Kimwipes™ was found with the best efficacy to collect environment samples (cage wipe), compared to other materials, including flocked swab, cotton swab and tissue (data not shown) and can be applied to all rodent housing systems with reliability and efficacy in environment sample collection. This material is cheap and could be easily pooled to detect the pathogen status in cage-, row-, rack- and room-levels for routine environmental monitoring or to trace back to the exact cage housing the infected animals. Furthermore, this sampling format could be performed outside of animal rooms with less possibility of pathogen outbreak and no interference with animals during collection.

In summary, the multiplex PCR assay established in this study is more sensitive and accurate than traditional diagnostic methods and can differentiate concurrent infestation of *Myocoptes musculinus*, *Myobia musculi*/*Radfordia* spp., and the novel unclassified fur mite, species A. Furthermore, there are many other advantages, including no animal sacrifice and suffer (3Rs), no ectoparasite expert needed, distinct decrease in false-negative results, and cost-effectiveness by pooling samples. This method could be applied not only to monitor the pathogen status of both live animals and various housing systems, but also to trace the early outbreak of fur mite infestations in rodent colonies.

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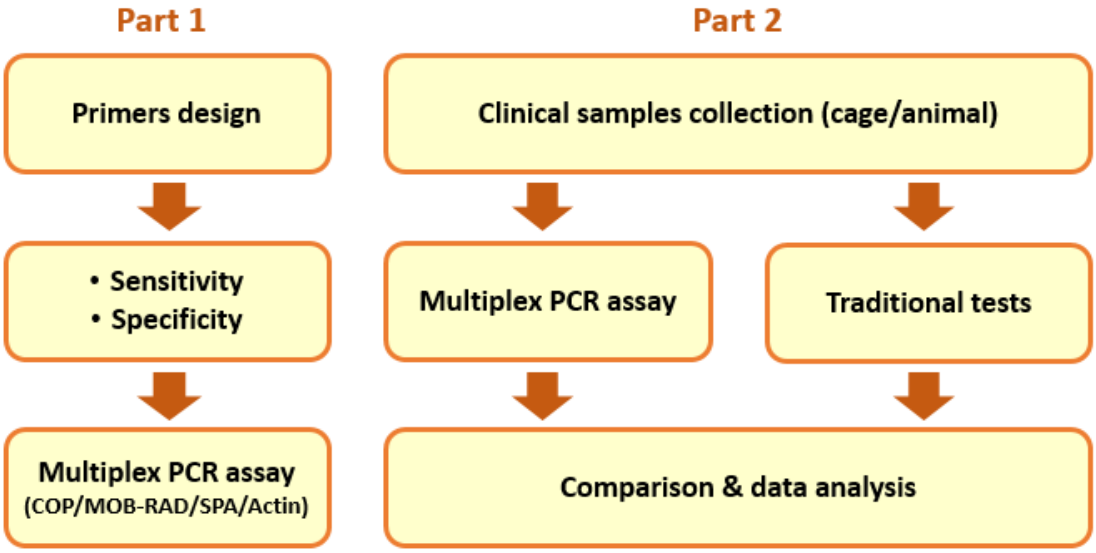

Figure 1. Flow chart of experimental design

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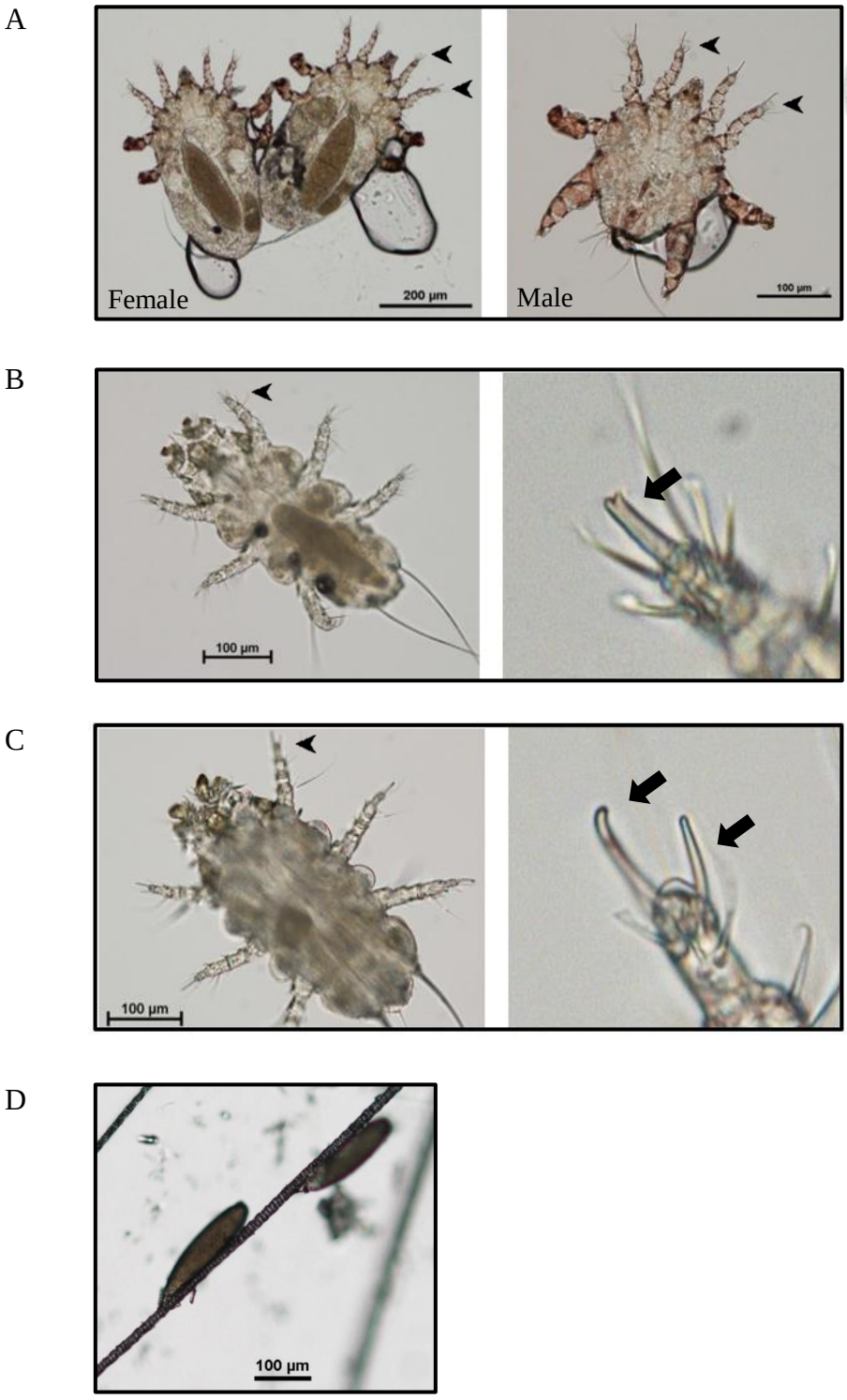

Figure 2. Fur mites and eggs under a light microscope at high-powered field.
(A) *Myocoptes musculinus* (left: adult female; right: adult male) and its first and second legs with ambulacral suckers (arrowhead). (B) *Radfordia* spp. and its second leg (left, arrowhead) with two tarsal claws (right, arrow). (C) Species A and its second leg (left, arrowhead) with two separate claws (right, arrow). (D) Eggs of fur mite.

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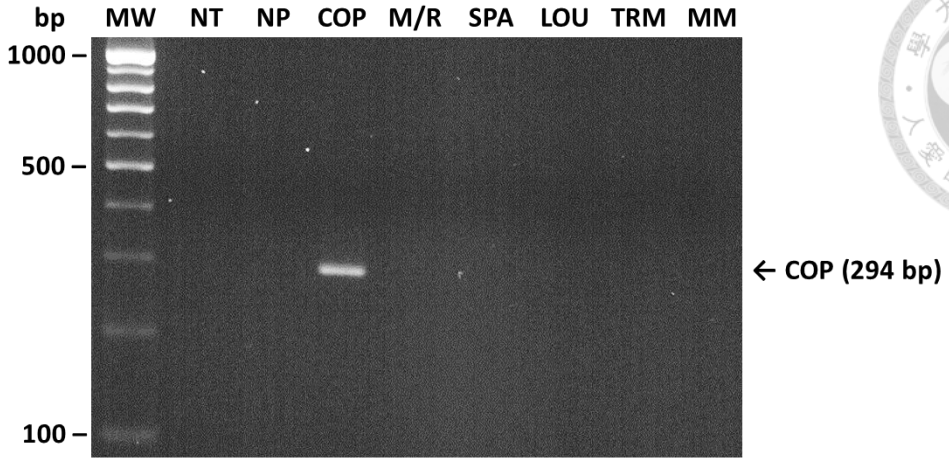

Figure 3. Specificity of *Myocoptes musculinus* specific PCR assay. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: 10^2 copies of *Myocoptes musculinus* DNA; M/R: 10^5 copies of *Radfordia* spp. DNA; SPA: 10^5 copies of species A DNA; LOU: 10^5 copies of louse DNA; TRM: 10^5 copies of tropical rat mite DNA; MM: 10^5 copies of mold mite DNA. All reactions were mixed with the no-parasite cage wipe samples except no template control.

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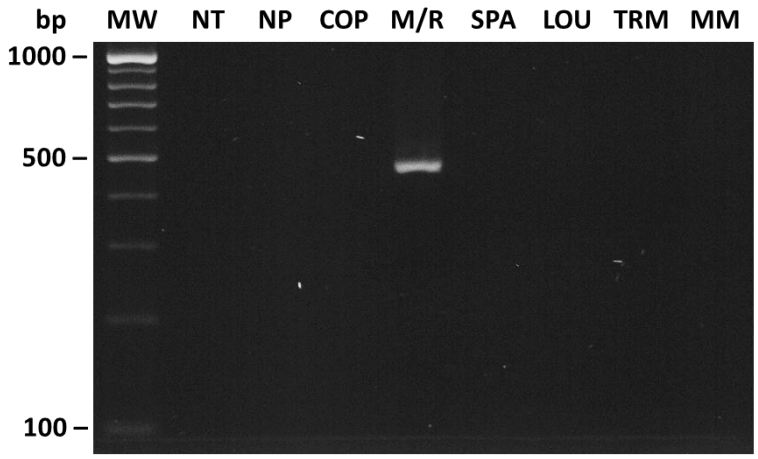

Figure 4. Specificity of *Myobia musculi*/*Radfordia* spp. specific PCR assay. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: 10^5 copies of *Myocoptes musculinus* DNA; M/R: 10^2 copies of *Radfordia* spp. DNA; SPA: 10^5 copies of species A DNA; LOU: 10^5 copies of louse DNA; TRM: 10^5 copies of tropical rat mite DNA; MM: 10^5 copies of mold mite DNA. All reactions were mixed with the no-parasite cage wipe samples except no template control.

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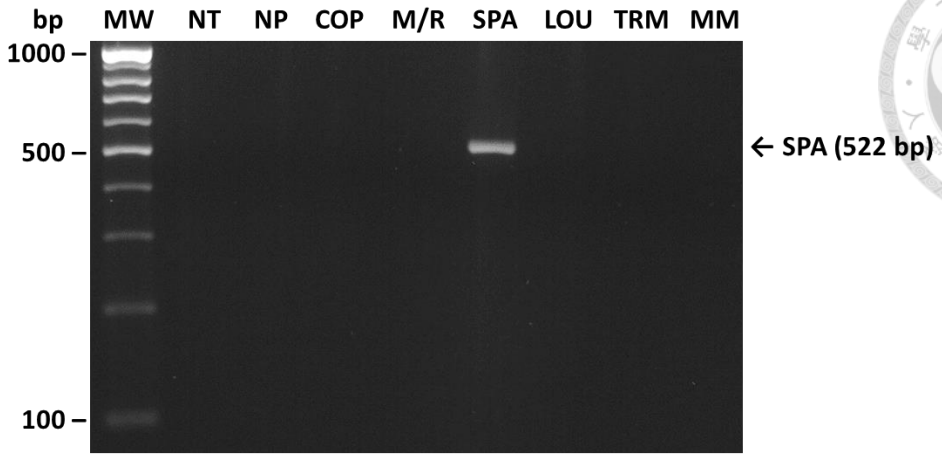

Figure 5. Specificity of species A specific PCR assay. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: 10^5 copies of *Myocoptes musculinus* DNA; M/R: 10^5 copies of *Radfordia* spp. DNA; SPA: 10^2 copies of species A DNA; LOU: 10^5 copies of louse DNA; TRM: 10^5 copies of tropical rat mite DNA; MM: 10^5 copies of mold mite DNA. All reactions were mixed with the no-parasite cage wipe samples except no template control.

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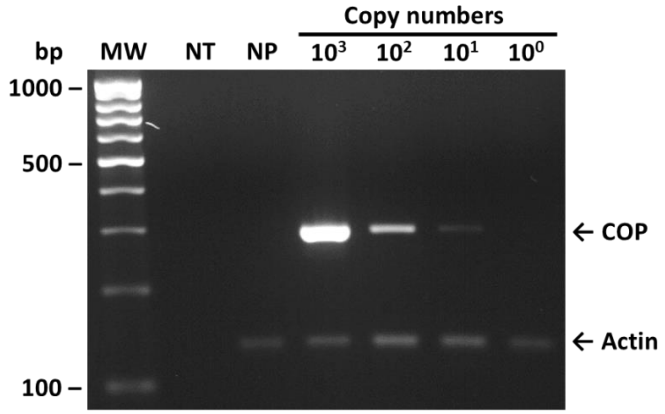
A



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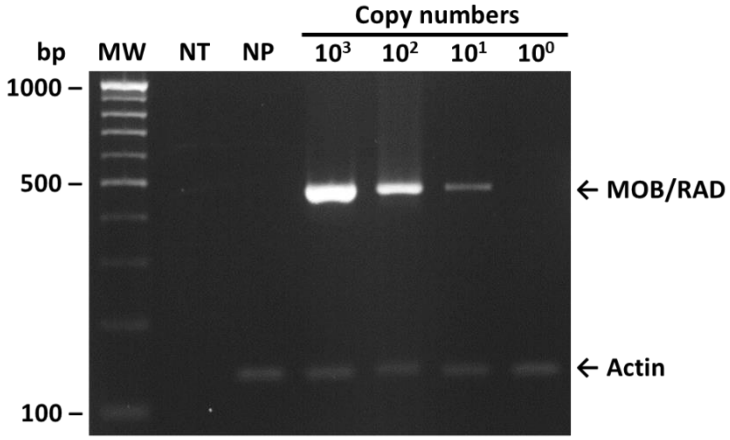

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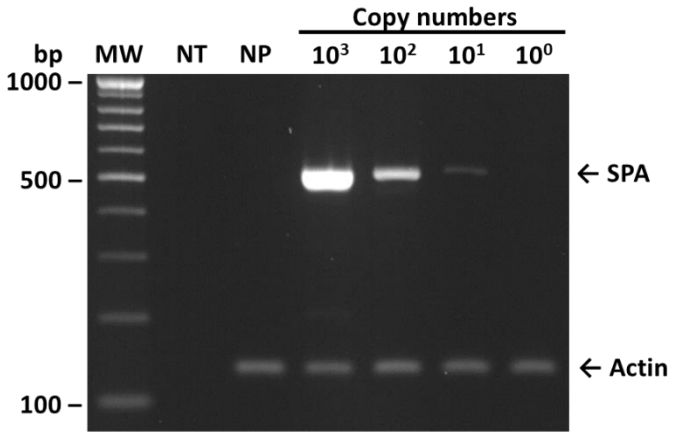

Figure 6. Sensitivity of the single specific PCR assays. Serial dilution of fur mite DNA was mixed with the no-parasite cage wipe sample. (A) The *Myocoptes musculinus* specific PCR assay. (B) The *Myobia musculi*/*Radfordia* spp. specific PCR assay. (C) The species A specific PCR assay. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: *Myocoptes musculinus*; MOB/RAD: *Myobia musculi*/*Radfordia* spp.; SPA: species A

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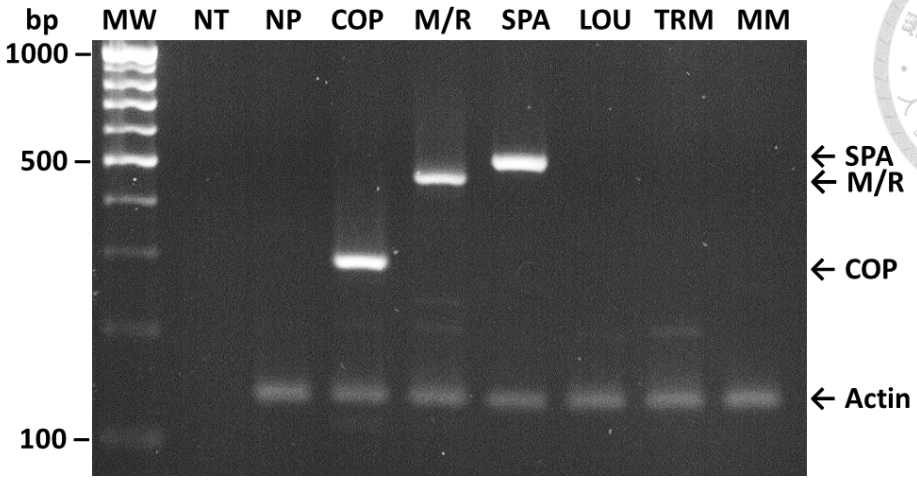

Figure 7. Specificity of the multiplex PCR assay. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: 10^2 copies of *Myocoptes musculinus* DNA; M/R: 10^2 copies of *Radfordia* spp. DNA; SPA: 10^2 copies of species A DNA; LOU: 10^5 copies of louse DNA; TRM: 10^5 copies of tropical rat mite DNA; MM: 10^5 copies of mold mite DNA. All reactions were mixed with the no-parasite cage wipe samples except no template control.

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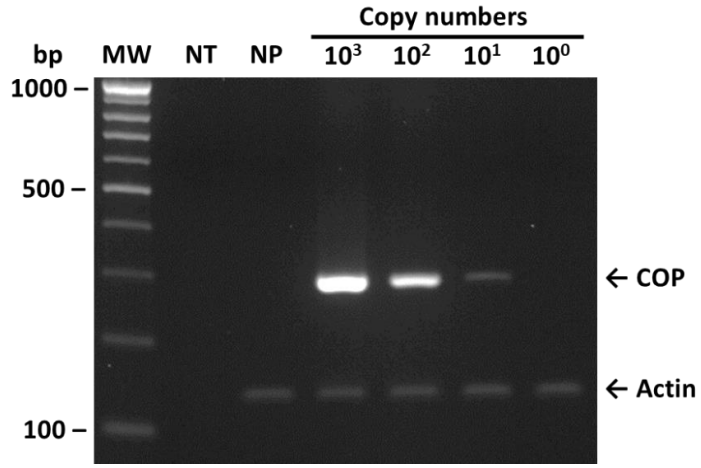
A



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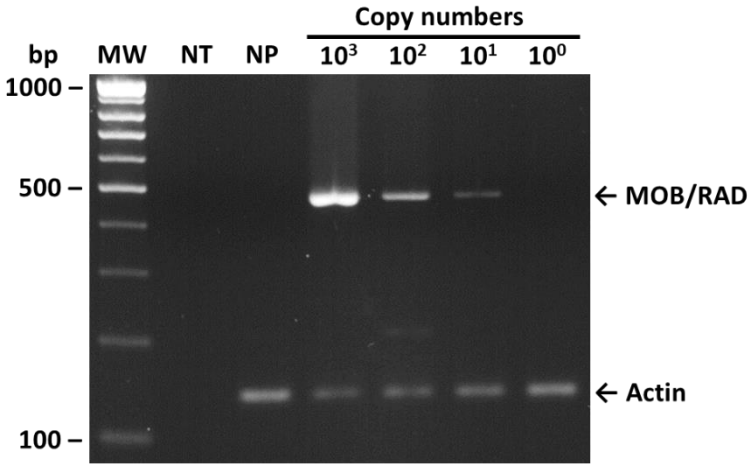

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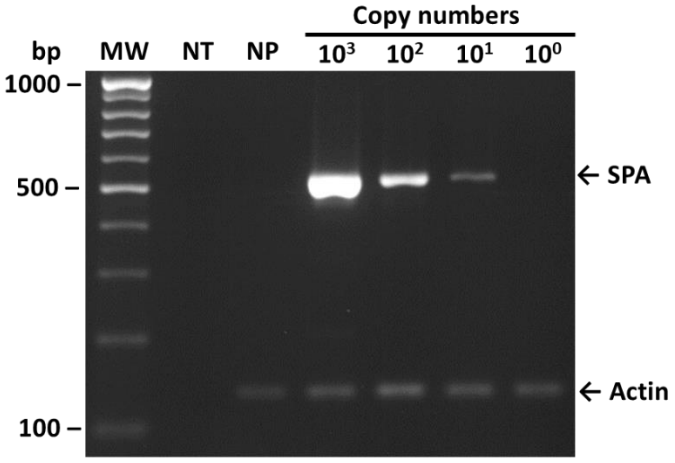

Figure 8. Sensitivity of the multiplex PCR assay in single infestation. Serial dilution of fur mite DNA was mixed with the no-parasite cage wipe sample. (A) *Myocoptes musculinus*. (B) *Myobia musculi*/*Radfordia* spp. (C) Species A. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: *Myocoptes musculinus*; MOB/RAD: *Myobia musculi*/*Radfordia* spp.; SPA: species A

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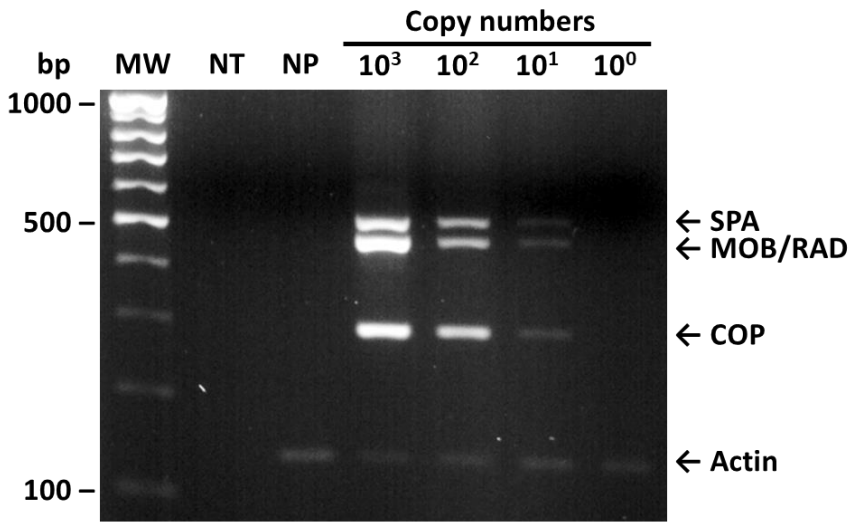

Figure 9. Sensitivity of the multiplex PCR assay in triple infestation. Serial dilutions of different fur mite DNA were mixed with the no-parasite cage wipe sample.
MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: *Myocoptes musculinus*; MOB/RAD: *Myobia musculi*/*Radfordia* spp.; SPA: species A

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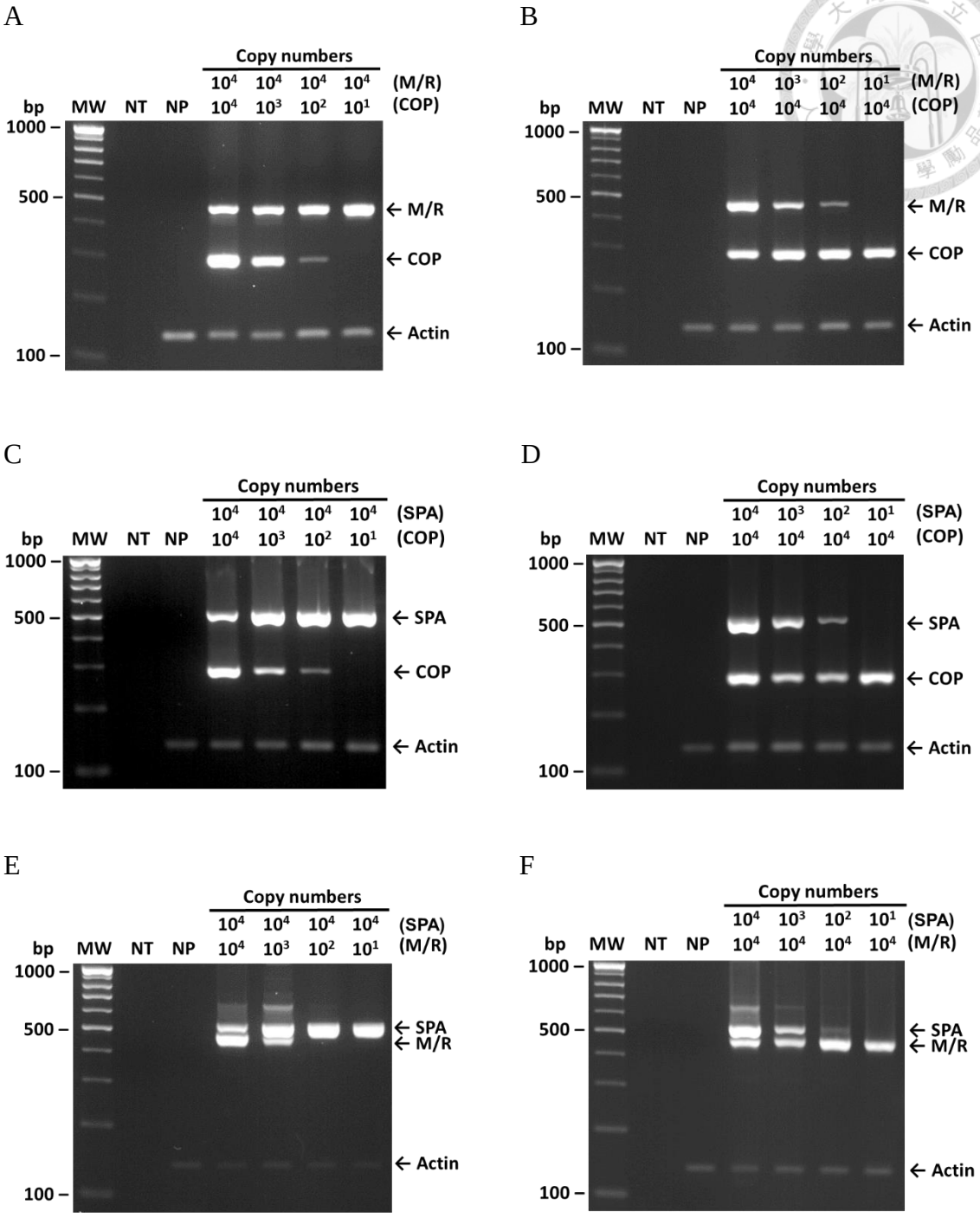

Figure 10. Detection of different combination in dual infestation. (A) 10^4 copies of *Myobia musculi* DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of *Myocoptes musculinus* DNA. (B) 10^4 copies of *Myocoptes musculinus* DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of *Radfordia* spp.DNA. (C) 10^4 copies of species A DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of *Myocoptes musculinus* DNA. (D) 10^4 copies of *Myocoptes musculinus* DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of species A DNA. (E) 10^4 copies of species A DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of *Radfordia* spp. DNA. (F) 10^4 copies of *Radfordia* spp. DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of species A DNA.

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All reactions were mixed with the ectoparasite-negative cage wipe samples except no template control. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: *Myocoptes musculinus*; M/R: *Myobia musculi*/*Radfordia* spp.; SPA: species A.

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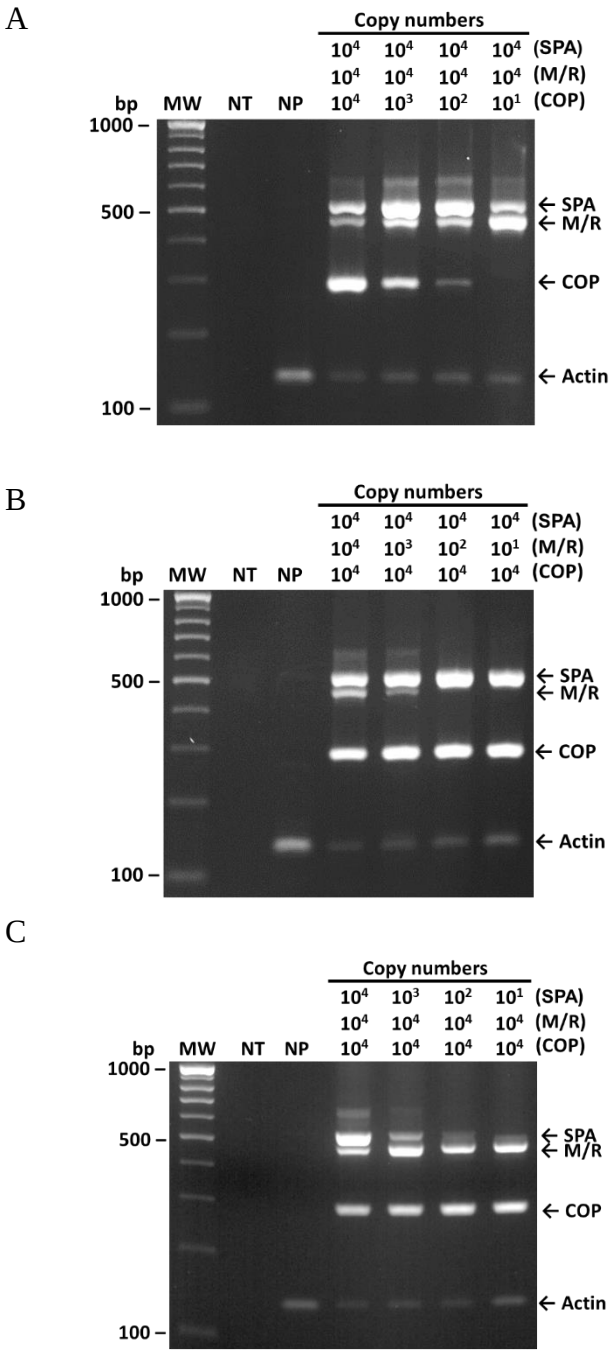

Figure 11. Detection of different combination in triple infestation. (A) 10^4 copies of *Radfordia* spp. and species A DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of *Myocoptes musculinus* DNA. (B) 10^4 copies of *Myocoptes musculinus* and species A DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of *Radfordia* spp. DNA. (C) 10^4 copies of *Myocoptes musculinus* and *Radfordia* spp. DNA mixed with 10^4, 10^3, 10^2, 10^1 copies of species A DNA. All reactions were mixed with the ectoparasite-negative cage wipe samples except no template control. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; COP: *Myocoptes musculinus*; M/R: *Myobia musculi*/*Radfordia* spp.; SPA: species A.

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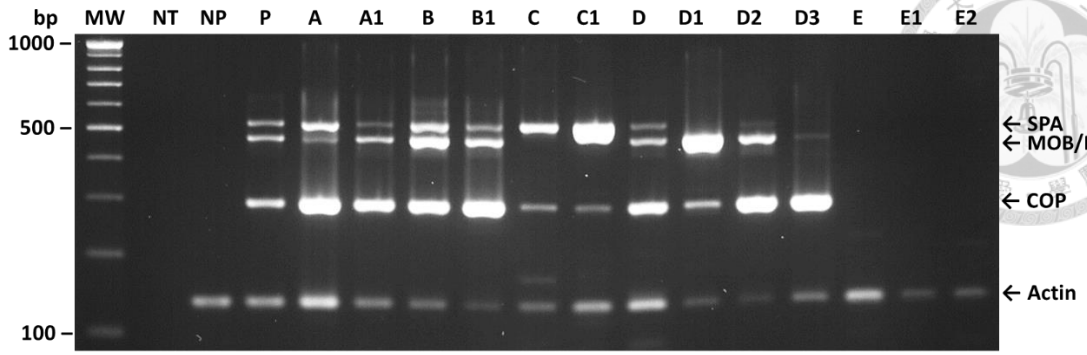

Figure 12. Detection of fur mites in clinical samples by the multiplex PCR assay. MW: molecular weight marker; NT: no-template negative control; NP: no-parasite cage wipe control; P: 10^2 copies of *Myocoptes musculinus, Radfordia* spp., and species A DNA mixed with the no-parasite cage wipe sample; A, B, C, D, E: clinical cage wipe samples from different 5 facilities; A1, B1, C1, D1, D2, D3, E1, E2 : clinical fur swab samples from different animals living in the corresponding A-E cages. COP: *Myocoptes musculinus*; MOB/RAD: *Myobia musculi*/*Radfordia* spp.; SPA: species A.

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Table 1. Information of animals and fur mite diagnostic results of various methods on clinical samples[1]

Animal ID	Cage #	Facility code	Facility classification[2]	Species	Age[3]	Infection status[4]	Traditional method			Multiplex PCR[5]	
							Pluck test	Tape test	Pelt exam	fur swab	cage wipe
1				Mouse	Adult	–[6]	–	–	–	–	
2	1	F1	SPF	Mouse	Adult	–	–	–	–	–	–
3				Mouse	Adult	–	–	–	–	–	
4				Mouse	Adult	–	–	–	–	–	
5	2	F1	SPF	Mouse	Adult	–	–	–	–	–	–
6				Mouse	Adult	–	–	–	–	–	
7	3	F2	SPF	Mouse	Elder	–	–	–	–	–	–
8	4	F2	SPF	Mouse	Elder	–	–	–	–	–	–
9				Mouse	Elder	–	–	–	–	–	
10	5	F3	SPF	Rat	Adult	–	–	–	–	–	–
11	6	F4	C	Mouse	Adult	–	–	–	–	–	–
12	7	F5	C	Mouse	Elder	**COP, M/R, SPA**[7]	**Egg**[7]	**COP**	**Egg, COP, non-COP**[7]	**COP, M/R**	**COP, M/R, SPA**
13	8	F5	C	Mouse	Elder	**COP, M/R, SPA**	**Egg, COP**	**COP**	**Egg, COP, RAD**[7]	**COP, M/R, SPA**	**COP, M/R, SPA**
14	9	F5	C	Mouse	Elder	**COP, M/R, SPA**	**Egg**	**COP**	**Egg, COP**	**COP, SPA**	**COP, M/R, SPA**
15	10	F5	C	Mouse	Elder	**COP, M/R, SPA**	–	**Egg, COP**	**Egg**	**COP, M/R**	**COP, M/R, SPA**
16	11	F6	C	Mouse	Elder	**COP, SPA**	**Egg**	–	**Egg, COP, non-COP**	**COP, SPA**	**COP, SPA**
17	12	F7	C	Mouse	Elder	–	–	–	–	–	–
18				Mouse	Elder	–	–	–	–	–	
19				Mouse	Elder	–	–	–	–	–	
20				Mouse	Elder	–	–	–	–	–	

doi:10.6342/NTU201903951

21	13	F8	C	Mouse	Adult	–	–	–	–	–	–
22	14	F8	C	Mouse	Adult	–	–	–	–	–	–
23					Adult	–	–	–	–	–	
24	15	F8	C	Mouse	Elder	–	–	–	–	–	–
25					Elder	–	–	–	–	–	
26	16	F9	C	Mouse	Elder	–	–	–	–	–	–
27					Elder	–	–	–	–	–	
28					Elder	–	–	–	–	–	
29					Elder	–	–	–	–	–	
30	17	F10	C	Mouse	Elder	–	–	–	–	–	–
31	18	F10	C	Mouse	Adult	–	–	–	–	–	–
32					Adult	–	–	–	–	–	
33	19	F11	C	Rat	Elder	–	–	–	–	–	–
34					Elder	–	–	–	–	–	
35	20	F12	P	Mouse	Young	**COP, M/R, SPA**	**Egg, COP**	**Egg, COP**	**Egg, COP, non-COP**	**COP, M/R, SPA**	**COP, M/R, SPA**
36					Young	**COP, M/R, SPA**	**Egg**	–	**Egg, COP**	**COP, M/R, SPA**	
37	21	F12	P	Mouse	Adult	**COP, M/R, SPA**	**Egg, COP**	**Egg, COP, non-COP**	**Egg, COP, non-COP**	**COP, M/R, SPA**	**COP, M/R, SPA**
38					Adult	**COP, M/R, SPA**	**Egg**	**Egg, COP**	**Egg, COP, non-COP**	**COP, M/R**	
39	22	F12	P	Mouse	Adult	**COP, M/R, SPA**	**Egg**	**Egg, COP, non-COP**	**Egg, COP, SPA**	**COP, M/R, SPA**	**COP, M/R, SPA**
40					Adult	**COP, M/R, SPA**	**Egg**	**Egg, non-COP**	**Egg, COP, SPA**	**COP, M/R, SPA**	
41	23	F13	P	Mouse	Young	–	–	–	–	–	–
42					Young	–	–	–	–	–	
43	24	F14	P	Mouse	Adult	**COP, M/R, SPA**	–	–	**Egg, COP, SPA**	**COP, M/R**	**COP, M/R, SPA**
44					Young	**COP, M/R, SPA**	**Egg**	**Egg, COP**	**Egg, COP, RAD**	**COP, M/R, SPA**	

doi:10.6342/NTU201903951

45					Young	**COP, M/R, SPA**	**Egg**	**COP**	**Egg, COP, RAD**	**COP, M/R**	
46					Young	**COP, M/R, SPA**	**Egg**	**–**	**Egg, COP, SPA**	**COP, M/R**	
47	25	F15	P	Mouse	Young	**COP, M/R, SPA**	**–**	**–**	**Egg, COP**	**COP, M/R, SPA**	**COP, M/R, SPA**
48					Adult	**COP, M/R, SPA**	**Egg**	**Egg**	**Egg, COP**	**COP, M/R, SPA**	

[1] A total of 48 animals from 15 facilities were tested by 3 traditional methods (pluck test, tape test and pelt exam) and the multiplex PCR for fur mite infestation.

[2] SPF: specific-pathogen free; C: conventional; P: pet store.

[3] Age range for the young (Young) is age < 4 weeks old; age range for the adult (Adult) is 4 weeks old ≤ age ≤ 16 weeks old; age range for the older adult (Elder) is age > 16 weeks old.

[4] The infection status of each animal was based on the results of three single specific PCR assays on the fur swab sample and/or traditional diagnostic methods.

[5] The multiplex PCR was applied to both fur swab and cage wipe samples collected from 48 animals and 25 cages. Each cage housed 1 to 4 animals.

[6] —: Negative result.

[7] COP: *Myocoptes musculinus*; M/R: *Myobia musculi* and/or *Radfordia* spp.; RAD: *Radfordia* spp.; SPA: species A; non-COP: non-*Myocoptes musculinus* fur mite(s); Egg: fur mite egg(s).

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Table 2. Comparison of the results of traditional diagnostics and multiplex PCR (fur swab)[1]

Diagnostics	Fur mite[2]	Infection status[3]	+	+	-	-	Total	Sensitivity[4]	Accuracy[5]
		Diagnostics	+	-	-	+			
Antemortem									
Multiplex PCR	COP		17	0	31	0	48	100 %	100 %
	MOB/RAD		15	1	32	0	48	93.8 %	97.9 %
	SPA		11	6	31	0	48	64.7 %	87.5 %
	Total		43	7	94	0	144	86 % (43/50)	95.1 % (137/144)
Pluck test	COP		3	14	31	0	48	17.7%	70.8 %
	MOB/RAD		0	16	32	0	48	0 %	66.7 %
	SPA		0	17	31	0	48	0 %	64.6 %
	Unclarified		0	NA[6]	NA	NA	NA		
	Total		3	47	94	0	144	6 % (3/50)	67.4 % (97/144)
Tape test	COP		10	7	31	0	48	58.8 %	85.4 %
	MOB/RAD		0	16	32	0	48	0 %	66.7 %
	SPA		0	17	31	0	48	0 %	64.6 %
	Unclarified		3	NA	NA	NA	NA		
	Total		10	40	94	0	144	20 % (10/50)	72.2 % (104/144)
Postmortem									
Pelt exam	COP		16	1	31	0	48	94.1%	97.9 %
	MOB/RAD		3[7]	13	32	0	48	18.8 %	72.9 %
	SPA		4	13	31	0	48	23.5 %	72.9 %
	Unclarified		5	NA	NA	NA	NA		
	Total		23	27	94	0	144	46 % (23/50)	81.3 % (117/144)

[1] All 48 animals were tested by three traditional methods (pluck test, tape test and pelt exam) and the multiplex PCR for fur mite infestation.

[2] COP: *Myocoptes musculinus*; MOB/RAD: *Myobia musculi* and/or *Radfordia* spp.; SPA: species A; Unclarified: non-*Myocoptes musculinus* unclarified fur mite(s).

[3] The infection status of each animal was based on the results of three single specific PCR assays on the fur swab sample and/or three traditional diagnostic methods.

[4] True positives / (true positives + false negatives)

[5] (True positives + true negatives) / (true positives + false positives + false negative + true negatives)

[6] NA: not available.

[7] Only *Radfordia* spp. was observed on the fur-mite infested mice.

doi:10.6342/NTU201903951

Table 3. The multiplex PCR results of the environment samples[1]

Fur mite[2]	Pathogen status[3]	+	+	-	-	Total
	Multiplex PCR[4]	+	-	-	+	
COP		10	0	15	0	25
MOB/RAD		9	0	16	0	25
SPA		10	0	15	0	25
Total		29	0	46	0	75

[1] The cage wipe samples were collected from 25 cages, housing 48 animals.

[2] COP: *Myocoptes musculinus*; MOB/RAD: *Myobia musculi* and/or *Radfordia* spp.; SPA: species A.

[3] The pathogen status of each cage was based on the results of three single specific PCR assays on the cage wipe sample.

[4] The results of the multiplex PCR on cage wipe samples.

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