The Likelihood of Misidentifying Rodent Pasteurellaceae by Using Results from a Single PCR Assay

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The precise identification of rodent Pasteurellaceae is known to be highly challenging. An unknown strain of Pasteurellaceae appeared and rapidly spread throughout our animal facilities. Standard microbiology, combined with biochemical analysis, suggested that the bacteria strain was *Rodentibacter pneumotropicus* or *R. heylII*. We submitted samples of the unknown bacteria and known isolates of *R. pneumotropicus*, *R. heylII*, and *Muribacter muris*, to 2 service laboratories that provide animal health monitoring. Results of microbiology tests performed by both laboratories, species-specific PCR analysis performed by one laboratory, and independent 16S rRNA gene sequencing yielded identical identification of the unknown bacteria as Pasteurellaceae (*Pasteurella* spp.) and not *R. pneumotropicus* or *R. heylII*. In contrast, the similarly intended PCR assay performed by the other laboratory identified the bacteria as *R. heylII*. Careful evaluation of all of the results led us to conclude that the correct identification of the bacteria is Pasteurellaceae. From our experience, we recommend that a combination of several methods should be used to achieve correct identification of rodent Pasteurellaceae. Specifically, we advise that all primer sets used should be disclosed when reporting PCR test results, including in health reports provided by service laboratories and animal vendors. Careful, correct, and informative health monitoring reports are most beneficial to animal researchers and caretakers who might encounter the presence and effects of rodent Pasteurellaceae.

Abbreviations: FELASA, Federation of European Laboratory Animal Science Associations; HRM, high-resolution melting; MALDI-TOF MS, matrix-assisted laser desorption-ionization–time-of-flight mass spectrometry

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The rodent Pasteurellaceae are considered among the most prevalent opportunistic bacterial pathogens isolated from experimental animals.^{15,30} Among members of the Pasteurellaceae, *Rodentibacter pneumotropicus* and *R. heylII* (formerly [*Pasteurella*] *pneumotropica* biotypes *Jawetz* and *Heyl*, respectively) are most often associated with rodent infections, although associated clinical symptoms, in general, are few. However, Pasteurellaceae infections in immunocompetent mice could cause experimental perturbations that have the potential to confound experimental studies and represent an unwanted experimental variable.²⁹ Pasteurellaceae infections cause even greater concern in experiments involving immunodeficient mice or potentially immunocompromised transgenic mouse strains.^{2,11,13,20,25,33}

The high prevalence of these bacteria and their potential importance to animal experiments led the Federation of European Laboratory Animal Science Associations (FELASA) to recommend monitoring and reporting of all Pasteurellaceae species.²⁶ This recommendation reflects the evidence that different laboratories come to different conclusions regarding the same strain of rodent Pasteurellaceae and that commercial identification kits fail to identify them correctly.²⁶ In a revised publication, FELASA indicate that monitoring for all Pasteurellaceae may be performed but recommend specific reporting of [*Pasteurella*] *pneumotropica* only (namely, *R. pneumotropicus* and *R. heylII*).²² This revision may reflect the emergence of more

reliable molecular identification methods and the fact that many animal facilities worldwide rely on external and service laboratories that use such methods.

Members of the Pasteurellaceae, including [*Pasteurella*] *pneumotropica* biotype *Jawetz*,¹⁹ [*Pasteurella*] *pneumotropica* biotype *Heyl*¹⁷ and [*Actinobacillus*] *muris*,⁶ were first described long ago, but until recently, they have not been formally classified under genera because the position of these species in taxonomy was unclear. This ambiguity was reflected by the use of brackets, indicating that these are not true members of the bacterial family. However, recently these species went through taxonomic revision, and [*Pasteurella*] *pneumotropica* and several other rodent Pasteurellaceae were reclassified within the new genus *Rodentibacter*.^{1,5} The biotype *Jawetz* of [*Pasteurella*] *pneumotropica* was reclassified as part of *R. pneumotropicus*, and the biotype *Heyl* of [*Pasteurella*] *pneumotropica* was reclassified as *R. heylII*.¹ [*Actinobacillus*] *muris* was reclassified as *Muribacter muris*.²⁷

Due to the unclear taxonomy, precise identification of rodent Pasteurellaceae has been highly challenging. Detection usually relies on culture isolation of samples (usually from the nasopharynx) and subsequent biochemical characterization of suspected colonies. Identification of rodent Pasteurellaceae species that were recently reclassified as *Rodentibacter* spp. and their differentiation from other rodent Pasteurellaceae is possible, due to laborious biochemical analysis.^{1,5} Commercially miniaturized biochemical tests kits are available, but these kits are optimized for human samples and do not include all of the various Pasteurellaceae species. Specifically, one commonly used system was reported as suitable for classifying Pasteurellaceae strains with the

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family but unreliable for identifying rodent Pasteurellaceae to the species level.^{8,14} Matrix-assisted laser desorption-ionization–time-of-flight mass spectrometry (MALDI-TOF MS) might achieve sufficient specificity of identification. Although this method currently lacks appropriate murine datasets, institutional and commercial laboratories can develop their own databases, and this approach is expected to become more useful for the identification of microorganisms.

In view of the limitations of other methods, PCR-based assays are becoming essential for the identification of rodent Pasteurellaceae. In addition, these assays are considered to be most reliable and sensitive options available. Most PCR assays target the 16S rRNA gene. Some detect all rodent Pasteurellaceae,^{9,10} whereas others are able to differentiate between species.^{21,28,34,35} Unique and stable nucleotide motifs within the 16S rRNA gene permitted the development of an RT-PCR assay incorporating species-specific fluorogenic probes, which resulted in successful confirmation of identification of bacteria isolated by culture and differentiation between *R. pneumotropicus* and *R. heyltii*.¹² The same approach also was used to detect *R. pneumotropicus* in exhaust air dust samples, showing that exhaust air dust sampling is superior to conventional soiled-bedding sentinel monitoring.²³ In addition, an RT-PCR assay using the same nucleotide motifs, combined with high-resolution melting (HRM) curve analysis, provided reliable identification and differentiation of murine Pasteurellaceae species.²⁴ Alternatives extending beyond the 16S rRNA gene include development of specific PCR assays based on the *gyrB* gene sequence and subsequent restriction fragment length polymorphism analysis¹⁶ and a multiplex PCR assay based on the 16S–23S rRNA internal transcribed spacer region.³

Here we report our struggles to identify a strain of bacteria that was new to our animal facilities and that we suspected as *R. pneumotropicus* or *R. heyltii*. We also describe our efforts to reconcile the discrepancy between test results from different service laboratories.

Materials and Methods

Animals. Experiments were performed in accordance with Israeli law and the guidelines of the Weizmann Institute of Science. All experimental protocols were reviewed and approved by the Weizmann Institutional Animal Care and Use Committee, as part of the institutional rodent health monitoring program. C57BL/6JOLA^{Hsd} female mice (Envigo, Jerusalem, Israel; age, 5 to 6 wk) served as soiled-bedding sentinels for 5 to 6 mo. CrI:DC1(ICR) female mice bred inhouse served as surrogate mothers or sentinels for rederived or quarantined strains, respectively, for 6 wk prior to screening and introduction of the new colony into our animal facilities. These mice were tested by the institutional diagnostic lab at the end of the exposure period. Additional C57BL/6JOLA^{Hsd} female mice were tested immediately on arrival from the vendor.

Health monitoring and pathogen status. Animal facilities at the Weizmann Institute of Science are barrier facilities. Entrance is card-restricted and requires personal protective equipment, including disposable masks, shoe covers, hair bonnets, gowns, and gloves. All equipment and supplies are sterilized or disinfected. The water is autoclaved or filtered and acidified. All rodents are housed in IVC (microisolation) racks. Cages are changed inside a biosafety cabinet (BSL2) only. Housing is allowed for rodents that originate from approved vendors (Envigo and the Jackson Laboratory); animals from other sources are housed only after rederivation or quarantine followed by health screening.

Each quarter, the institutional diagnostic lab performs complete inhouse health monitoring for rodents, by using soiled-bedding sentinels and according to the recommendation of FELASA.^{22,26} Each side of a mouse cage rack (approximately 70 cages) has 2 sentinel cages, each containing 2 female C57BL/6JOLA^{Hsd} mice, which are introduced at 5 to 6 wk of age. At every cage change, both sentinel cages receive soiled bedding from a consecutive row of cages. Quarterly, the older sentinel cage (after 5 to 6 mo exposure to soiled bedding) is sent for testing; the other cage remains for another quarter, and a new sentinel cage is added. In addition to sentinels, randomly sampled stock mice are tested for ectoparasites.

During health monitoring, mice are euthanized by using CO₂ and evaluated for gross pathology, blood is collected retroorbitally for serology, the nasopharynx and cecum are swabbed for bacteriology, and rectal fecal pellets are collected for evaluation of *Helicobacter* spp. by PCR analysis. Ecto- and endoparasites are evaluated microscopically, and internal organs are taken for histopathology. We frequently detect mouse norovirus, Pasteurellaceae, *Helicobacter* spp., and nonpathogenic protozoa. Less frequently we also find *Klebsiella* spp., *Proteus mirabilis*, and fur mites.

Bacterial isolates and culture. Reference strains for *R. pneumotropicus* (ATCC 35149; NCTC 8141), *R. heyltii* (ATCC 12555; C57), and *M. muris* (ATCC 49577; CCUG 16938, NCTC 12432) were obtained from American Type Culture Collection (Manassas, VA).

Bacterial isolates were isolated from nasopharyngeal swab specimens from mice during routine health monitoring procedures. The swabs were cultured on tryptic soy agar with defibrinated sheep blood (blood agar; Hy Laboratories, Rehovot, Israel) and MacConkey agar (Becton Dickinson, Sparks, MD) for 24 h at 37 °C. Colonies growing on blood agar were gram stained (Gram Stain Kit, Becton Dickinson) and tested for oxidase reaction (Hy-Oxidase Test; Hy Laboratories). We attempted to identify oxidase-positive, gram-negative rods by using the API 20 NE biochemical kit (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's protocol. Single colonies were picked and propagated in tryptic soy broth (Becton Dickinson) for 24 h at 37 °C for DNA extraction. An aliquot was stored in 50% glycerol at –80 °C for subcloning and validation assays.

Identification and sequencing by service laboratories. Bacterial isolates were subcloned on blood agar plates for 24 h at 37 °C in duplicate. Single colonies were picked from the first set of plates and sent on cool packs to Charles River Laboratories (Lab 1; Wilmington, MA) and IDEXX BioResearch (Lab 2; Columbia, MO). Colonies were picked by using Amies transport swabs (Copan Italia, Brescia, Italy) for general microbiology and by using oral swabs (provided by Lab 1) for PCR analysis.

Both Lab 1 and Lab 2 confirm microbiologic identification of microorganisms through MALDI-TOF MS (specifications of the assays were not provided). Lab 1 results are provided only for the list of bacteria recommended by FELASA. Lab 2 results are not limited to the FELASA recommended list and instead are provided for any bacterial growth that were included in the MALDI-TOF dataset. In addition, we asked both labs to perform single-agent PCR assays that specifically target *R. pneumotropicus* and *R. heyltii*. Neither company provides the sequences of the primers or PCR products.

The second set of plates was collected and analyzed by Lab 3 (Hy Laboratories). Identification of bacteria by this company involved extraction of genomic DNA, amplification of the 16S rRNA gene, and sequencing of the PCR product(s). The results for the tested sample represented the highest homology available in

databases. We then requested and received the actual sequences obtained and performed BLAST analysis¹⁸ to find homologies and align sequences.

DNA extraction. DNA was extracted from 1 mL of liquid culture (or subculture after thawing) by using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and used for PCR assays (the kit is in regular use in the lab for other specimens). Another round of DNA extraction of the same isolates was performed by using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR results were equivalent for both extraction methods.

RT-PCR and HRM targeting the 16S rRNA gene. RT-PCR assay combined with HRM analysis followed a previously described protocol²⁴ with minor modifications. The forward (5' CGG GTT GTA AAG TTC TTT CGG T 3') and reverse (5' GGA GTT AGC CGG TGC TTC TTC 3') primers were purchased from Sigma-Aldrich (Rehovot, Israel). The reaction mixture consisted of 6 µL 2× MeltDoctor HRM Master Mix (Applied Biosystems), 0.3 µM of each primer, 20 ng of bacterial genomic DNA template, and ultrapure water (Biologic Industries, Beit-Haemek, Israel) to a final reaction volume of 12 µL.

Assays were performed in a StepOnePlus RT-PCR System (Applied Biosystems) with initial denaturation at 95 °C for 10 min, followed by 40 cycles comprising denaturation at 95 °C for 15 s, annealing–extension at 58 °C for 30 s, and HRM ramping from 60 to 95 °C. Fluorescence data were acquired in a continuous mode set to 0.3% (approximately 0.1 °C) increments at a ramp rate of 0.0075 °C/s (according to the manufacturer's manual) to generate specific melting curves. Sequencing-confirmed ATCC references were included as melting curve standards. Reactions were performed in triplicate. Data analysis was performed by using High Resolution Melt Software version 3.0.1 (Applied Biosystems).

The resulting melting curves were normalized by using pre-melting phase as 100% and post-melting phase as 0%, thus eliminating differences in fluorescence intensity and background fluorescence between wells. A no-template control was included in each experiment, to reveal the presence of contaminants in the reaction mixture.

Multiplex PCR assay targeting the 16S–23S rRNA internal transcribed spacer sequences. The multiplex PCR assay followed a previously described protocol³ with minor modifications. We used a common forward primer (conserved among all rodent Pasteurellaceae) on the 16S rRNA gene with different reverse primers targeting the 16S–23S rRNA internal transcribed spacer sequences. Primer sequences include: common forward primer (PastF), 5' ATG GGA GTG GGT TGT ACC A 3'; common reverse primer (PastR), 5' CAA TCT GTG TGR ACA CTT RC 3'; *M. muris*-specific primer a (A.m. R 101–120a), 5'-AAG CGG TCG GTT TTA GCT GT 3'; *M. muris*-specific primer b (A.m. R 101–120b), 5'-AAG CAG TTG GTT TTG GCT GT 3'; *R. heyliae*-specific primer (HeylR), 5' TTG CAG ATA CTT GCC CTT AC 3'; and *R. pneumotropica*-specific primer (JawR), 5'-GGC ATC CTA AAA TAC CCA TCC 3'. All primers were purchased from Sigma-Aldrich.

The reaction mixture consisted of 12 µL 2× GoTaq Green Master Mix (Promega, Madison, WI), the primers at appropriate concentrations (PastF, 1 µM; PastR, HeylR, and JawR, 0.5 µM each; A.m. R 101–120a and A.m. R 101–120b, 0.25 µM each; alternatively, only one of the A.m. R primers was used at 0.5 µM), 20 ng of bacterial genomic DNA template, and ultrapure water to a final reaction volume of 24 µL.

Assays were performed in a StepOnePlus RT-PCR System with initial denaturation at 95 °C for 5 min; 35 cycles comprising denaturation at 95 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 4 min. Amplification products were analyzed through electrophoresis of 4 µL of the reaction mixture in a GelRed (Biotium, Fremont, CA)-containing agarose gel (1.5%, Amresco, Solon, OH) and photographed under UV exposure (Bio-Rad Laboratories, Hercules, CA). A 100-bp DNA ladder (GeneRuler, ThermoFisher Scientific, Waltham, MA) was used as a molecular size standard.

Results

Detection of new strain of Pasteurellaceae. The Weizmann Institute of Science performs full inhouse health monitoring of rodents according to FELASA recommendations.^{22,26} During our routine health monitoring, we occasionally detect *R. pneumotropica* (isolate type 1) in swab samples from the nasopharynx of mice. The bacteria grow on blood agar as shiny gray colonies but do not grow on MacConkey agar. Gram staining yields gram-negative rods, and the oxidase test is positive. Final confirmation routinely involves using a biochemical test kit (API 20 NE, bioMérieux), which profiles the bacteria with strong identification (exceeding 99%) as '*P. pneumotropica*' (namely *R. pneumotropica* or *R. heyliae*). The colony appearance of this bacteria and its biochemical results are similar to those of the reference *R. pneumotropica* (ATCC 35149; NCTC 8141). Less frequently, we detect *R. heyliae* (isolate type 2) during the quarantine period of incoming mice colonies. The color of *R. heyliae* colonies differs slightly from that of *R. pneumotropica* but the biochemical identification is similar.

In mid 2013, we detected a new strain of bacteria in our sentinel mice (isolate type 3). Isolate type 3 grew on blood agar as small, yellowish β-hemolytic colonies, which were rod-shaped on Gram stains. Biochemical test kit (API 20 NE, bioMérieux) identified the bacteria as '*P. pneumotropica*' (exceeding 99%) as well. Within one quarterly health monitoring cycle, the bacteria had spread throughout our animal facilities, and positive results reached approximately 60% in sentinels. During 2017, we detected isolate type 3 in approximately 90% of our sentinels (we probably missed the remaining approximately 10% due to overgrowth of other bacteria). For comparison with other Pasteurellaceae findings, we regularly detect *R. pneumotropica* (isolate type 1) and *M. muris* (isolates type 4 and 5, for which API 20 NE identification is not valid) in about 3.5% and 2.5% of our sentinels respectively (localized to specific racks). These percentages might underestimate the actual prevalence because Pasteurellaceae are not transmitted well to soiled-bedding sentinels.^{23,32}

Due to the rapid spread and high prevalence, we suspected that the origin of isolate type 3 was the purchased sentinel animals. To prove this suspicion, we purchased additional mice together with mice designated as sentinels. These mice were shipped directly to our diagnostic lab without entering any of our animal facilities and were tested immediately on arrival. As we suspected, isolate type 3 was detected in nasopharynx swab samples obtained from these extra sentinel mice.

Verification of identification of Pasteurellaceae by service laboratories. For further identification of isolate type 3, we submitted several reference and bacterial isolates for testing at external service laboratories (Table 1).

Microbiology (Table 1) performed by both Lab 1 and Lab 2 identified the *R. pneumotropica* reference and isolate type 1 as *P. pneumotropica*; in addition, Lab 2 provided the biotype (Jawetz, namely *R. pneumotropica*). Lab 1 microbiology results for

Table 1. Identification of isolates by different laboratories and methods

	Microbiology		PCR		BLAST
	Lab 1	Lab 2	Lab 1	Lab 2	
References					
<i>R. pneumotropicus</i> (ATCC 35149; <i>n</i> = 2)	<i>R. pneumotropicus</i> or <i>R. heyltii</i>	<i>R. pneumotropicus</i>	<i>R. pneumotropicus</i>	<i>R. pneumotropicus</i>	<i>R. pneumotropicus</i> M75083.1 99% (nt 49–762)
<i>R. heyltii</i> (ATCC 12555; <i>n</i> = 1)	NS	NS	<i>R. heyltii</i>	<i>R. heyltii</i>	<i>R. heyltii</i> KX858303.1 100% (nt 11–745) AF012090.1 99% (nt 4–738)
<i>M. muris</i> (ATCC 49577; <i>n</i> = 1)	NS	NS	—	—	<i>M. muris</i> AF024526.1 99% (nt 70–762)
Isolates					
Type 1 (<i>n</i> = 5)	<i>R. pneumotropicus</i> or <i>R. heyltii</i>	<i>R. pneumotropicus</i>	<i>R. pneumotropicus</i>	<i>R. pneumotropicus</i>	<i>R. pneumotropicus</i> M75083.1 99% (nt 27–763)
Type 2 (<i>n</i> = 1)	NS	NS	<i>R. heyltii</i>	<i>R. heyltii</i>	<i>R. heyltii</i> KX858334.1 100% (nt 3–737) AF012090.1 99% (nt 4–738)
Type 3 (<i>n</i> = 6)	FELASA –	<i>Pasteurella</i> sp. not <i>pneumotropica</i>	<i>R. heyltii</i>	—	<i>P. pneumotropicus</i> JQ346058.1 100% (nt 14–762) Pasteurellaceae HF912264.1 99% (nt 1–708)
Type 4 (<i>n</i> = 2)	NS	NS	—	—	<i>M. muris</i> AF024526.1 99% (nt 76–745)
Type 5 (<i>n</i> = 3)	FELASA –	<i>M. muris</i>	—	—	<i>M. muris</i> KP278081.1 100% (nt 3–746) AF024526.1 98% (nt 13–759)

BLAST, BLAST analysis for 16s rRNA gene PCR and sequencing (approximately 700–750 bp; GenBank accession number for highest homology found in the BLAST databases is listed with the percentage similarity and the DNA nucleotide range that was covered); –, negative results; FELASA –, negative for the FELASA-recommended list of bacteria; NS, not submitted; nt, nucleotides.

isolates type 3 and 5 were negative (that is, not included in the list of bacteria recommended by FELASA), whereas Lab 2 results designated isolate type 3 as “*Pasteurella* sp. not *pneumotropica*” and isolate type 5 as “*A. muris*” (*M. muris*). Isolates type 2 and 4 were not submitted for microbiology.

Species-specific PCR assays (Table 1) by both Lab 1 and Lab 2 confirmed the relevant references, and isolate types 1 and 2 were identified as *R. pneumotropicus* and *R. heyltii*, respectively. PCR results for the other isolates were negative, except that isolate type 3 was identified only by Lab 1, as “*P. pneumotropica* biotype Heyl” (namely, *R. heyltii*).

The results of partial 16s rRNA gene sequencing and BLAST analysis (Table 1) was in line with the earlier described results. The *R. pneumotropicus* reference and isolate type 1 matched GenBank accession no. M75083.1 (99%). The *R. heyltii* reference and isolate type 2 matched GenBank accession no. AF012090.1 (99%). Isolates type 4 and 5 matched the *M. muris* reference AF024526.1 (98% and 99%, respectively, with 99% identity between the isolate types in the section that was sequenced). Isolate type 3 matched both *P. pneumotropica* (JQ346058.1) and Pasteurellaceae (HF912264.1) with 100% identity (HF912264.1 is identical to JQ346058.1 for the shared sequenced region with

Figure 1. High-resolution melting curve analysis. Bacterial isolates: type 1, matching *R. pneumotropicus* (M75083.1; $n = 9$); type 2, matching *R. heyltii* (AF012090.1; $n = 2$); type 3, matching Pasteurellaceae (HF912264.1; $n = 7$); types 4 and 5, matching *M. muris* (AF024526.1, $n = 2$; KP278081.1, $n = 5$, respectively). (A) Alignment of expected amplicons of the 16S rRNA gene according to the noted accession numbers. The sequence of M75083.1 is displayed on top, corresponding to the color code. (B) Derivative melting temperature curves. (C) Difference curves derived from normalized data by using representative isolate type 5 as baseline. Each isolate was run in triplicate. Note that isolate type 4 is indistinguishable from isolate type 3.

only one [N-to-A] change at the beginning of the HF912264.1 sequence). Isolate type 3 was only 96% identical to other *R. pneumotropicus* and *R. heyltii* entries in GenBank. In addition, extended 16S rRNA gene sequencing of isolate type 3 (GenBank accession no., MH628087) matched JQ346058.1 (nucleotides covered, 31 to 1471; identity, 99%) and HF912264.1 (nucleotides covered, 1 through 876; identity, 99%).

Evaluation of literature-suggested PCR assays for identification of Pasteurellaceae. We next tested the isolates with 2 literature-suggested PCR assays for identification of rodent Pasteurellaceae. From the sequencing results, we expected the RT-PCR HRM analysis to detect all of the references and isolates (Figure 1 A). The melting profile of all isolates and matching references resulted in a single melting peak (Table 2). Isolate types 1, 2, 3, and 5, each exhibited a distinct HRM profile. However, the HRM profile of isolate type 4, the amplicon for which differed from isolate type 5 at only one nucleotide (A/G), was indistinguishable from isolate type 3 (Figure 1 B and C).

In the multiplex PCR analysis, all isolates showed the common Pasteurellaceae band at 165 to 170 bp (Figure 2). The *R. pneumotropicus* reference and isolate type 1 yielded an additional band of 451 bp. The *R. heyltii* reference and isolate type 2 produced an additional band of 326 bp. Isolate types 4 and 5 showed additional bands at 255 bp, as did the *M. muris* reference. Isolate type 3 yielded no additional bands (Figure 2 A). Furthermore, the efficiency of the assay for the additional *M. muris* band differed according to the primers used. In particular, A.m. R 101–120a had higher affinity for isolate type 4, whereas A.m. R 101–120b (targeting a 3 single-nucleotide mutations in comparison to the type strain⁴) had higher affinity for isolate type 5 (Figure 2 B and C).

Each PCR assay was repeated at least twice for each DNA extraction method, with equivalent results.

Discussion

We described here our struggle to reliably identify isolates of a bacteria that appeared to be a member of Pasteurellaceae. We and several service laboratories used different identification methods, which produced puzzling and contradicting results. Initially, the biochemical test kit (API 20 NE, bioMérieux) and the first BLAST hit of the 16S rRNA gene sequencing (GenBank accession no. JQ346058.1) suggested that the bacteria is *R. pneumotropicus* or *R. heyltii*. However, the biochemical system we used is known to be unreliable for identification of Pasteurellaceae.^{8,14} In addition, a closer look at the BLAST results showed that the second hit (omitting sequences from uncultured bacteria) was equally suitable (HF912264.1, essentially identical to JQ346058.1) and identified the bacteria as Pasteurellaceae. This second hit is also better documented than the first and is referred to as 'β-hemolytic Pasteurellaceae isolated from mouse',^{3,4} which is consistent with our own observation of hemolysis.

Isolates of this unknown Pasteurellaceae strain, together with 4 other isolate types, were submitted to service laboratories. Microbiology performed by both Lab 1 and Lab 2, results of PCR analysis performed by Lab 2, and sequencing data performed by Lab 3 were in good agreement, confirming that the bacteria in question was a Pasteurellaceae and not *R. pneumotropicus* or *R. heyltii*. In contrast, PCR analysis performed by Lab 1 identified the bacterial species as *R. heyltii*. As long as the exact details of this PCR assay and the sequences of primers and amplicons are unavailable, this discrepancy cannot be reconciled.

Table 2. High-resolution melting curve analysis of bacterial isolates

Isolate type	Species according to sequencing	ATCC reference	GenBank accession no.	No. of isolates analyzed	HRM assignment	T _m
1	<i>R. pneumotropicus</i>	35149	M75083.1	9	<i>R. pneumotropicus</i>	75.7 ± 0.2
2	<i>R. heyltii</i>	12555	AF012090.1	2	<i>R. heyltii</i>	78.5 ± 0.1
3	Pasteurellaceae	not applicable	HF912264.1	7	Pasteurellaceae or <i>M. muris</i>	77.0 ± 0.1
4	<i>M. muris</i>	49577	AF024526.1	2	Pasteurellaceae or <i>M. muris</i>	77.1 ± 0.0
5	<i>M. muris</i>	not applicable	KP278081.1	5	<i>M. muris</i>	76.6 ± 0.1

T_m, peak melting temperature (°C; mean ± 1 SD).

Figure 2. Multiplex PCR assay targeting the 16S-23S rRNA internal transcribed spacer region. (A) Assay including all primers (PastF, PastR, HeylR, JawR, and 1:1 mix of A.m. R 101–120a and A.m. R 101–120b). Lanes 1 and 17, 100-bp ladder; lane 2, *R. pneumotropicus* reference (ATCC 35149); lane 3, *R. heyltii* reference (ATCC 12555); lane 4, *M. muris* reference (ATCC 49577); lane 5, isolate type 1; lane 6, isolate type 2; lanes 7–11, isolate type 3; lanes 12 and 13, isolate type 4; lanes 14 and 15, isolate type 5; and lane 16, no-template control. (B and C) Assay including all primers except (B) A.m. R 101–120b or (C) A.m. R 101–120a. Lane 1, 100-bp ladder; lane 2, *M. muris* reference (ATCC 49577); lanes 3 and 4, isolate type 4; and lanes 5–8, isolate type 5.

RT-PCR assay combined with HRM analysis of a variable region in the 16S rRNA gene sequence distinguished between our isolates according to sequencing results. The one exception was that one of the *M. muris* strains showed the same HRM profile as Pasteurellaceae in the amplified region. The authors who suggested this method acknowledge this major limitation of HRM—that an unknown specimen not included in the reference library might have the same T_m value as a nonassociated reference strain and thus allow incorrect assignment of the species.²⁴

The multiplex PCR assay targeting the 16S–23S rRNA internal transcribed spacer sequences provided additional evidence, supporting the identification of our unknown bacteria as Pasteurellaceae. In addition, the authors who proposed the assay deposited the matching HF912264.1 sequence into GenBank.^{3,4}

Furthermore, in contrast to the HRM assay, the multiplex PCR assay correctly identified both *M. muris* strains in concordance with 16S rRNA gene sequencing results.

We conclude that our new bacteria is a Pasteurellaceae species and not *R. pneumotropicus* or *R. heyltii*. In addition, this case supports the advice to use a combination of several methods to achieve correct identification of rodent Pasteurellaceae.^{5,14,31} Although more time-consuming and expensive compared with a single PCR analysis, sequencing of the 16S rRNA gene is considered the most reliable method for identification and differentiation of Pasteurellaceae. However, even this method should be used with caution, because GenBank might include misidentified sequences of insufficiently characterized bacteria, as we believe is the case for accession no. JQ346058.1. Several companies provide *R. pneumotropicus*- and *R. heyltii*-targeted PCR services as stand-alone methods for detecting the bacteria directly from animal specimens and indirectly from exhaust air dust of animal cage racks. However, the contradicting results from the service laboratories again support the use of multiple methods. Specifically, due to limited diversity, the 16S rRNA gene sequence alone cannot be used to discriminate among the *Haemophilus*–*Pasteurella*–*Actinobacillus* complex.³¹ PCR assays with other targets than the 16S rRNA gene have been reported^{3,16} and could be used as an alternative or to enhance identification efforts. In addition, the recent reclassification of several members of Pasteurellaceae^{1,5,27} likely will promote improved identification and expand the databases for sequencing–PCR assays and MALDI-TOF MS.⁵

Finally, we suggest that the previous recommendation of FELASA²⁶ that advocated reporting of all Pasteurellaceae should be adopted for health monitoring reports. The report should include all Pasteurellaceae species that were detected and identified and should specify any primer sets that were used for PCR assays.^{7,9} This information should be included in all health monitoring reports provided by animal vendors, service laboratories, and experimental animal facilities and likely will be particularly informative to animal researchers who encounter these bacteria or must account for the effects of bacterial infections during their experiments.

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