# *Pasteurella multocida* subsp. *multocida* and *P. multocida* subsp. *septica* Differentiation by PCR Fingerprinting and α-Glucosidase Activity

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*Pasteurella multocida* **is composed of three subspecies that are often differentiated by fermentation of sorbitol and dulcitol. We studied 35 dulcitol-negative** *P. multocida* **isolates from infected dog and cat bite wounds, 16 of which yielded weak and/or conflicting fermentation reactions in Andrades sorbitol, thus making it difficult to distinguish between the two dulcitol-negative subspecies of** *P. multocida,* **i.e.,** *P. multocida* **subsp.** *multocida* **and** *P. multocida* **subsp.** *septica.* **All isolates and two control strains were further analyzed using a PCR fingerprinting technique with a single primer (M13 core) and assessed for α-glucosidase (α-Glu) activity. Although the PCR fingerprint patterns and α-Glu activity did not correlate well with the sorbitol fermentation reactions, they did correlate well with each other. All strains identified as** *P. multocida* **subsp.** *septica* **were positive for α-Glu activity and exhibited the group I PCR fingerprint profile. All strains categorized as** *P. multocida* **subsp.** *multocida* **displayed either the group II or group III PCR fingerprint profile; 9 of 11 of these isolates were α-Glu negative. These data suggest that both PCR fingerprinting and α-Glu activity provide reliable means for differentiating** *P. multocida* **subsp.** *multocida* **from** *P. multocida* **subsp.** *septica,* **particularly in strains that produce weak and/or discrepant sorbitol fermentation reactions.**

*Pasteurella* species have been isolated from various animals, either as saprophytes in the nasopharynx or gastrointestinal tract or as primary pathogens (reviewed in reference 17). Human disease is generally associated with some form of animal contact, most commonly a dog bite or cat bite or scratch (13, 14, 27, 30). Taxonomic relationships and nomenclature for this genus have undergone considerable change throughout the years. In 1985, DNA hybridization studies performed by Mutters et al. (22) revealed three homology groups of *Pasteurella multocida* that differed sufficiently enough from each other that they qualified as different species of *Pasteurella*. However, because the recommended therapy for human infection with *Pasteurella* is generally the same regardless of the species involved (reviewed in reference 17), the three groups of *P. multocida* were assigned different subspecies names for epidemiological purposes. *P. multocida* subsp. *multocida* includes the dulcitol-negative, sorbitol-positive isolates; *P. multocida* subsp. *septica* includes the dulcitol-negative, sorbitol-negative isolates; and *P. multocida* subsp. *gallicida* includes the dulcitol-positive isolates.

Other authors (3, 14) have suggested different ecological niches, as well as potential differences in pathogenicity, for the various *Pasteurella* species. *Pasteurella multocida* subsp. *multocida* and *P. multocida* subsp. *septica* are more frequently recovered from "more serious cases of infection" (14), including bacteremia. Whereas *P. multocida* subsp. *multocida* can be isolated from both dog- and cat-associated injuries, *P. multocida* subsp. *septica* is more frequently isolated from cases with cat contact and may have a greater affinity for the central nervous system (3). Therefore, there may be both epidemiological and clinical importance to the correct identification of these subspecies.

In our ongoing studies of organisms isolated from animal bite wound infections in humans, we have cultured numerous *Pasteurella* isolates, including *P. multocida*. In this study, we attempted to differentiate 35 dulcitol-negative *P. multocida* clinical isolates to the subspecies level using sorbitol fermentation as the differentiating test. We found that a significant number of our clinical isolates gave variable results when assayed for sorbitol and dulcitol fermentation. This is problematic in that sorbitol and dulcitol fermentation are key biochemical tests for the differentiation of *P. multocida* subspecies as originally described by Mutters et al. (22). To our knowledge, variation in the ability of a single *P. multocida* isolate to ferment sorbitol and dulcitol has not been addressed elsewhere in the literature.

Since the studies of Mutters et al. (22), there have been many studies on the biochemical characterization of *P. multocida* subspecies. However, most (if not all) of these studies rely heavily on the use of sorbitol fermentation to differentiate *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*. In the studies of Bisgaard and colleagues (4, 21), sorbitol fermentation is the only biochemical characteristic which clearly and consistently differentiates *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*. Likewise, the studies of Blackall and colleagues (5, 12), show that sorbitol fermentation is also the only biochemical test that consistently differentiates *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*. In other work by that group (6, 7), the authors differentiated *P. multocida* subsp. *multocida* from *P. multocida* subsp. *gallicida*.

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Based on citation of their previous work for the differentiation of these organisms, we conclude that sorbitol, as well as dulcitol, fermentation reactions were likely used as key differentiating reactions for these subspecies.

A few exceptions to the use of sorbitol fermentation to differentiate the two dulcitol-negative subspecies of *P. multocida* have been reported in the literature. Seven sorbitol-negative strains of *P. multocida* have been described which have not been classified as either *P. multocida* subsp. *multocida* or *P. multocida* subsp. *septica* due to differences in their trehalose and/or xylose fermentation reactions relative to the *P. multocida* subsp. *septica* type strain (12). In their discussion, the authors note that these could be either sorbitol-negative variants of *P. multocida* subsp. *multocida* or trehalose-negative *P. multocida* subsp. *septica* strains. In another study (25), eight sorbitol-negative strains have apparently been identified as *P. multocida* subsp. *multocida* as deduced from the difference between the number of *P. multocida* subsp. *multocida* strains and the number of sorbitol-positive strains of *P. multocida* isolated from dead turkeys cited in the tables. However, because the authors cite the original work of Mutters et al. (22) for methods on biotype and subspecies identification, i.e., methods which rely on sorbitol fermentation for the differentiation of *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*, it is not clear on what basis these eight sorbitol-negative strains were identified as *P. multocida* subsp. *multocida*. Thus, based on our review of the literature, it appears that sorbitol fermentation is still used as a key test for the phenotypic differentiation of *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*.

In an effort to ascertain if our sorbitol-variable isolates could be classified as *P. multocida* subsp. *multocida* or *P. multocida* subsp. *septica*, we used a PCR fingerprinting technique. In addition, we screened our isolates for preformed enzyme activity using the API-ZYM panel to determine if another biochemical marker that would clearly differentiate these isolates could be found. Our results suggest that both PCR fingerprinting and α-glucosidase (α-Glu) activity provide reliable means for differentiating *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*, particularly in strains that produce weak and/or discrepant sorbitol fermentation reactions.

## MATERIALS AND METHODS

**Strains.** Thirty-five clinical isolates of *P. multocida* cultured from different sources were obtained from the R. M. Alden Research Laboratory culture collection. Thirty-one of these strains were isolated from infected cat bite wounds in humans; four were isolated from dog bite wounds in humans. All strains had been previously identified as *P. multocida* using the API-20E test system (BioMerieux, Hazelwood, Mo.). Moeller's ornithine decarboxylase broth (Carr Scarborough Microbiologicals, Decatur, Ga.) and urease Wee-Tabs (Key Scientific, Round Rock, Tex.) were used to confirm ornithine decarboxylase and urease reactions, respectively. Indole reactions were confirmed using the spot indole test (*para*-dimethylaminocinnamaldehyde). All isolates were positive for catalase, oxidase, indole production, and ornithine decarboxylase; were negative for urease; reduced nitrate to nitrite; and fermented glucose, sucrose, xylose, and mannitol. None of the isolates fermented arabinose, inositol, mellibiose, rhamnose, or amygdalin. The isolates were subcultured from stock cultures (frozen at −70°C in 20% skim milk) onto tryptic soy agar plates supplemented with 5% sheep blood (Hardy Diagnostics, Santa Maria, Calif.) twice before further biochemical or PCR fingerprinting analyses. Control strains included *P. multocida* subsp. *multocida* ATCC 12947 (dog isolate) and *P. multocida* subsp. *septica* ATCC 51688 (human wound isolate).

**Biochemical tests.** The isolates were tested for sorbitol and dulcitol fermentation in Andrades media obtained from various sources (Carr Scarborough Microbiologicals; Remel, Lenexa, Kans.; and Hardy Diagnostics) and in prereduced anaerobically sterilized (PRAS) medium (Anaerobe Systems, Morgan Hill, Calif.) that had been open to the ambient air for inoculation of the cultures. Cultures were incubated at 37°C for up to 14 days. α-Glu activity was determined using the API-ZYM test system (BioMerieux) and Wee-Tabs (Key Scientific) as per the manufacturers' instructions.

**PCR fingerprinting analysis.** Several colonies of each isolate were suspended in 1 ml of sterile distilled water to a turbidity approximately equal to a number 3 McFarland standard. Two hundred microliters of the cell suspension was removed, pelleted, resuspended in 100 to 200 μl of Insta-Gene Matrix (Bio-Rad, Hercules, Calif.), and then incubated at 50°C for 15 to 30 min. Cell solutions were vortexed and then heated for 8 to 10 min at 100°C. Cell lysate supernatants containing the DNA extract were centrifuged to remove cellular debris and stored at −20°C until use.

Each cell lysate supernatant was subjected to PCR amplification in 50-μl volumes containing 25 μl of cell lysate supernatant; PCR buffer with 1.5 mM MgCl$_2$ (final concentration) (Perkin-Elmer Cetus, Norwalk, Conn.); 200 μM dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, Piscataway, N.J.); 25 pmol of the single primer, M13 core (5′-GAGGGTGGCGGTTCT-3′) (13); and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer). Amplification reactions were performed as follows: 40 s at 93°C, 1 min at 50°C, and 40 s at 72°C for 35 cycles, followed by a final extension cycle of 6 min at 72°C. Reaction tubes were held at 4°C prior to analysis. Samples were concentrated to approximately 20 to 25 μl each (Speed-Vac; Savant, Halbrook, N.Y.) prior to electrophoretic separation in 1.2% agarose gels (0.5 by 25 by 20 cm) for 5 h at 3V/cm. Amplified products were detected by staining with ethidium bromide (2 μg/ml). Data were evaluated as described previously (10). Each isolate was analyzed a minimum of three times, twice from the same extract and once from a separate extract, to ensure reproducibility of the results.

## RESULTS

**Sorbitol fermentation reactions provided ambiguous results for differentiating dulcitol-negative *P. multocida* subspecies.** Sorbitol and dulcitol fermentation reactions were performed to differentiate *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. The control strains reacted as expected in both the Andrades and PRAS fermentation reactions; i.e., *P. multocida* subsp. *multocida* ATCC 12947 was positive for sorbitol fermentation and negative for dulcitol fermentation, whereas *P. multocida* subsp. *septica* ATCC 51688 was negative for both sorbitol and dulcitol fermentation. All isolates tested negative for dulcitol fermentation when tested using the PRAS medium; i.e., the pH was ≥6.0. In contrast, dulcitol fermentation results were variable using the Andrades medium. Three of the isolates (9%) tested both clearly positive (rose to deep rose; pH, <5.8) and negative (no color change; pH, ≥6.5) for dulcitol fermentation on repeat testing with this medium. Fourteen of the 35 clinical isolates (40%) gave at least one weak (pale pink to very pale pink; pH, 6.0 to 6.4) dulcitol fermentation reaction when tested with the Andrades medium, although repeat testing of the weak fermenters generally yielded a clear negative reaction. Thus, when data from the PRAS and Andrades media were examined together to establish a consensus, all 35 isolates were determined to be dulcitol nonfermenters.

Based on the API-20E sorbitol fermentation results, 18 isolates (51%) were sorbitol fermenters and 14 (40%) were nonfermenters (Table 1). The remaining three isolates (9%) gave discrepant results, i.e., positive and negative results on repeat testing using the API-20E system. Using the PRAS sorbitol fermentation medium, 10 isolates (29%) were sorbitol fermenters (pH, ≤5.5), 12 (34%) were nonfermenters (pH, ≥6.0), and 13 isolates (37%) tested weakly positive (pH, 5.6 to

TABLE 1. Comparison of sorbitol fermentation results obtained using different test media

| Sorbitol fermentation result | No. of isolates as determined by: | | |
|---|---|---|---|
| | APF-20E | PRAS | Andrades |
| Positive | 18 | 10 | 16 |
| Negative | 14 | 12 | 4 |
| Weak | 0 | 13 | 11[a] |
| Discrepant reaction on repeat testing | 3 | ND[b] | 7[a] |

[a] Overlapping groups.
[b] ND, not done.

5.9) in at least one test assay. With the Andrades medium, 16 isolates (46%) tested positive for sorbitol fermentation and 4 isolates (11%) were nonfermenters. Eleven isolates (31%) gave at least one weak sorbitol fermentation reaction. Seven isolates (20%) yielded at least one clear positive and negative sorbitol fermentation reaction on repeat testing. When the data from the API-20E, PRAS medium, and Andrades medium were examined together to establish a consensus, 13 isolates (37%) were determined as nonfermenters and 19 (54%) were sorbitol fermenters. The sorbitol fermentation reactions for three of the isolates (9%) remained uncertain.

**PCR fingerprinting patterns did not correlate with the sorbitol fermentation reactions.** PCR fingerprinting analysis with the single primer, M13 core, was used to group the clinical isolates. Based on the presence or absence of four major bands (band A at $\approx$1,420 bp, band B at $\approx$1,130 bp, band C at $\approx$975 bp, and band D at $\approx$885 bp), the clinical isolates separated into three groups. Group I isolates (24 of 35) and the control strain *P. multocida* subsp. *septica* ATCC 51688 shared bands A, B, and C (Fig. 1 [23 strains are shown]). Group II isolates (4 of

35) and the control strain *P. multocida* subsp. *multocida* ATCC 12947 shared bands A, C, and D (Fig. 2A). Group III isolates (7 of 35) were characterized by bands A and C (Fig. 2).

The PCR fingerprint patterns did not appear to correlate well with the sorbitol fermentation reactions (Table 2). All of the sorbitol nonfermenters shared bands A, B, and C, i.e., the group I pattern characteristic of the *P. multocida* subsp. *septica* control strain. However, eight sorbitol fermenters and the three uncertain fermenters also fell within PCR fingerprint group I. Eleven additional sorbitol fermenters had PCR fingerprint patterns characteristic of either group II or group III.

**$\alpha$-Glu activity did not correlate with the sorbitol fermentation reactions but did correlate with the PCR fingerprint patterns.** API-ZYM results from the control and clinical isolates demonstrated that the isolates could be differentiated based on $\alpha$-Glu activity. *P. multocida* subsp. *multocida* ATCC 12947 showed negative $\alpha$-Glu activity, whereas *P. multocida* subsp. *septica* ATCC 51688 was positive for $\alpha$-Glu activity. Twenty-six of the clinical isolates (74%) showed positive $\alpha$-Glu activity; nine of the isolates (26%) were negative for this enzyme activity. Because the Wee-Tabs double test tablets ($\alpha$-Glu and phenylalanine deaminase) often produced negative or weakly positive results for isolates that tested positive using the API-ZYM test system (data not shown), we used the Wee-Tabs single test tablet to test for $\alpha$-Glu activity (nitrophenol substrate test system). Subsequent testing of 20 select clinical isolates (16 strains that showed a weak or discrepant reaction with the Wee-Tabs double test tablets and four randomly selected isolates) and the two reference strains using the Wee-Tabs single test tablets for $\alpha$-Glu activity confirmed the API-ZYM results.

As shown in Table 2, the $\alpha$-Glu activity did not always correlate with the sorbitol fermentation reactions. Although all
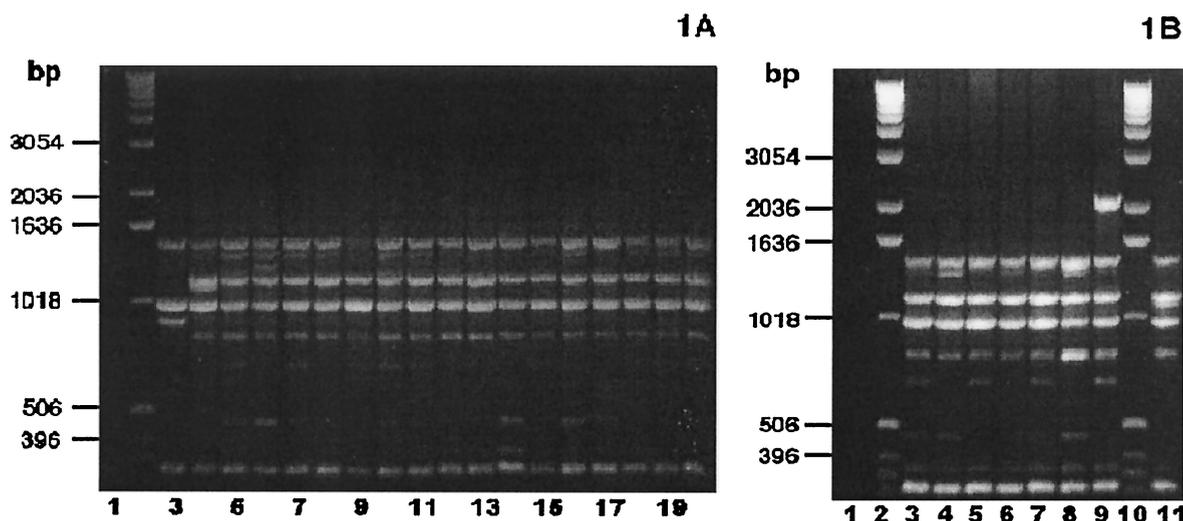


FIG. 1. *P. multocida* subsp. *septica* PCR fingerprint profiles. (A) Thirteen sorbitol-negative, $\alpha$-Glu-positive and three sorbitol-uncertain, $\alpha$-Glu-positive *P. multocida* bite wound isolates, examined by PCR fingerprint analyses using the single primer, M13 core, exhibited the group I PCR fingerprint profile characteristic of *P. multocida* subsp. *septica*. (Sorbitol fermentation was based on a consensus of API-20E, PRAS, and Andrades sorbitol fermentation reactions.) Lane 1, negative control (no DNA template); lane 2, DNA ladder; lane 3, *P. multocida* subsp. *multocida* ATCC 12947; lane 4, *P. multocida* subsp. *septica* ATCC 51688; lanes 5 to 20, bite wound isolates. (B) Seven sorbitol-positive, $\alpha$-Glu-positive *P. multocida* bite wound isolates, examined by PCR fingerprint analyses using the single primer, M13 core, showed the group I PCR fingerprint profile characteristic of the *P. multocida* subsp. *septica* control strain. Lane 1, negative control (no DNA template); lanes 2 and 10, DNA ladder; lanes 3 to 9, bite wound isolates; lane 11, *P. multocida* subsp. *septica* ATCC 51688.
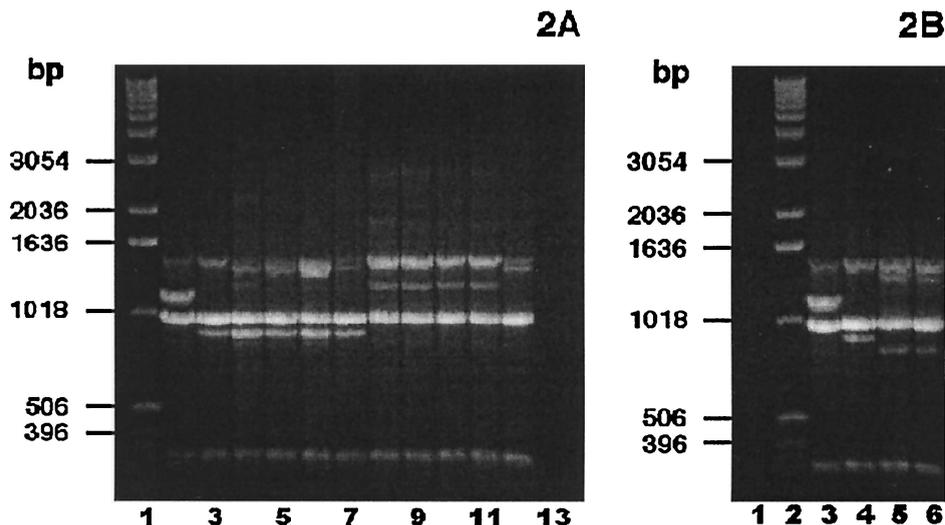
FIG. 2. *P. multocida* subsp. *multocida* PCR fingerprint analyses. (A) Nine sorbitol-positive, α-Glu-negative *P. multocida* subsp. *multocida* bite wound isolates, examined by PCR fingerprint analyses using the single primer, M13 core, displayed either the group II or group III PCR fingerprint profile. (Sorbitol fermentation was based on a consensus of API-20E, PRAS, and Andrades sorbitol fermentation reactions.) Lane 1, DNA ladder; lane 2, *P. multocida* subsp. *septica* ATCC 51688; lane 3, *P. multocida* subsp. *multocida* ATCC 12947; lanes 4 to 7, bite wound isolates demonstrating the group II PCR fingerprint pattern; lanes 8 to 12, bite wound isolates showing the group III PCR fingerprint profile; lane 13, negative control (no DNA template). (B) Two sorbitol-positive, α-Glu-positive *P. multocida* bite wound isolates, examined by PCR fingerprinting analyses using the single primer, M13 core, exhibited the group III PCR fingerprint profile characteristic of other *P. multocida* subsp. *multocida* clinical isolates. Lane 1, negative control (no DNA template); lane 2, DNA ladder; lane 3, *P. multocida* subsp. *septica* ATCC 51688; lane 4, *P. multocida* subsp. *multocida* ATCC 12947; lanes 5 and 6, bite wound isolates.

sorbitol nonfermenters were positive for α-Glu, 10 of the 19 positive sorbitol fermenters and the 3 isolates whose sorbitol fermentation reactions were unclear were also positive for this enzyme. In contrast, the α-Glu activity did correlate well with the PCR fingerprint profiles (Table 3). All of the α-Glu-negative isolates expressed either the group II or group III PCR fingerprint pattern. Twenty-four of the 26 α-Glu-positive isolates exhibited the group I PCR fingerprint pattern; two α-Glu-positive isolates had the group III fingerprint pattern.

## DISCUSSION

The results of this study suggest that sorbitol fermentation may not provide the ideal marker for the differentiation of the two dulcitol-negative *P. multocida* subspecies, *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. We have found that a significant number of the *P. multocida* isolates examined in this study gave weak and/or discrepant results for sorbitol fermentation, even when sorbitol fermentation tubes from different manufacturers were used. Furthermore, when

the results of sorbitol fermentation using all three of the test media (API-20E, PRAS, and Andrades) were considered, 3 of the 35 strains still failed to give a clear sorbitol fermentation reaction. Unfortunately, this made it difficult to categorize these organisms, as the sorbitol and dulcitol fermentation reactions have been the basis for differentiating *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica* (22).

To assist us in distinguishing between these two subspecies, we used a single-primer (M13 core) PCR fingerprinting technique. In other studies that we and others have performed, this and similar techniques have been successfully employed to differentiate species, including *Porphyromonas*, *Bacteroides fragilis* group, *Leptospira*, *Staphylococcus*, and *Streptococcus* (9, 10, 24, 31, 32). In this study, the dulcitol-negative *P. multocida* isolates clearly separated into three PCR fingerprint groups based on the presence or absence of four primary bands. The group I fingerprint profile was characteristic of the control strain *P. multocida* subsp. *septica* ATCC 51688; the group II fingerprint profile was characteristic of the control strain *P. multocida* subsp. *multocida* ATCC 12497. Although the group III fingerprint pattern was different from that of each of the control strains used in this study, we regarded the group III

TABLE 2. Sorbitol fermentation reactions show poor correlation with PCR fingerprint analyses and α-Glu activity

| Sorbitol fermentation result[a] | No. of isolates in PCR fingerprint group: | | | No. of isolates with α-Glu activity: | |
|---|---|---|---|---|---|
| | I | II | III | Positive | Negative |
| Negative | 13 | 0 | 0 | 13 | 0 |
| Positive | 8 | 4 | 7 | 10 | 9 |
| Uncertain | 3 | 0 | 0 | 3 | 0 |

[a] Based on consensus of API-20E, PRAS, and Andrades sorbitol fermentation reactions.

TABLE 3. α-Glu activity shows good correlation with the PCR fingerprint profiles

| α-Glucosidase activity | No. of isolates in PCR fingerprint group: | | |
|---|---|---|---|
| | I | II | III |
| Positive | 24 | 0 | 2 |
| Negative | 0 | 4 | 5 |

isolates as a subgroup of *P. multocida* subsp. *multocida* because the majority of these isolates were positive for sorbitol fermentation and negative for α-Glu activity in accordance with the control strain. We also noted that these three fingerprint patterns were substantially different from the fingerprint patterns generated by the American Type Culture Collection type strains of *P. canis, P. dagmatis, P. stomatis, P. haemolytica, P. testudinis,* and *P. pneumotropica* (S. Hunt Gerardo, unpublished observations).

Other investigators (33) have used the M13 core primer to distinguish strain-to-strain variation among *P. multocida* subsp. *multocida* pig respiratory isolates. The same four primary bands detected in our studies were also found by these investigators. However, none of their fingerprint profiles matched the combination of primary bands expressed by the control strains or clinical isolates analyzed in our study. One possible explanation for these differences may be the differences in the sources of these *Pasteurella* isolates, i.e., pig respiratory isolates versus isolates from infected dog and cat bite wounds in humans. Another likely contributing factor is the difference in magnesium ion concentrations used in the PCR amplification reaction mixtures. The magnesium ion concentration is known to affect primer annealing, the strand dissociation temperature of the template and the PCR product, product specificity and yield, and the polymerase activity and fidelity (1, 19). It is notable that we used 1.5 mM $MgCl_2$, whereas Zucker et al. (33) used a significantly higher concentration (4.5 mM $Mg^{2+}$) in their reaction mixture. Thus, differences in assay conditions provide a reasonable explanation for the differences in our results. As both of our assay systems provided reproducible results in our respective laboratories, it is possible that the higher magnesium ion concentration is useful for detecting strain-to-strain variation, whereas the lower concentration is more useful for analyzing the subspecies (species) differences. As Zucker et al. (33) restricted their analysis to *P. multocida* subsp. *multocida*, it would have been quite interesting to see what PCR fingerprint profile(s) was produced by *P. multocida* subsp. *septica* isolates under the assay conditions used in their laboratory.

Additional PCR methods have been used for the diagnosis and identification of *P. multocida* clinical isolates (reviewed in reference 15). However, these methods have been specifically designed to detect either strain-to-strain variation among *P. multocida* isolates or toxin-producing strains of *P. multocida*, rather than for the subspecies identification of these organisms. For the detection and identification of *P. multocida* in mixed cultures or clinical specimens by specific PCR, two approaches have been developed. One approach uses the *psl* gene, which codes for the P6-like protein of *P. multocida* (20). The other approach uses a sequence unique to *P. multocida* that was originally detected by subtractive hybridization (29). Although each of these PCR assays is capable of distinguishing *P. multocida* from other *Pasteurella* species and closely related genera, neither of them appears to distinguish among the subspecies of *P. multocida*. Two additional specific PCR approaches have proven useful for distinguishing *P. multocida* type B, the causative agent of hemorrhagic septicemia (8, 28). These assays are specific for serogroup B of *P. multocida* and have not been reported to differentiate *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. Similarly, several

PCR assays have been developed for the detection of toxigenic strains of *P. multocida* (reviewed in reference 15), but these do not differentiate *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*.

There have also been a number of other molecular assays used in the diagnosis and identification of *P. multocida* (reviewed in reference 15). Specifically, restriction endonuclease analysis, ribotyping, pulsed-field gel electrophoresis, and PCR fingerprinting have all been used for the differentiation of *P. multocida* isolates. Although each of these methods is useful for detecting strain-to-strain variation among *P. multocida* isolates, we have found no mention of their utility in specifically differentiating *P. multocida* subsp. *multocida* from *P. multocida* subsp. *septica*. In fact, Snipes et al. (26) demonstrated that *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* overlap in serotype, restriction endonuclease analysis type, and ribotype expression; i.e., both subspecies could be found within a given serotype, restriction endonuclease analysis type, or ribotype.

In addition to these molecular approaches for differentiating *P. multocida* isolates, a variety of biochemical reactions have also proven to be useful for their characterization. In particular, lactose, maltose, trehalose, and xylose fermentation reactions have been used to further characterize *P. multocida* species into biovars. Based on these biochemical characteristics, a number of variants within both *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* have been noted (6, 7, 12). Interestingly, trehalose and xylose fermentation reactions can be variable for both *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* (4, 6, 7, 12, 21). Similarly, although most isolates are maltose negative, maltose-positive strains of both *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* have been identified (23). Although there is no question that these variations have proven to be useful in epidemiological studies of *P. multocida* infections, these variants have, nevertheless, exhibited the expected sorbitol fermentation reaction for their respective subspecies, with the possible exception of the strains described above (12, 25). Therefore, these observations appear to confirm Mutters' original statement that "Variations in raffinose, lactose, maltose, trehalose, and D-xylose fermentation (are) of no taxonomic consequence" (22).

One of the interesting observations of our study is the difficulty in accurately assessing sorbitol fermentation among our *P. multocida* isolates. As mentioned above, this poses a significant problem in distinguishing the two dulcitol-negative *P. multocida* subspecies, as this is the critical biochemical test for their differentiation. In addition to being negative for dulcitol fermentation, all of our isolates were positive for xylose fermentation and negative for arabinose fermentation, further ruling out classification as *P. multocida* subsp. *gallicida*. It is also noteworthy that we identified 10 seemingly discrepant isolates (Fig. 1B and 2B), all of which were positive for sorbitol fermentation and α-Glu activity, i.e., 8 with the *P. multocida* subsp. *septica* PCR fingerprint pattern (sorbitol fermentation discrepancy) and 2 with the *P. multocida* subsp. *multocida* PCR fingerprint pattern (α-Glu discrepancy). In every case that we have seen in the literature, sorbitol-positive, dulcitol-negative isolates have been identified as *P. multocida* subsp. *multocida*, irrespective of their xylose, maltose, and/or trehalose fermentation reactions. Therefore, our proposal that eight sorbitol-

positive strains might actually be *P. multocida* subsp. *septica*, based on their positive α-Glu activity and their PCR fingerprint pattern, is novel.

We acknowledge that the PCR fingerprinting data alone may not provide sufficient evidence to support our hypothesis that this method can be used to differentiate between the two dulcitol-negative *P. multocida* subspecies. However, the differentiation of isolates based on α-Glu activity and the strong correlation of that activity with the PCR fingerprinting profiles does strengthen our hypothesis. It is noteworthy that Holst et al. (14) reported that 39 of 95 (41%) of their *P. multocida* subsp. *multocida* isolates (all sorbitol positive) were positive for α-Glu activity, in contrast to 100% (21 of 21) of the *P. multocida* subsp. *septica* isolates (sorbitol negative). Their results suggest that our eight "discrepant" sorbitol-positive, α-Glu-positive strains are *P. multocida* subsp. *multocida* rather than *P. multocida* subsp. *septica* as we have suggested. However, we find it highly unlikely that strains of different species, e.g., *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* (22), would present with the same polymorphic genomic fingerprint. If anything, when using the M13 core primer, one finds different, distinct PCR fingerprint patterns within a species, as was seen in this and other studies (1, 11, 16, 18, 31). Furthermore, in the study in which Mutters et al. (22) separated the three *P. multocida* subspecies based on DNA-DNA homology, only 4 dulcitol-negative, sorbitol-negative isolates (*P. multocida* subsp. *septica*) were examined, in contrast to 11 dulcitol-negative, sorbitol-positive isolates (*P. multocida* subsp. *multocida*). Our data suggest that examination of a larger number of isolates might have revealed that sorbitol fermentation was not a consistent marker for the differentiation of isolates into these two subspecies. Therefore, we believe that the PCR fingerprinting technique provides a more reliable means for differentiating between these two subspecies than the fermentation reactions.

In conclusion, we found that PCR fingerprinting analyses and α-Glu activity gave more consistent results than did sorbitol fermentation reactions for differentiating *P. multocida* dulcitol-negative isolates. Furthermore, the PCR fingerprinting profiles and α-Glu activity correlated much better with each other than did either one of these results with the sorbitol fermentation reactions. Thus, we propose that PCR fingerprint analysis (using the M13 core primer) and α-Glu activity more accurately reflect the differences in the two subspecies, *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*, than does sorbitol fermentation.

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