



# Evaluation of Pyrrolidonyl Arylamidase Activity in *Staphylococcus delphini*

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**ABSTRACT** Clinical reference textbooks lack data for pyrrolidonyl arylamidase (PYR) activity in *Staphylococcus delphini*. This study evaluated PYR activities of 21 *S. delphini* strains by reference broth, rapid disc, and rapid slide methods. Species and subgroup identifications were confirmed by nucleic acid-based methods and included nine group A and 12 group B strains. Testing by rapid PYR methods with products from four manufacturers was performed at two testing locations, and, with the exception of one strain tested at one location using reagents from one manufacturer, each *S. delphini* strain tested positive for PYR activity. Therefore, PYR may be a useful single-test adjunct for distinguishing *Staphylococcus aureus* from *S. delphini* and other members of the *Staphylococcus intermedius* group.

**KEYWORDS** *Staphylococcus delphini*, *Staphylococcus intermedius* group, pyrrolidonyl arylamidase

Pyrrolidonyl arylamidase (PYR), also known as pyrrolidonyl aminopeptidase, is a bacterial enzyme that hydrolyzes L-pyrroglutamic acid- $\beta$ -naphthylamide to produce  $\beta$ -naphthylamine, which combines with *N,N*-dimethylaminocinnamaldehyde reagent to form a red color (1). Rapid disc or slide tests for PYR activity are used to differentiate *Enterococcus* species from *Streptococcus* species (1) but can also be used to presumptively differentiate *Staphylococcus aureus* from phenotypically similar staphylococci (2, 3). One such group of staphylococci is the *Staphylococcus intermedius* group (SIG), which includes the species *Staphylococcus delphini*, *Staphylococcus intermedius*, and *Staphylococcus pseudintermedius* (4). *S. delphini* has been further subdivided into two distinct phylogenetic clades referred to as groups A and B (5). PYR test results were not provided in the original description of *S. delphini* (6); however, a subsequent report indicated that 100% of 22 *S. delphini* isolates (17 group A and 5 group B) were PYR positive according to a commercial multibiochemical test strip (Rapid ID 32 Staph [bioMérieux, Durham, NC, USA]) (5). As highlighted in Table 1, the PYR activity of *S. delphini* is listed in some clinical reference texts as “not determined” or “not available,” and in other references either *S. delphini* or the PYR test result is excluded from *Staphylococcus* species identification tables (7–10). In comparison, the PYR activities of *S. intermedius* and *S. pseudintermedius* are clearly documented as positive (7–9). Furthermore, rather than using multibiochemical test strips, PYR activity is typically evaluated in the routine clinical microbiology setting with rapid PYR disc or slide kits, which can be used in less than 5 min. Therefore, the purpose of this study was to determine the PYR activities of *S. delphini* strains using the reference standard broth method (3) to complete an important piece of “missing” biochemical data and to evaluate the performance of four rapid PYR test kits produced by different manufacturers to assist

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**TABLE 1** Documented PYR activities of *S. aureus* and members of the *S. intermedius* group represented in clinical reference texts

Clinical reference text	Documented PYR activity <sup>a</sup>			
	<i>S. aureus</i>	<i>S. delphini</i>	<i>S. intermedius</i>	<i>S. pseudintermedius</i>
Manual of Clinical Microbiology, 11th ed (7)	NEG	Not determined	POS	POS
Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 7th ed (8)	NEG	Not available	POS	POS
Bailey and Scott's Diagnostic Microbiology, 13th ed (9)	NEG	Excluded	POS	POS
Clinical Veterinary Microbiology, 2nd ed (10)	Excluded from identification table			

<sup>a</sup>PYR, pyrrolidonyl arylamidase; NEG, negative; POS, positive.

clinical microbiologists in the rapid differentiation of *S. aureus* from the phenotypically similar coagulase-positive SIG members.

**RESULTS**

Each PYR test kit reacted as expected with the control organisms (*Streptococcus agalactiae*, negative; *Enterococcus faecalis*, positive). Only one manufacturer (Key Scientific Products) included expected reactions for *Staphylococcus* species in its instructions. Nonetheless, the selected *S. aureus* (negative) and *S. pseudintermedius* (positive) controls performed consistently with complete agreement for each test kit, at each test point, and, in the case of the rapid PYR disc/slide tests, between laboratories. Positive PYR reactions observed in the reference broth test were indicated with a brilliant red-fuchsia color, while negative reactions were indicated by yellow or weakly orange color (Fig. 1). Positive PYR reactions observed with the rapid disc and rapid slide tests were of a similar red-pink color but were generally weaker than those observed with the reference broth, and in these tests, *Staphylococcus* species (*S. pseudintermedius* and *S. delphini*) produced weaker reactions than the control *Enterococcus* species. Results of PYR tests for the 21 *S. delphini* and control strains are summarized in Table 2. Our results show excellent agreement among the test systems (broth, rapid disc, and rapid slide),



**FIG 1** Observed broth PYR reactions for the control and *S. delphini* DSM 20771<sup>T</sup> strains. 1, *Enterococcus faecalis* ATCC 29212; 2, *Streptococcus agalactiae* ATCC 12386; 3, *Staphylococcus aureus* ATCC 25923; 4, *Staphylococcus pseudintermedius* LMG 22219<sup>T</sup>; 5, *Staphylococcus delphini* DSM 20771<sup>T</sup>.

**TABLE 2** Results of PYR broth and rapid PYR disc/slide testing at the two testing sites

Organism <sup>a</sup>	No. of strains tested	Reference PYR broth <sup>b</sup> (% of positive tests)	Rapid PYR disc/slide test results by manufacturer <sup>c</sup> (% of positive tests)			
			A	B	C	D
<i>E. faecalis</i> ATCC 29212	1	100	100	100	100	100
<i>S. agalactiae</i> ATCC 12386	1	0	0	0	0	0
<i>S. aureus</i> ATCC 25923	1	0	0	0	0	0
<i>S. pseudintermedius</i> LMG 22219 <sup>T</sup>	1	100	100	100	100	100
<i>S. delphini</i> (group A)	9	100	100	100	100	94.4 <sup>d</sup>
<i>S. delphini</i> (group B)	12	100	100	100	100	100
<i>S. delphini</i> (groups A and B)	21	100	100	100	100	97.6 <sup>e</sup>

<sup>a</sup>LMG, Collection of the Laboratorium voor Microbiologie en Microbiële Genetica.

<sup>b</sup>Thermo Fisher Scientific (Remel); tested only at the University of Tennessee.

<sup>c</sup>Manufacturers are A, Thermo Fisher Scientific (Remel); B, Becton, Dickinson and Company; C, Hardy Diagnostics; and D, Key Scientific Products. All rapid test kits were tested once in each of two laboratories.

<sup>d</sup>One *S. delphini* group A isolate, *S. delphini* MI 09-2894, tested negative using the Key Scientific Products PYR disc kit at Weill Cornell Medical College. Of a total of 18 tests results available from both testing sites for the *S. delphini* group A isolates (9 results from each site), 94.4% (17/18) were positive.

<sup>e</sup>Of 42 test results from both testing sites available for the *S. delphini* group A and B isolates combined (21 results from each testing site), 97.6% (41/42) were positive.

manufacturers, and laboratories, with all *S. delphini* strains testing positive for PYR activity except for one strain that tested negative with one rapid disc test kit in one laboratory. Interlaboratory agreement, based on total number of rapid disc and rapid slide tests (42 rapid disc/slide test results in total for both testing sites) performed on all *S. delphini* strains was 97.6% (41/42 rapid disc/slide test results) (Table 2).

## DISCUSSION

Of the tested *S. delphini* isolates in this study, 100% tested positive using the reference PYR broth method, while >95% of the rapid disc/slide test results for the 21 *S. delphini* isolates were positive. The ability to accurately differentiate *Staphylococcus aureus* from other phenotypically similar coagulase-positive staphylococci is important for accurate interpretation of antimicrobial susceptibility test results that may influence therapy selection. This has been demonstrated most clearly with respect to the detection of methicillin susceptibility in *S. aureus* versus *S. pseudintermedius* (11). While some reference and larger hospital-based microbiology laboratories have turned to DNA sequencing- or mass spectrometry-based technologies to rapidly and accurately identify *Staphylococcus* species (12–14), many laboratories will continue to utilize single rapid tests and short test sets for the identification of *S. aureus* and SIG members. The use of PYR testing to distinguish some coagulase-positive *Staphylococcus* species from *S. aureus* is well established (7, 8, 9; see also <http://www.aavmc.org/data/files/case-study/burnham%20-%20staph%20pseudintermedius%20-%20student%20materials.pdf>). The rapid PYR disc methods took <5 min to complete, while the reference broth method required 4 h. It should be noted that strict adherence to manufacturer recommendations is required for all PYR-based tests. It is especially important that PYR test reactions be read promptly after adding the development reagent (*N,N*-dimethylaminocinnamaldehyde), since some negative reactions may appear positive after increased time. Yellow-, salmon-, or orange-colored reactions are generally considered by all manufacturers to be negative. It is recommended that isolates exhibiting pale-pink or weak reactions be retested either with a longer substrate incubation of 5 min, directly with reagent alone to determine if the reactions are specific to the development reagent rather than the substrate, or using the reference tube method.

The purpose of this study was to complete “missing” biochemical data for *S. delphini* to facilitate the rapid differentiation of *S. aureus* and members of the SIG isolated from animal and human clinical specimens. While *S. pseudintermedius* is increasingly recognized as a potential human pathogen (11, 15), *S. delphini* and *S. intermedius* are primarily animal pathogens. To the best of the authors’ knowledge, neither *S. delphini* nor *S. intermedius sensu stricto* has been reported as a cause of human infection; likewise, methicillin resistance has not been reported in these species. Each of the *S. delphini* isolates used in this study was presumed to be susceptible to oxacillin by the

**TABLE 3** *Staphylococcus delphini* strains used in this study

Strain	Group	Host species	Geographic origin	Reference(s) or source
DSM 20771 <sup>Ta</sup>	A	Dolphin	Italy	6
VPP 12 D	A	Horse	Switzerland	This study
MI 09-8445	A	Sea otter	USA	This study
MI 09-2894	A	Raccoon	USA	This study
MI 16-1129	A	Ferret	USA	This study
19-039	A	Avian	USA	11
52-006	A	Horse	USA	This study
56-021	A	Llama	USA	This study
62-031	A	Horse	USA	This study
8086	B	Horse	UK	20
AV8047	B	Pigeon	Japan	20, 21
H4A	B	Horse	Japan	5, 20
HT2003-0674	B	Camel	France	20, 22
P27B	B	Pigeon	Japan	5, 20
P50	B	Pigeon	Japan	5, 20
26-037	B	Horse	USA	11
33-090	B	Horse	USA	11
35-016	B	Horse	USA	11
49-046	B	Horse	USA	This study
53-033	B	Horse	USA	This study
56-039	B	Horse	USA	This study

<sup>Ta</sup>DSM 20771<sup>T</sup>, Deutsch Sammlung von Mikroorganismen und Zellkulturen GmbH, *S. delphini* type strain.

standard disk diffusion method (range of zone of inhibition diameters, 22 to 28 mm; arithmetic mean, 26 mm) according to interpretive criteria used for *S. pseudintermedius* (11), and the *mecA* gene was not detected by PCR (data not shown). Perhaps due to misidentifications as *S. aureus* (12, 16) and the lack of routine species differentiation within the SIG in some laboratories, the true epidemiology and distribution of *S. delphini* infections in the human population may not be known. *S. delphini* has been isolated from many different animal species (17–19), including some, such as horses, that may have close human contact. Therefore, methods that can be used for quick presumptive recognition of all members of the SIG (including *S. delphini*), such as PYR, will be very useful in medical and veterinary clinical microbiology laboratories.

## MATERIALS AND METHODS

**Bacterial strains.** We used four control strains: *Enterococcus faecalis* strain ATCC 29212, *Streptococcus agalactiae* strain ATCC 12386, *Staphylococcus aureus* strain ATCC 25923 (American Type Culture Collection, Manassas, VA, USA), and *Staphylococcus pseudintermedius* strain LMG 22219<sup>T</sup> (received from Freddy Haesebrouck, Ghent University, Ghent, Belgium). The *Staphylococcus delphini* strains used in this study are summarized in Table 3. The *S. delphini* strain collection included seven previously published strains, including the *S. delphini* type strain (*S. delphini* DSM 20771<sup>T</sup>) (received from Vincent Perreten, University of Bern, Bern, Switzerland), and 14 clinical strains identified in this study. All isolates were cultured on tryptic soy agar with 5% sheep blood (TSAB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Initially, isolates (21 test strains and four control strains) were subcultured from long-term stocks and incubated at 35 ± 2°C in 5% carbon dioxide (CO<sub>2</sub>) for 18 to 24 h. Subsequently, these cultures were subcultured and incubated at 35 ± 2°C in 5% CO<sub>2</sub> for 18 to 24 h prior to PYR testing.

**Molecular identification of *Staphylococcus delphini* isolates.** Bacterial cell lysates were obtained from colonies grown on TSAB following incubation at 35 ± 2°C in 5% CO<sub>2</sub> for 18 to 24 h. A single isolated colony was suspended in 0.5 ml of a 1:1 mix (vol/vol) containing Tris-EDTA buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and 0.1-mm zirconium beads (Biospec Products, Bartlesville, OK, USA) and subsequently vortexed to disrupt bacterial cells. The species and subgroup identification of *S. delphini* strains were confirmed by thermonuclease (*nuc*) gene PCR using previously described primers (23). The total reaction mixture for each PCR was 25 μl, containing 2.5 μl of supernatant from the bacterial cell lysate, 1 μl of each primer (final concentration, 10 pmol), 8 μl of water, and 12.5 μl of 2× PCR solution with a final concentration of 0.625 U rTaq polymerase, 0.2 mM deoxynucleoside triphosphate mixture, and 1× reaction buffer (Premix Taq, TaKaRa Bio Inc., Kyoto, Japan). The thermocycler parameters were 1 cycle at 95°C for 1 min 30 s, 30 cycles at 56°C for 30 s, 72°C for 2 min 30 s and 94°C for 1 min, 1 cycle at 50°C for 2 min, and 1 cycle at 72°C for 5 min, followed by holding at 4°C. Resultant PCR products were resolved by agarose gel electrophoresis (1.0%) and visualized by staining with ethidium bromide (0.5 μg/ml).

**Antimicrobial susceptibility testing.** Each *S. delphini* isolate was tested for oxacillin susceptibility using the standard disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (24). Diameters of the zone of growth inhibition were recorded and, because no interpretive breakpoints that apply specifically to this species exist, comparisons were made to the

current breakpoints used for *S. pseudintermedius* (11, 24). Bacterial cell lysates, obtained as described above, were subjected to PCR for the *mecA* gene using previously described primers, thermocycling conditions, and agarose gel electrophoresis method (25).

**PYR testing.** Four control strains were used to validate products each time *S. delphini* strains were tested. Reference broth testing was performed using Remel PYR broth (catalog number 062085; Thermo Fisher Scientific [Remel], Lenexa, KS, USA) at one location (University of Tennessee). Each isolate was tested only once, and testing was performed and interpreted by a single individual according to manufacturer instructions. PYR broth was inoculated with 4 colonies from an overnight TSAB plate culture and incubated in an aerobic atmosphere for 4 h at 37°C. After incubation, 1 drop of PYR reagent (catalog number R21258) was added to the tube. A red (positive) color reaction was recorded after 1 min and checked for significant change at 2 min. The following rapid disc or rapid slide tests were tested: manufacturer A, PYR disc with reagent (catalog number R30854301; Thermo Fisher Scientific [Remel]); manufacturer B, BBL DrySlide PYR kit (catalog number 231747; Becton, Dickinson and Company); manufacturer C, PYR test kit and reagent (catalog number Z175; Hardy Diagnostics, Santa Maria, CA, USA); and manufacturer D, PYR discs (with PEP) (catalog number K1538B; Key Scientific Products, Stamford, TX, USA). For the rapid disc or rapid slide tests, testing was conducted independently at two locations (University of Tennessee and Weill Cornell Medical College) using kits from identical lots. At each site, isolates were assayed only once by each of the different disc or slide tests. Furthermore, testing was performed and interpreted by a single individual at each site according to manufacturer instructions. The sites were not blinded to the PYR activity of the control strains for the rapid PYR tests, as this served as quality control prior to testing. However, all other testing factors were blinded. Discs and slides were moistened with a 10- $\mu$ l loopful of distilled water, the incubation time of the organism on discs and slides for all kits prior to addition of reagent was 2 min (including Key Scientific Products, which indicates that the incubation time can be between 2 and 5 min), and the color reaction was recorded 1 min after addition of the reagent for all kits tested (including Key Scientific Products, which instructs at least 1 min but not more than 2 min before recording).

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