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Whole-cell protein profiles are useful for distinguishing enterococcal species recovered from clinical specimens

R. MASSA¹, C. BANTAR²†, H. LOPARDO³, C. VAY⁴, G. GUTKIND¹*¹

¹Cátedra de Microbiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Junín 956 (1113) Ciudad Autónoma de Buenos Aires;²Centro de Educación Médica e Investigación Clínica "Norberto Quirno" (CEMIC). Galván 4102 (1431) Ciudad Autónoma de Buenos Aires;³Servicio de Microbiología, Hospital de Pediatría "Prof. Dr. Juan P. Garrahan". Combate de los Pozos 1881 (1245) Ciudad Autónoma de Buenos Aires;⁴Departamento de Bioquímica Clínica, Hospital de Clínicas "José de San Martín" Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Córdoba 2150 (1113) Ciudad Autónoma de Buenos Aires. Argentina.

†Present address: Hospital San Martín, Paraná, Entre Ríos, Argentina
*Correspondence. E-mail: ggutkind@ffyb.uba.ar

ABSTRACT

Whole-cell protein analysis was performed for differentiating 150 enterococcal isolates to the species level, which had previously been identified by extended phenotypic conventional tests. Whole-cell protein profile (WCPP) showed a high degree of similarity within species and comparison between species revealed important differences in band profiles. All Enterococcus faecalis and Enterococcus faecium isolates were properly located into their corresponding species, regardless of their clinical source and susceptibility pattern. Moreover, WCPP allowed relocation of some isolates that had erroneously been identified by the usual conventional scheme (i.e. two atypical arginine-negative E. faecalis isolates). WCPP proved to be a simple method to ascertain the various enterococcal species, especially those other than E. faecalis, and may be a suitable tool for high-complexity or reference clinical laboratories.

Key words: enterococci, whole-cell protein profile

RESUMEN

Los perfiles de proteínas totales son útiles para distinguir especies de enterococos recuperados de muestras clínicas. La comparación del perfil de proteínas totales permitió agrupar 150 aislamientos de enterococos dentro de la especie en la que habían sido ubicados por el esquema convencional de pruebas bioquímicas. Los patrones de proteínas totales, comparados visualmente, se mantuvieron con alto grado de similitud intraespecie y revelaron diferencias notorias en la comparación interespecie. Todos los aislamientos de Enterococcus faecalis y Enterococcus faecium, independientemente de los sitios de aislamiento, cuadro clínico del paciente, biotipo o antibiotipo, fueron fácilmente encuadrados en su especie. Asimismo, el estudio del perfil de proteínas totales de enterococos permitió reubicar taxonómicamente aislamientos que habían sido incorrectamente identificados por los métodos bioquímicos convencionales, como por ejemplo dos aislamientos atípicos de E. faecalis arginina negativos. Dado que la metodología empleada es económica y rápida, la comparación de perfiles de proteínas totales en SDS-PAGE podría ser considerada una herramienta confirmatoria útil en la identificación de especies de enterococos.

Palabras clave: enterococos, perfil de proteínas totales

Enterococcal identification, in most cases, can be properly reached by using a considerable number of physiological tests. However, some isolates with atypical features may be erroneously characterized to the species level.

In fact, certain relatively common atypical phenotypic features can lead to mismatches in the classification even at the genus level, given that enterococci can be confused with lactococci or Vagococcus fluvialis.

Even if classification of enterococci was clarified by DNA:DNA hybridization studies and 16S rRNA gene sequence similarity, the number of species and their taxonomic position are subject of continuous revision. At present, more than 25 Enterococcus species have been described, including from E. faecalis, E. faecium, E. durans, E. avium, E. casseliflavus, E. malodoratus, E. gallinarum, E. hirae, E. mundtii, E. raffinosus and E. pseudoavium (12) to other less frequent species such as E. cecorum, E. columbae, E. saccharolyticus, E. dispars, E. sulfureus and E. flavescens. In addition, according to the analysis of 16S rRNA sequence, the former species, E. solitarius and E. seriolicida have been proposed to belong to the Tetragenococcus and Lactococcus genus, respectively (13) and later studies suggested that E. flavescens and E. casseliflavus are synonymous (5, 8).

More recently, a number of novel species has been described: E. asini (2), E. haemoperoxidus, E. moraviensis (10), E. villorum (15) and E. porcinus (probably a sin-
Diverse species (1), E. ratti (11), E. gilvus, E. pallens (14), E. canis (1) and E. phoeniculicola (4), and others are described every year.

Despite the high diversity of Enterococcus species, most human infections are mainly limited to E. faecalis and E. faecium and to a lesser degree, to E. raffinosa, E. casseliflavus, E. avium, E. hirae and E. gallinarum, albeit other more rare species, such as E. cecorum, E. dispar, E. durans, E. gilvus, E. mundtii and E. pallens, have also been reported in humans. On the other hand, E. columbae, E. haemoperoxidans, E. malodoratus, E. moraviensis, E. porcinus, E. pseudoavium, E. ratti, E. saccharolyticus and E. sulfureus have never been recovered from humans (12).

Considering the clinical and epidemiologic relevance of identification to the species level among enterococci, as they may differ in both inherent and acquired resistance to several antimicrobial agents, we sought to assess the performance of the whole-cell protein profile in the identification of species belonging to the Enterococcus genus, including isolates displaying atypical phenotypic features, in order to provide a suitable tool for reference or high-complexity clinical laboratories.

One hundred and fifty unique isolates recovered from clinical specimens were collected from three different hospitals, and included in this study. E. faecalis ATCC 29212, E. faecium ATCC 19434, E. durans ATCC 19432, E. avium ATCC 14025, E. malodoratus ATCC 43197, E. hirae ATCC 9790, and E. pseudoavium ATCC 49372 were also included as SDS-PAGE controls.

Organisms were identified by conventional tests. Briefly, isolates were assumed to belong to the Enterococcus genus if they showed gram-positive cocci growing in chains, were catalase-negative, exhibited any inhi-

**Table 1. Value of the whole cell protein profile in the identification of enterococci.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Proportion of typeable isolates (ratio of isolates)</th>
<th>Type of profile of the total proteins</th>
<th>% of not typeable proteins (ratio of isolates)</th>
<th>Ratio: profile of total proteins vs. convencional biochemical scheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis</td>
<td>89.6 (42/48)</td>
<td>Profile A: shared by all of the typeable isolates of this species</td>
<td>10.4 (6/48)</td>
<td>43/48</td>
</tr>
<tr>
<td>E. faecium</td>
<td>73.8 (32/42)</td>
<td>Profile B: shared by all of the typeable isolates of this species</td>
<td>26.2 (10/42)</td>
<td>31/42</td>
</tr>
<tr>
<td>E. raffinosa</td>
<td>68.7 (11/16)</td>
<td>Three profiles; C_E: 9 isolates C_C: 1 isolate C_CC: 1 isolate</td>
<td>31.2 (5/16)</td>
<td>11/16</td>
</tr>
<tr>
<td>E. avium</td>
<td>75 (6/9)</td>
<td>Profile D: shared by all of the typeable isolates of this species</td>
<td>25 (2/9)</td>
<td>6/9 [3]</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>100 (12/12)</td>
<td>Profile E: shared by all of the typeable isolates of this species</td>
<td>-</td>
<td>12/14 [4]</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>100 (5/5)</td>
<td>Profile F: shared by all of the typeable isolates of this species</td>
<td>-</td>
<td>6/5 [3]</td>
</tr>
<tr>
<td>E. flavescens</td>
<td>100 (1/1)</td>
<td>Profile G: shared by all of the typeable isolates of this species</td>
<td>-</td>
<td>3/1 [4]</td>
</tr>
<tr>
<td>E. hirae</td>
<td>88 (8/9)</td>
<td>Two profiles; H_E: 8 isolates H_C: 1 isolate</td>
<td>11 (1/9)</td>
<td>8/9</td>
</tr>
<tr>
<td>E. mundtii</td>
<td>33.3 (1/3)</td>
<td>Profile I: represented by the only typeable isolate that could be analyzed.</td>
<td>66.6 (2/3)</td>
<td>1/3</td>
</tr>
<tr>
<td>E. durans</td>
<td>100 (3/3)</td>
<td>Profile J: shared by all of the typeable isolates of this species</td>
<td>-</td>
<td>3/3</td>
</tr>
</tbody>
</table>

(1): Proportion of available isolates from their whole-cell protein profiles. (2): Percentages of isolates that could not be analyzed from their whole-cell protein profiles. (3): one of the nine identified isolate as E. avium, according the pattern of total proteins was relocated as E. casseliflavus; (4): two of the fourteen that had been identified as E. gallinarum, according to their pattern of proteins were relocated as E. flavescens.
bition zone around a 30-µg vancomycin disk (relative criterion), grew in 6.5% ClNa broth, grew both at 10 °C and at 45 °C, were positive to the bile-esculin test and hydrolyzed L-pyrrolidonyl-β-naphthylamide (PYR) and leucin-β-naphthlamide (LAP). The identification to the species level was performed by following the scheme proposed by Texeira and Facklam. Any isolate showing discrepancy with the protein profile was reidentified with further biochemical tests (Table 1).

Whole-cell protein extracts were obtained, collected from 18 h-old cultures in 50 ml of Casoy Broth (Merck Química Argentina), by centrifugation (8000xg, 20 min, 4 °C), resuspended in 500 µl of phosphate buffer pH 7.0, 0.1 M; proteins were solubilized with sample buffer (0.25 M tris-HCl pH 6.8, 8% sodium dodecyl sulfate, 20% 2-mercapto-ethanol, 0.008% bromophenol blue, 40% glycerol) by boiling at 100 °C during 10 minutes. Bacterial debris was separated by centrifugation (13500 rpm, 1 minute). Solubilized proteins were separated by SDS-PAGE (3), by using resolving gels with 10% T and 2.7% C acrylamide-bisacrylamide, at 90 V on a Bio-Rad Mini Protein cell during 2 hours. Gels were stained with Coomassie Brilliant Blue R 0.25% in methanol: acetic acid: water, 45:10:45, and destained by transference on the methanol: acetic acid: water, 45:10:45. Most isolates were processed by following this treatment. However, some isolates required minor protocol modifications such as different volumes of culture processed (1/4, 1/2 and 5x of volume), 2x sample buffer concentration and boiling for 30 minutes. Profiles were visually evaluated.

Microorganisms were identified as *E. faecalis* (48), *E. faecium* (42), *E. hirae* (9), *E. raffinosus* (16), *E. avium* (9), *E. gallinarum* (14), *E. casseliflavus* (5), *E. durans* (3), *E. mundtii* (3) and *E. flavescens* (1).

**Figure 1 A.** Comparison of patterns from isolates belonging group I of Facklam’s classification. Lanes 1, 2, 4, 5: whole-cell protein profiles of *E. avium* isolates. Lane 3: whole-cell protein profile of *E. casseliflavus* isolate that has been identified as *E. avium*. Lane 5: *E. avium* ATCC 14025. Lanes 6 to 14: whole-cell protein profiles of *E. raffinosus* isolates. Lane 15: *E. malodoratus* ATCC 43197. **B.** Comparison of patterns from isolates belonging group II of Facklam’s classification. Lanes 1 and 2: whole-cell protein profiles of *E. casseliflavus* isolates. Lanes 3 to 6: whole-cell protein profiles of *E. gallinarum* isolates. Lanes 7, 8 and 9: whole-cell protein profiles *E. flavescens* isolates. Lane 10: whole-cell protein profile of Vagococcus sp. isolate. Lane 11: whole-cell protein profile of *E. faecalis* isolate. Lane 12: whole-cell protein profile of *E. faecium* isolate.
All *E. faecalis* isolates showed similar profiles, regardless of the antibiotype and including 2 isolates that, atypically, did not hydrolyze arginine. The same was observed for *E. faecium*. *E. faecalis* and *E. faecium* typical patterns are shown in Figure 1B and Figure 2.

Some isolates could not be analyzed, as they did not yield enough protein after extraction. This phenomenon was observed for 6 *E. faecalis* isolates, 10 of *E. faecium*, 1 of *E. hirae*, 5 of *E. raffinosus*, 3 of *E. avium* and 2 of *E. mundtii* (Table 1). This table, displays the proportion of isolates that could be analyzed for their protein profile (Proportion of typeable isolates) and the percentages of isolates that could not be analyzed (% of not typeable).

Whole-cell protein profile allowed differentiation among between the species included in group I of Facklam’s classification: *E. avium*, *E. malodoratus*, *E. raffinosus*, *E. pseudoavium*. Most *E. raffinosus* isolates shared a common protein pattern (Table 1). However, two isolates gave another profile, which was different between themselves (Figure 1A, lanes 6 and 8). *E. avium*, *E. gallinarum*, *E. casseliflavus* and *E. flavescens* showed a distinctive pattern (Figure 1A and 1B), although the last two species displayed close profiles. (Figure 1B, lanes 1 and 2: whole-cell protein profiles of *E. casseliflavus* isolates). Lanes 7, 8 and 9: whole-cell protein profiles of *E. flavescens* isolates). The only available *E. mundtii* isolate showed a protein profile different from any of the remaining species (data not shown).

*E. durans* required a concentration of total protein extract five times higher than the other species. Furthermore, cellular break down and proteins solubilization required boiling in the presence of 4% SDS (2x sample buffer) during 30 minutes. The three available isolates belonging to this species shared the same protein profile, but each one showed different relative band intensities (Figure 2). Eight out of the nine available *E. hirae* isolates, showed the same protein profile (Figure 2).

Whole-cell protein profile determined by SDS-PAGE, represents the expression of an important proportion of the genome. However, this expression may be modified by different factors. Therefore, this technique needs to be carefully controlled and standardized in order to obtain reproducible results. Although some isolates did not yield suitable protein extracts by this procedure, we believe that this may be solved by the inclusion of additional steps, such as an incubation step with mutanolysin/lysozyme or mechanical disruption. Even so, most isolates could still be easily located into the respective species.

Although *E. flavescens* has been proposed as a new species by Pompei and coworkers in 1992 (6), whole-cell protein profiles of *E. casseliflavus* and *E. flavescens* were in very close agreement with those authors that suggested it is closely related to *E. casseliflavus*, constituting a new subspecies (7), on the basis of the PCR amplification of the corresponding ligase genes (D-Ala-D-Ser o D-Ala-D-Lactate).

Reaching the species level by using only conventional physiological tests is difficult to achieve in the presence of atypical isolates, even in the median-complexity clinical microbiology laboratory setting (12). The whole-cell protein profile method showed a high assignment index (124/150; 83%), as stated by the formula T=Nt/N, where Nt is the number of isolates assigned to one species and N is the total number of isolates tested (9). Furthermore, this technique was highly specific, as shown by the high degree of intraspecies similarity.

The whole-cell protein profile analysis was able to detect some isolates that had erroneously been identified by the conventional scheme. This simple method allowed for relocating three isolates (1 *E. avium* and 2 *E. gallinarum*), previously and erroneously considered in other species. Two *E. raffinosus* isolates showed different profiles from their corresponding type strain. Only further analysis, including DNA-characterization, would determine if these isolates represent different subgroups within *E. raffinosus* or constitute a new species.

Ideally, this is a method that may prove useful both for relatively high-complexity clinical microbiology and reference laboratories. SDS-PAGE patterns can be obtained in very short times, are reproducible and do not require any sophisticated and expensive reagents or equipment, as compared with molecular biology kits.
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