Polish Journal of Microbiology
2011, Vol. 60, No 4, 335 – 339

SHORT COMMUNICATION

Comparison of Multilocus Variable-Number Tandem-Repeat Analysis with Multilocus Sequence Typing and Pulsed-Field Gel Electrophoresis for *Enterococcus faecalis*

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Received 2 August 2011, revised 6 October 2011, accepted 10 October 2011

Abstract

Enterococcus faecalis represents recently an important etiological agent of health care-associated infections (HAIs) and there is a need for evaluation and comparison of typing methods available for this microorganism. We tested multilocus VNTR (variable-number tandem repeats) analysis (MLVA) on a well-characterized collection of 153 clinical isolates of *E. faecalis,* corresponding to 52 multilocus sequence types and 67 pulsed-field gel electrophoresis (PFGE) profiles. MLVA showed high discriminatory power, discerning 111 different types (diversity index equal 98.9%). The concordance MLVA/MLST and MLVA/PFGE was 0.95 and 0.74, respectively. High discriminatory power of MLVA indicates its utility for local epidemiology such as outbreak investigation, and for differentiation of clones defined by other methods.

Key words: *E. faecalis* clones, MLST, PFGE, VNTR

Enterococci, common and harmless colonizers of human and animal gastrointestinal tract, nowadays represent an important factor of health care-associated infections (HAIs) including invasive infections (mostly endocarditis and bacteraemia) and infections of urinary tract and post-operative site (European Centre for Disease Prevention and Control, 2010; Sydnor and Perl, 2011). The number of patients at risk of enterococcal HAIs is currently increasing, including especially persons with haematological malignancies, and receiving bone marrow and solid-organ transplants (Sydnor and Perl, 2011). Among enterococci causing HAIs, *Enterococcus faecalis* is the predominant species (Jett *et al.*, 1994) and its intrinsic lack of susceptibility to several antimicrobial agents, together with an acquisition of additional resistance traits posses an increasing challenge to therapy (Arias and Murray, 2008). There is an obvious need for the development and evaluation of molecular typing methods for both epidemiological studies of hospital *E. faecalis* outbreaks, and fast identification of high-risk enterococcal complexes, associated with elevated epidemic properties and antimicrobial resistance (Leavis *et al.*, 2006; Ruiz-Garbajosa *et al.*, 2007).

For *E. faecalis*, the "gold-standard" technique of pulsed-field gel electrophoresis (PFGE) of macrorestricted bacterial DNA is widely used in outbreak studies. In global epidemiology, PFGE is now being more and more widely replaced by sequence-based methods that allow for unambiguous identification of isolates, easy data accumulation and comparison. A few such schemes have been described, including the most popular approach of multilocus sequence typing (MLST) with seven house-keeping loci (Ruiz-Garbajosa *et al.*, 2007). Another typing method, multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) has also been proposed for *E. faecalis* (Titze-de-Almeida *et al.*, 2004) as a fast and cheap alternative to PFGE and MLST. MLVA indexes variation of number of repeats present in bacterial genomes and thus also provides an unambiguous, portable identification of an isolate (van Belkum, 2007). The aim of this study was to evaluate the discriminatory power and typability of MLVA using an extensive collection of isolates characterized previously by MLST and PFGE (Kawalec *et al.*, 2007).

A hundred and fifty three isolates of *E. faecalis* derived from colonization, as well as from invasive and non-invasive infections of hospitalized patients from 42 medical centres in Poland during 1996–2005 (Kawalec *et al.*, 2007) were used in the current analysis. These isolates represented 67 different PFGE profiles and 52 different sequence types (STs); the latter were

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grouped into five clonal groups (CC21, CC 40, CC87, group of ST88/89, group of ST132/141) and 42 singletons by the eBURST analysis (Feil *et al.*, 2004). MLVA was performed as described (Titze-de-Almeida *et al.*, 2004), according to the scheme including seven VNTR loci: *ace* (the B region), *esp* (the A and C regions), *efa2*, *efa3*, *efa5*, and *efa6*. The data were then analyzed by the eBURST analysis (Feil *et al.*, 2004). The diversity index (DI) with 95% confidence intervals was calculated as described by others (Grundman *et al.*, 2001); the Wallace coefficient was determined using the site http://darwin.phyloviz.net/ComparingPartitions/ (last accessed on the 4th of July 2011).

From four to eight variants were found for particular VNTR loci and among these only the *aceB* locus was 100% typable (Table I). This gene is known to be ubiquitous in *E. faecalis* (Nallapareddy *et al.*, 2000). A very good typability was found also for *efa6* (99.3% *i.e.* a single isolate was negative). For the other five loci *espC, espA, efa2, efa3* and *efa5,* typability was 73.9%, 68.0%, 50.3%, 46.4% and 86.3%, respectively; such incomplete MLVA profiles were found also in other studies on *E. faecalis* (Titze-de-Almeida *et al.*, 2004; Wałecka *et al.*, 2009; Xavier *et al.*, 2010). Interestingly, the complete MLVA profiles were observed mostly for isolates from infections than from carriage or hospital environment (Fisher exact one-tailed test, $p=0.035$), which is in agreement with the fact that the former isolates are enriched in virulence factors (Shankar *et al.*, 1999). All strains negative for both *espC* and *espA* VNTR lacked the *esp* gene (Kawalec *et al.*, 2007). Apart from the total absence of the gene, the observed lack of PCR product for certain VNTR loci may also be due to sequence polymorphisms that hinder annealing of primers, a deletion of the whole repeat region from a gene, as reported for esp_{Efm} (Leavis *et al.*, 2004), or insertion of a mobile element into a locus (Koeck *et al.*, 2005). Incomplete MLVA profiles were observed also for other species, *e.g.* for *Enterococcus faecium* (Top *et al.*, 2008; Werner *et al.*, 2007) and *Streptococcus pneumoniae* (Koeck *et al.*, 2005).

A hundred and eleven different combinations of VNTR variants were observed in the studied group, corresponding to 111 MLVA types (MTs), numbered consecutively 39–149 (*i.e.*, 1.4 isolate/MT; Table I) that all represented new profiles compared to the 38 MTs described in the earlier study (Titze-de-Almeida *et al.*, 2004). The DI for MLVA was equal 98.9% (CI; 98.2–99.6%); the DIs for MLST and PFGE calculated on the basis of previous results (Kawalec *et al.*, 2007) were both significantly lower and showed similar values of 94.0% (CI, 92.2–95.9%) and 92.4% (CI, 89.7–95.0%), respectively. A lower diversity of isolates was observed in the study on *E. faecalis* in four Brazilian hospitals where 38 MTs and 31 PFGE types were found among 83

Table I
MTs and VNTR loci profiles of Polish clinical isolates of *E. faecalis*

MT[a]	*aceB*	*espC*	*espA*	*efa2*	*efa3*	*efa5*	*efa6*
38	4	nt	nt	6	3	4	4
39	4	6	2	6	3	5	4
40	4	7	nt	6	3	5	4
41	4	5	nt	6	3	5	4
42	4	1	2	6	3	5	4
43	4	4	2	6	3	5	4
44	4	3	2	6	3	5	4
45	4	nt	2	6	3	5	4
46	4	nt	2	5	3	5	4
47 (3)	4	7	2	5	3	5	3
48	2	6	2	5	3	5	3
49	2	6	2	5	nt	3	3
50	4	7	1	5	2	4	3
51	4	nt	nt	5	1	4	3
52 (2)	4	7	1	5	nt	4	3
53	4	7	1	5	2	3	3
54	4	nt	2.5	5	nt	5	3
55	2	nt	3	8	nt	7	3
56	2	5	1	nt	nt	7	3
57 (2)	2	6	1	nt	nt	7	3
58 (6)	4	6	2	nt	nt	5	3
59 (13)	4	7	2	nt	nt	5	3
60 (3)	2	7	2	nt	2	5	3
61	2	4	2	nt	2	5	3
62	2	7	2	nt	2	3	3
63	2	7	2	nt	2	4	3
64	4	5	2	nt	nt	5	3
65	4	5	2	nt	nt	6	3
66	4	7	2	nt	nt	5	nt
68	4	7	nt	nt	nt	4	3
69	4	7	nt	6	3	4	4
70 (3)	4	7	2	nt	nt	4	3
71	4	6	nt	nt	nt	5	3
72	2	5	nt	7	nt	7	3
73	5	7	2	nt	nt	3	3
74	4	8	2	5	3	5	3
75	4	6	2	4	3	5	3
76	2	6	nt	7	2	3	3
77	3	4	2	nt	nt	nt	3
78	4	5	2	nt	nt	4	3
79	4	6	nt	7	2	5	2
80	4	5	nt	6	2	6	3
81	4	6	nt	8	1	3	3
82	1	nt	nt	7	1	5	3
83	4	8	2	nt	nt	3	4
84	2	nt	nt	8	nt	6	2
85	2	nt	nt	nt	nt	6	3
86	2	nt	nt	nt	3	6	3

Table I continued

MT[a]	*aceB*	*espC*	*espA*	*efa2*	*efa3*	*efa5*	*efa6*
87	4	nt	nt	6	3	5	2
88	4	nt	nt	6	3	nt	3
89	3	nt	nt	nt	nt	6	3
90	3	7	nt	nt	3	6	3
91	3	9	4	nt	3	6	3
92	3	9	4	nt	nt	6	3
93 (2)	3	7	4	nt	nt	6	3
94 (4)	3	7	1	7	nt	5	3
95 (2)	3	7	2	8	nt	5	3
96	3	nt	2	7	nt	5	3
97 (3)	3	6	2	8	nt	5	3
98 (2)	3	nt	nt	7	nt	5	3
99	3	nt	2.5	6	nt	5	3
100	3	nt	2	6	nt	5	3
101 (2)	3	nt	nt	6	nt	5	3
102 (2)	3	nt	nt	5	nt	5	3
103	3	6	nt	5	nt	5	3
104 (2)	4	6	3	nt	3	nt	3
105	4	6	3	2.5	3	7	3
106	5	6	1	nt	3	7	3
107	5	6	3	nt	3	nt	3
108	3	6	3	7	3	nt	3
109	3	6	3	9	4	nt	3
110	4	6	3	9	3	nt	3
111	4	nt	5	nt	3	nt	3
112	4	5	4	8	3	7	3
113	3	nt	nt	nt	3	nt	3
114	3	3	4	nt	3	5	3
115	3	nt	2	nt	3	5	3
116	3	6	5	nt	3	5	3
117	3	5	5	2.5	3	nt	3
118	3	6	3	nt	3	5	3
119 (2)	3	5	nt	nt	3	nt	3
120 (2)	3	5	4	nt	3	nt	3
121	3	5	4	2.5	3	nt	3
122 (2)	3	5	5	nt	3	nt	3
123	3	6	4	nt	3	3	3
124 (2)	3	3	2	nt	nt	1	3
125	4	nt	nt	6	2	7	3
126 (2)	4	nt	nt	nt	2	7	4
127	4	6	nt	nt	2	7	4
128	4	6	2	5	nt	5	3
129	4	7	2	5	nt	5	3
130	3	7	2	nt	nt	5	3
131	3	5	nt	5	nt	5	3
132	2	6	nt	nt	2	3	3
133	2	5	3	nt	2	3	4
134	4	nt	nt	6	nt	7	3

Table I continued

MT[a]	*aceB*	*espC*	*espA*	*efa2*	*efa3*	*efa5*	*efa6*
135	4	nt	nt	nt	nt	5	4
136	3	nt	nt	5	nt	nt	2
137	2	7	2	3.5	nt	3	1
138	4	6	nt	3.5	2	6	2
139	4	6	nt	3.5	nt	6	2
140	3	nt	2	5	1	4	3
141	3	nt	nt	nt	1	nt	3
142	3	nt	nt	6	nt	7	3
143 (2)	3	nt	nt	8	nt	5	3
144	3	nt	nt	6	3	5	3
145	3	6	2	nt	nt	5	3
146	4	nt	nt	8	nt	5	3
147	3	nt	nt	5	2	5	3
148	4	nt	nt	3.5	2	5	4
149	2	5	2	5	nt	nt	3

[a] Number of isolates, if bigger than one, given in brackets; nt, nontypable.

isolates (2.4 isolates/MT); the DI for MLVA of this group was 93.3% (Titze-de-Almeida *et al.*, 2004). A similar study revealed 40 MTs among 56 isolates (*i.e.*, 1.4 isolate/MT) from two Polish hospitals (Wałecka *et al.*, 2009). The overall concordance of the compared typing methods, measured by the Wallace coefficient was 0.95 for MLVA/MLST and 0.74 for MLVA/PFGE. In an earlier study, the concordance for MLVA/PFGE was reported as 0.90 for main five clusters of isolates (Titze-de-Almeida *et al.*, 2004).

The eBURST analysis performed on the MLVA profiles delimited 15 clonal groups and 37 singletons (Fig. 1). Three major groups such as MLVA-59 (the naming of the MLVA-defined groups according to the presumable ancestral MT), MLVA-101 and MLVA-119 included 17, 14 and 7 MTs, respectively, and 41, 18 and 10 isolates, respectively. The comparison of MLVA and MLST grouping (Fig. 1) revealed certain cases of incongruence between the two methods. The principal group MLVA-59 was associated with three clonal groups defined by MLST (CC40, CC21, group of ST88/89) and singleton STs 26, 55, 59 and 136. Another major group, MLVA-101, grouped isolates belonging to four groups defines by MLST and five singleton STs. On the other hand, known HIRECCs of *E. faecalis* (Ruiz-Garbajosa *et al.*, 2006; Kawalec *et al.*, 2007) such as CC2 (represented here by ST6), CC9 and CC87 each included several MTs; the most divergent CC87 contained three MLVA groups and 10 MLVA singletons, *i.e.* altogether 20 different MTs. Such high variability of MTs within established enterococcal HIRECCs makes it difficult to use MLVA for their identification. Although other typing methods of *E. faecalis*, such as PFGE and

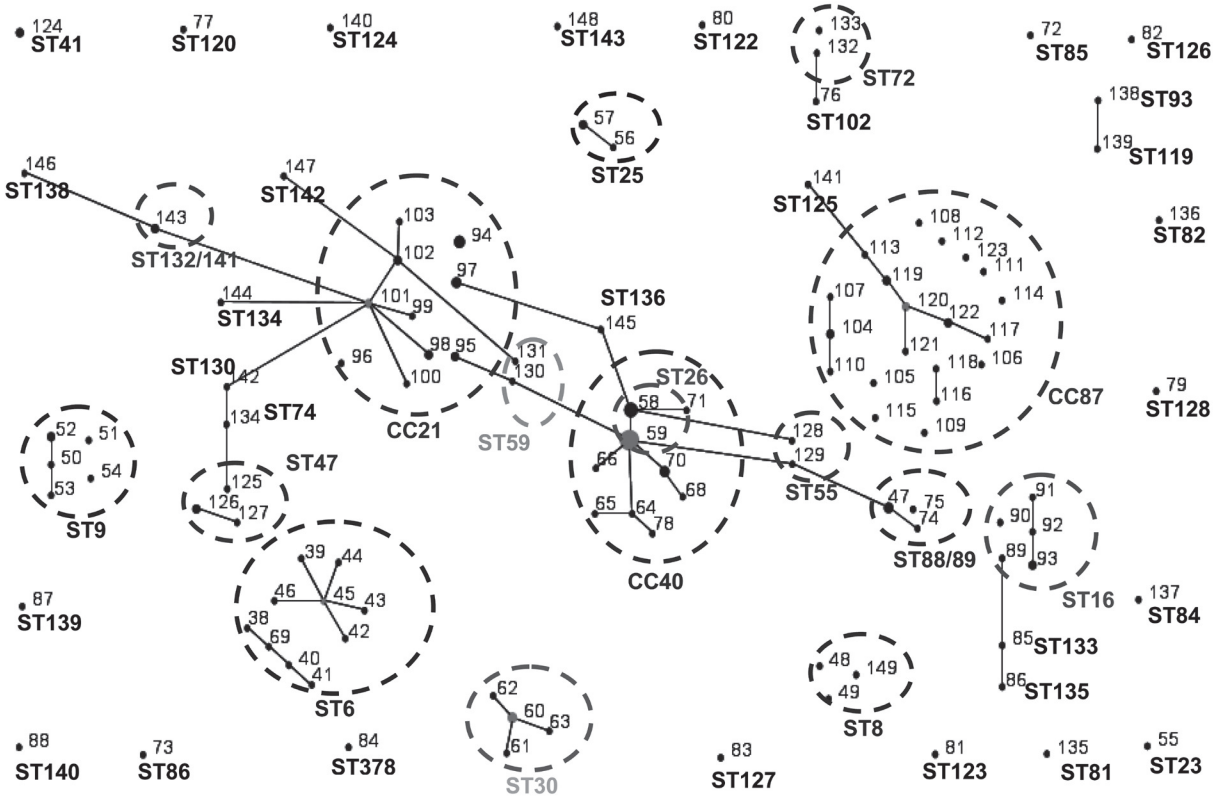

Fig. 1. Clonal relationships among *E. faecalis* according to MLVA and eBURST analysis, compared to MLST results.
Solid circles, MTs with size proportional to the number of isolates; light grey circles, presumable ancestors of clonal groups; lines, SLV links; open coloured circles, main STs and clonal groups as defined by MLST. The eBURST output was extensively manually edited.

sequence-based typing methods generally show a good agreement, some examples of their incongruence, presumably resulting from recombination typical for this species were also found (Nallapareddy *et al.*, 2002; Kawalec *et al.*, 2007; Chowdhury *et al.*, 2009).

Very high discriminatory power of MLVA and observed incongruence with other typing methods, such as PFGE and MLST, observed in the current study may have resulted from the selection of isolates. Our collection was derived from several centres over a long period of time while other studies (Titze-de-Almeida *et al.*, 2004; Wałecka *et al.*, 2009) used isolates from more limited areas and time span. MLVA markers are likely the ones evolving faster than house-keeping loci in MLST. The VNTR loci, selected for the current *E. faecalis* MLVA scheme encode surface-located factors that presumably are under strong selective pressure. The change of repeat number during infection was observed for the alpha C protein of the Alp-like protein family in *Streptococcus agalactiae,* resulting in the escape from the host immunological system (Madoff *et al.*, 1996). The VNTR loci used for *E. faecalis* MLVA encode Esp (enterococcal surface protein), a partial homolog of the Alp-like proteins (Hendrickx *et al.*, 2009), Ace (adhesin to collagen from *E. faecalis*; Hendrickx *et al.*, 2009), the Efa2, Efa3 and Efa5 proteins that belong to the ABC superfamily of membrane channel-forming proteins (Khwaja *et al.*, 2005) and the Efa6 protein of unknown function that is also a surface protein (da Silva Ruivo, 2008). It has to be also considered that in the case of MLVA some similarities in the typing patterns, including the loss of typability at a given loci might have arisen by convergent evolution, thus resulting in incongruence with other methods. In conclusion, this is, to our knowledge, a first report on comparison of MLVA and MLST for *E. faecalis*. Our study indicates MLST as the method of choice for long-term epidemiology while MLVA appears to be over-discriminatory for such studies. The MLVA-based identification of enterococcal HIRECCs should be treated with caution as examples of incongruence between MLVA and MLST as well as high variability of MTs within known HIRECCs were observed. The high discriminatory power of MLVA indicates the utility of this method for studies on hospital outbreaks and for further differentiation of the most important clones, which may be difficult with other typing methods.

Acknowledgements

This work was supported by a grant ACE from the European Union VI Framework Program under the contract LSHE-CT-2007-037410 and a complementary founding from the Ministry of Science and Higher Education, Poland (decision 937/6. PR UE/2009/7).

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