Evaluation of High-Resolution Melting Curve Analysis of Ligation-Mediated Real-Time PCR, a Rapid Method for Epidemiological Typing of ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* Species) Pathogens

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A single-tube method, ligation-mediated real-time PCR high-resolution melt analysis (LMqPCR HRMA), was modified for the rapid typing of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. (ESKAPE) pathogens. A 97% agreement (60/62 isolates) was achieved in comparison to pulsed-field gel electrophoresis (PFGE) results, which indicates that LMqPCR HRMA is a rapid and accurate screening tool for monitoring nosocomial outbreaks.

Increasing antibiotic resistance in bacterial pathogens presents a great threat to human health, and the prevention of the spread of such pathogens is important (1). The ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. [2]) pathogens are especially problematic because of their antibiotic resistance mechanisms and nosocomial spread (2, 3). By using new strategies for the rapid typing of isolates, the possibility of preventing the uncontrolled spread of these pathogens is greatly increased. Pulsed-field gel electrophoresis (PFGE) has been considered the gold standard for epidemiological typing (4, 5). However, next-generation sequencing with whole-genome sequencing (WGS) is likely to become the new standard (6–10). The disadvantages of these methods are that they require both special equipment and standard protocols and training (4–6, 8–10). Thus, there is a need for a simple and fast typing method enabling real-time monitoring of nosocomial outbreaks. Most of the already-existing molecular typing methods, such as multilocus sequence typing (MLST), repetitive-sequence-based PCR (rep-PCR) (such as the commercial platform DiversiLab), and comparative genomic hybridization, although more rapid than PFGE still are expensive and species specific, and they exhibit insufficient resolution (11).

In this study, we further modified ligation-mediated PCR (LM PCR) with polyacrylamide gel analysis (12, 13) for use in a single-tube real-time PCR platform with high-resolution melt analysis (LMqPCR HRMA) for rapid epidemiological typing of the multiresistant ESKAPE pathogens. Following optimization, the method was evaluated blindly using a selection of ESKAPE pathogens that were previously defined by PFGE analysis as being identical, closely related, or unrelated (see the supplemental material).

Briefly, LMqPCR HRMA is based on fragmentation by a restriction enzyme (HindIII), followed by ligation of adaptor oligonucleotides to create amplicons. Analysis is done by HRM analysis, resulting in specific melt patterns depending on the number of amplified oligonucleotides, DNA sequence, G+C content, and length. By altering the denaturation temperature (T_D) during PCR, the method was optimized for ESKAPE pathogens and performed in duplicate in three separate experiments. For a detailed description of the method, see the supplemental Materials and Methods. A complete list of the isolates, their origins, and resistance genes is presented in Table 1. All isolates had been analyzed with PFGE at the Public Health Agency of Sweden (see the supplemental Materials and Methods). A standardized protocol was developed based on several parameters of the melting curve shape to determine if two or more isolates were different within the same run (see the supplemental Materials and Methods).

Overall, 59 out of 62 of the ESKAPE pathogen isolates were identified with full agreement between LMqPCR HRMA and PFGE (≥90% as the cutoff for similarity) (Table 1). The LMqPCR HRMA and PFGE results did not correlate for *E. faecium* strain Ef09 and *A. baumannii* strains Ab02 and Ab03 (Fig. 1). Ef09 clustered with other isolates using LMqPCR HRMA but was defined as unique by PFGE. When adding agarose gel electrophoresis analysis after HRMA, 60 out of 62 isolates (97%) were identified with full agreement with PFGE, with Ef09 being classified as unique. In contrast to PFGE, for which Ab02 and Ab03 were defined as being

TABLE 1 Complete list of isolates included in the study

Species and isolate	Origin[a]	PFGE results[b]	LMqPCR HRMA results[c]	Resistance mechanism(s)[d]
E. faecium				
Ef01	1	A	Ef-I	*vanB*
Ef02	1	A1	Ef-I[f]	*vanB*
Ef03	1	A	Ef-I[f]	*vanB*
Ef04	1	B	Ef-II	*vanA*
Ef05	1	B	Ef-II	*vanA*
Ef06	1	B1	Ef-II[f]	*vanA*
Ef07	1	C	Ef-III	*vanA*
Ef08	1	C	Ef-III	*vanA*
Ef09	1	Unique	Similar to Ef-II[e,f]	*vanA*
Ef10	1	Unique	Unique	*vanA*
S. aureus				
Sa01	3	A1	Sa-I[f]	*mecA*
Sa02	3	B	Sa-II	*mecA*
Sa03	3	B	Sa-II	*mecA*
Sa04	3	Unique	Unique	*mecA*
Sa05	3	C1	Sa-III	*mecA*
Sa06	3	C2	Sa-III[f]	*mecA*
Sa07	3	A2	Sa-I	*mecA*
Sa08	3	D	Sa-IV	*mecA*
Sa09	3	D	Sa-IV	*mecA*
Sa10	3	A2	Sa-I	*mecA*
Sa11	3	A2	Sa-I	*mecA*
Sa12	3	A3	Sa-I[f]	*mecA*
K. pneumoniae				
Kp01	1	A	Kp-I	CTX-M-1
Kp02	1	A	Kp-I	CTX-M-1
Kp03	1	A	Kp-I	CTX-M-1
Kp04	1	B	Kp-II	CTX-M-1
Kp05	1	B	Kp-II	CTX-M-1
Kp06	1	B	Kp-II	CTX-M-1
Kp07	1	C	Kp-III	CTX-M-1
Kp08	1	C	Kp-III	CTX-M-1
Kp09	1	Unique	Unique	CTX-M-9
Kp10	1	Unique	Unique	CTX-M-1
A. baumannii				
Ab01	2	Unique	Unique	OXA-23, OXA-51
Ab02	2	A1	Ab-I[e]	OXA-23, OXA-51
Ab03	2	A2	Ab-I[e,f]	OXA-23, OXA-51
Ab04	3	B	Ab-II	OXA-23, OXA-51
Ab05	2	B	Ab-II	OXA-23, OXA-51
Ab06	2	B	Ab-II	OXA-23, OXA-51
Ab07	1	Unique	Unique	OXA-51
Ab08	1	A	Ab-III	OXA-23, OXA-51
Ab09	1	A	Ab-III	OXA-23, OXA-51
Ab10	1	Unique	Unique	No OXA
P. aeruginosa				
Pa01	1	A	Pa-I	IMP
Pa02	1	A	Pa-I	IMP
Pa03	1	A	Pa-I	IMP
Pa04	1	B	Pa-II	No MBL
Pa05	1	B	Pa-II	No MBL
Pa06	1	C	Pa-III	VIM
Pa07	1	C1	Pa-III[f]	VIM
Pa08	1	C2	Pa-III[f]	VIM
Pa09	1	Unique	Unique	No MBL
Pa10	1	Unique	Unique	No MBL

TABLE 1 (Continued)

Species and isolate	Origin[a]	PFGE results[b]	LMqPCR HRMA results[c]	Resistance mechanism(s)[d]
E. cloacae				
EnC01	1	A	EnC-I	cAmpC
EnC02	1	A	EnC-I	cAmpC
EnC03	1	Unique	Unique	cAmpC
EnC04	1	B	EnC-II	cAmpC
EnC05	1	B	EnC-II	cAmpC
EnC06	1	B	EnC-II	cAmpC
EnC07	1	Unique	Unique	cAmpC
EnC08	1	Unique	Unique	cAmpC
EnC09	1	Unique	Unique	cAmpC
EnC10	1	Unique	Unique	cAmpC

[a] Origins 1 to 3 are defined as follows: 1, the Public Health Agency of Sweden, Solna, Sweden; 2, Department of Clinical Microbiology, County Council of Östergötland, Linköping, Sweden; and 3, Department of Clinical Microbiology, Kalmar County Hospital, Kalmar, Sweden.
[b] Arbitrary classification of PFGE cluster within each species are as follows: letters denote isolates within a cluster with >90 to 97% similarity, numbers denote closely related patterns with >97% similarity.
[c] Classification of HRMA clusters I, II, III, etc. Ef, *E. faecium*; Sa, *S. aureus*; Kp, *K. pneumonia*; Ab, *A. baumannii*; Pa, *P. aeruginosa*; EnC, *E. cloacae*.
[d] *vanA* and *vanB* indicate vancomycin resistance; *mecA* indicates methicillin resistance; CTX-M phylogenetic groups 1 and 9, species-specific OXA enzymes, and metallo-β-lactamases (MBL) IMP and VIM indicate β-lactamases; cAmpC indicates chromosomal β-lactamases.
[e] Isolates for which the results from PFGE (with 90 to 97% similarity) and HRMA do not correspond.
[f] Isolates for which the results from PFGE (with >97% similarity) and HRMA do not correspond.

similar to *A. baumannii* strains Ab08 and Ab09, these two groups of isolates were not defined as similar by LMqPCR HRMA.

Using >97% as the similarity cutoff for the PFGE results, the methods were in agreement for 53 out of 62 isolates (85%), with discrepancies for *E. faecium* strains Ef02, Ef06, and Ef09, *S. aureus* strains Sa01, Sa06, and Sa12, *A. baumannii* Ab03, and *P. aeruginosa* strains Pa07 and Pa08 (Table 1). No discrepancies were found for *K. pneumoniae* and *Enterobacter cloacae* strains. None of the isolates determined as being unique according to LMqPCR HRMA were evaluated as being similar in the PFGE analysis (cutoff for PFGE, >97%). The HRMA method is therefore suitable for confirming that isolates in suspected nosocomial outbreaks are not related. The complete list of results is presented in Table 1.

We describe a rapid method based on restriction cleavage and ligation-mediated real-time PCR (qPCR) with high-resolution melt analysis, which can be used as a primary method for epidemiological typing in the investigation of suspected nosocomial outbreaks caused by pathogens from the ESKAPE group and as a definitive method for excluding unrelated isolates. This method has already been shown to identify a nosocomial outbreak of O25b-sequence type 131 (ST131)-associated extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* isolates with a resolution comparable to that found with PFGE (14). Hence, in the evaluation of two or more isolates from a suspected outbreak in the routine microbiological laboratory, this method can confirm whether such isolates are different and to a very high certainty if they are similar. LMqPCR HRMA does not require any prior knowledge of the DNA sequence, although the typeability is dependent on the G+C content of the species. In order to determine the similarities and differences between isolates, it is possible to
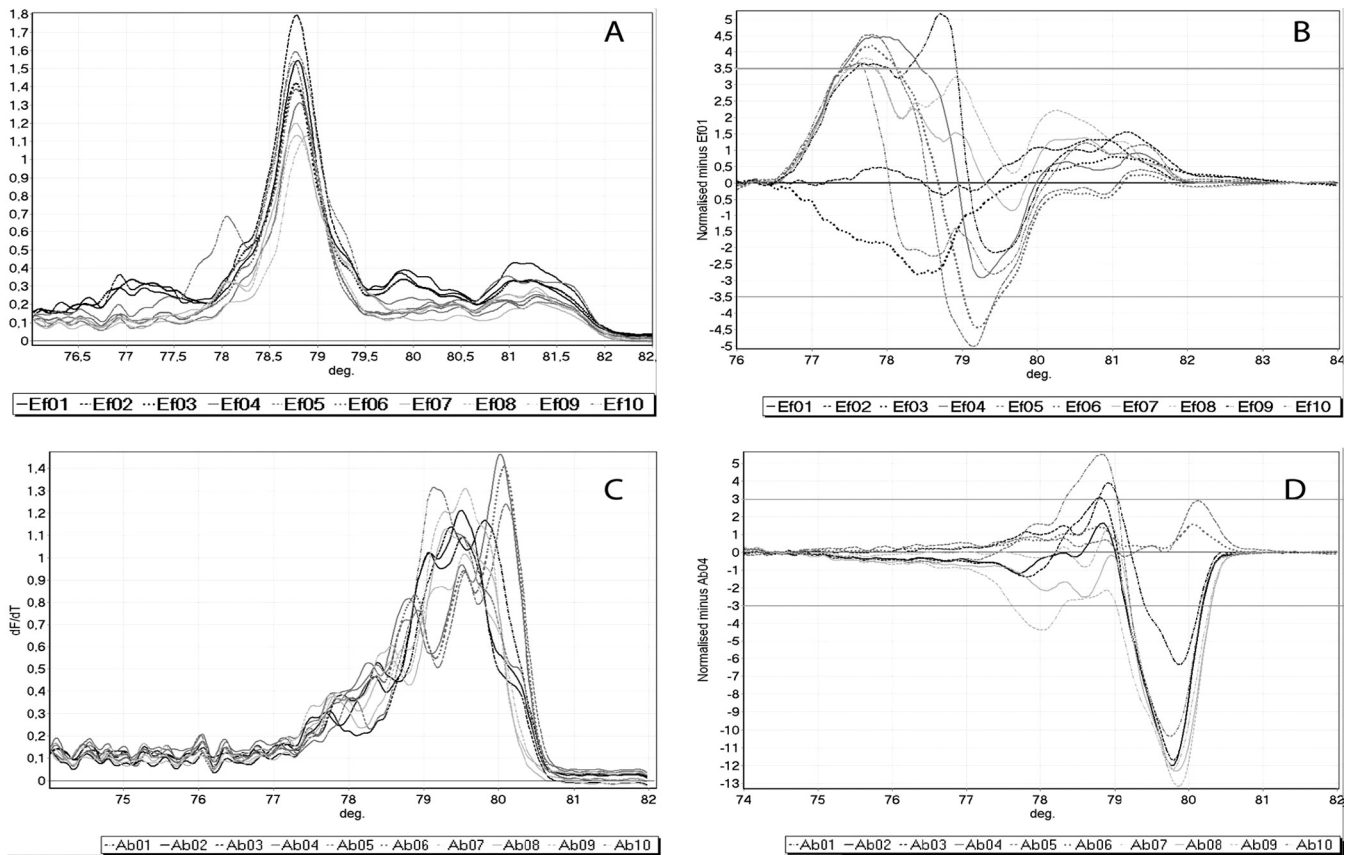
FIG 1 LMqPCR HRMA results for *E. faecium* (A and B) and *A. baumannii* (C and D). (A and C) Complete melting curves for all isolates are visualized plotting *dF/dt* against temperature (°C). The algorithm from Woksepp et al. (14) was used on the complete melting curves visualized in panels A and C, together with a subjective evaluation of the discrepancies between the melting curves for clustering analysis of the isolates. (B) Isolate *E. faecium* Ef01 is set as reference, plotting the difference of all other isolates to Ef01. The isolates within ±3.5 U of the *y* axis (Ef02 and Ef03) are considered to cluster with Ef01. (D) Isolate *A. baumannii* Ab04 is set as reference, plotting the difference of all other isolates to Ab04. Isolates within ±3 U of the *y* axis (Ab05 and Ab06) are considered to cluster with Ab04. For *E. faecium*, Ef01, Ef02, and Ef03 clustered together, Ef04, Ef05, and Ef06 clustered together, and Ef07 and Ef08 clustered together, with Ef09 being similar to the isolates Ef04, Ef05, and Ef06. Ef10 was unique. For *A. baumannii*, Ab02 and Ab03 clustered together, Ab04, Ab05, and Ab06 clustered together, and Ab08 and Ab09 clustered together. Ab01, Ab07, and Ab10 were unique. deg, degrees Celsius.

use the Rotor-Gene software and the algorithm previously developed by Woksepp et al. (14). Among the ESKAPE pathogens, the HRM patterns from the Gram-negative bacteria were more straightforward to evaluate than were the Gram-positive bacteria. When analyzing the HRMA results for Gram-negative bacteria, a visual inspection of the HRM curves was enough for all but *A. baumannii* to determine discrepancies between the isolates (see Fig. S1A in the supplemental material). For Gram-positive bacteria, our results show that additional agarose electrophoresis might be of benefit, especially in cases where the isolates are similar by HRM analysis only. The species that gave the highest resolution in LMqPCR HRMA were *K. pneumoniae*, *P. aeruginosa*, *E. coli* (14), and *E. cloacae*. All of these species have G+C contents between 51 and 66%, in contrast to *E. faecium*, *S. aureus*, and *A. baumannii*, which have G+C contents between 33% and 39% (http://www.ncbi.nlm.nih.gov/genome).

Our study has limitations, one being that it was performed on a limited number of isolates for each of the chosen species, although the isolates were carefully selected to represent identical, closely related, and unrelated types, based on the PFGE results. Another limitation is that despite the algorithm used, the HRMA data interpretation has a subjective component that is shared with other methods for epidemiological typing.

The LMqPCR HRMA method was primarily developed for the rapid investigation of clonal dissemination. For long-term surveillance, other methods, such as PFGE or WGS, should be used. However, the results confirm the usefulness of this rapid method, both as a screening method to exclude a potential nosocomial outbreak of ESKAPE pathogens but also to indicate relatedness to a high degree of certainty. It is important, however, to remember that typing results should be correlated to clinical and epidemiological data.

In conclusion, LMqPCR HRMA is a rapid and simple method for investigating nosocomial outbreaks of resistant ESKAPE pathogens. Results comparable to those from PFGE can be achieved within 1 day in a single-tube system.

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