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Development of a Novel Real-Time PCR Assay with High-Resolution Melt Analysis To Detect and Differentiate OXA-48-Like β-Lactamases in Carbapenem-Resistant *Enterobacteriaceae*

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The rapid global spread of carbapenem-resistant *Enterobacteriaceae* (CRE) poses an urgent threat to public health. More than 250 class D β-lactamases (OXAs) have been described in recent years, with variations in hydrolytic activity for β-lactams. The plasmid-borne OXA-48 β-lactamase and its variants are identified only sporadically in the United States but are common in Europe. Recognition of these OXA-48-like carbapenemases is vital in order to control their dissemination. We developed a real-time PCR assay based on high-resolution melt analysis, using $bla_{OXA-48-like}$-specific primers coupled with an unlabeled 3′-phosphorylated oligonucleotide probe (LunaProbe) homologous to OXA-48-like carbapenemase genes. The assay was validated using genomic DNA from 48 clinical isolates carrying a variety of carbapenemase genes, including bla_{KPC}, bla_{SME}, bla_{IMP}, bla_{NDM-1}, bla_{VIM}, bla_{OXA-48}, $bla_{OXA-162}$, $bla_{OXA-181}$, $bla_{OXA-204}$, $bla_{OXA-244}$, $bla_{OXA-245}$, and $bla_{OXA-232}$. Our assay identified the presence of $bla_{OXA-48-like}$ β-lactamase genes and clearly distinguished between bla_{OXA-48} and its variants in control strains, including between $bla_{OXA-181}$ and $bla_{OXA-232}$, which differ by only a single base pair in the assay target region. This approach has potential for use in epidemiological investigations and continuous surveillance to help control the spread of CRE strains producing OXA-48-like enzymes.

The *Enterobacteriaceae* account for up to 25% of health care-associated infections (HAIs) reported to the U.S. National Healthcare Safety Network (1). Over the past 5 years, the prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) has increased dramatically in the United States (2–4). CRE are a particular threat to patient safety because there are limited or no treatment options for such infections and they are associated with high mortality rates (5). Several health care-associated CRE outbreaks have been reported, highlighting the need for CRE resistance mechanism testing at the hospital, regional, and national levels (6). Because carbapenem resistance in *Enterobacteriaceae* is often associated with carbapenemases located on plasmids or other mobile genetic structures, efficient transmission between strains and across species of the same family is not only possible but is well described (7). A recent report from the National Institutes of Health Clinical Center (8) provided insight regarding how outbreaks caused by CRE may lead to devastating patient outcomes and can be exceptionally difficult and costly to investigate and to control.

Testing for CRE resistance mechanisms is infrequently performed by clinical laboratories in the United States, due in part to the lack of FDA-cleared tests for this purpose and the complex and evolving nature of carbapenem resistance among the *Enterobacteriaceae* (9–11). Prior to the 21st century, carbapenem resistance in CRE was almost exclusively attributable to overexpression of an intrinsic cephalosporinase (*ampC*) or production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) such as TEM, SHV, and CTX-M, combined with modification of outer membrane porins (7). However, since the description of *Klebsiella pneumoniae* carbapenemase (KPC) in 2001 (12), several classes of carbapenemases have been identified and characterized in the *Enterobacteriaceae* (13). Further complicating laboratory detection is the alarming rate at which new variants of these carbapenemases are identified (14).

A prime example of this rapid expansion is the OXA family of β-lactamases. OXA-48, encoded by the gene bla_{OXA-48}, is a plasmid-borne OXA enzyme, characterized in 2004, with hydrolytic activity against penicillins and low-level activity against carbapenems but no activity against third- or fourth-generation cephalosporins (15, 16). In the past decade, several variants of OXA-48, including OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, and OXA-370, have been described (17–19). These enzymes possess similar activities against β-lactams with the exception of OXA-163, which is highly active against broad-spectrum cephalosporins but lacks the ability to hydrolyze carbapenems efficiently (17). The bla_{OXA-48} gene was first observed in the United States in 2012 (20), and an isolate carrying $bla_{OXA-232}$ was described in 2014 (21). Other than these two reports, the incidence of the OXA-48 family of carbapenemases in the United States is largely unknown.

Our laboratory periodically screens all CRE isolates for the presence of carbapenemases, using a laboratory-developed multiplex real-time PCR assay that is designed to detect the presence of

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TABLE 1 Performance of LunaProbe HRMA with previously characterized CRE isolates and genomic DNA prepared from isolates carrying bla_{OXA-48}-like genes

Organism	Strain	Carbapenemase gene	Assay result[a]				MIC (μg/ml)[b]		
			MHT	Carba NP	LunaProbe HRMA	CRE PCR	IPM	MEM	ETP
K. pneumoniae	ATCC BAA-1705	bla_{KPC}	+	+	−	bla_{KPC}	8	>16	>2
K. pneumoniae	UCLA 14-36-04	bla_{KPC}	+	+	−	bla_{KPC}	>8	>16	>2
K. pneumoniae	ATCC BAA-2146	bla_{NDM-1}	+	+	−	bla_{NDM-1}	4	16	>2
K. pneumoniae	UCLA 11-05-2	bla_{IMP}	+	+	−	bla_{IMP}	1	2	1
K. pneumoniae	UCLA 11-05-3	bla_{VIM}	+	+	−	bla_{VIM}	>8	>16	>2
Serratia marcescens	CACMLE 213-1	bla_{SME}	+	+	−	bla_{SME}	>8	>16	>2
K. pneumoniae	CAV 1543	bla_{OXA-48}	+	−	$bla_{OXA-48/245}$	bla_{OXA-48}	4	4	>2
K. pneumoniae	CAV 1636	$bla_{OXA-181}$	+	−	$bla_{OXA-181/204}$	−	4	8	>2
K. pneumoniae	UCLA 14-36-06[c]	$bla_{OXA-232}$	+	−	$bla_{OXA-232}$	−	4	16	>2
K. pneumoniae	UCLA 14-36-87	bla_{OXA-48}	+	−	$bla_{OXA-48/245}$	bla_{OXA-48}	>8	>16	>2
K. pneumoniae	UCLA 14-36-88	$bla_{OXA-181}$	+	−	$bla_{OXA-181/204}$	−	4	4	>2
K. pneumoniae	UCLA 14-36-89	$bla_{OXA-162}$	+	−	$bla_{OXA-162}$	bla_{OXA-48}	4	8	>2
K. pneumoniae	UCLA 14-36-91	$bla_{OXA-162}$	+	−	$bla_{OXA-162}$	bla_{OXA-48}	8	>16	>2
K. pneumoniae	UCLA 14-36-95	$bla_{OXA-232}$	+	−	$bla_{OXA-232}$	−	4	16	>2
K. pneumoniae	UCLA 14-36-96	$bla_{OXA-232}$	+	−	$bla_{OXA-232}$	−	4	>16	>2
K. pneumoniae	UCLA 14-36-99	$bla_{OXA-181}$	+	−	$bla_{OXA-181/204}$	−	>8	>16	>2
A. baumannii	CDC CarbaNP-4	$bla_{OXA-24-like}$	+	−	−	−	>8	>16	>2
A. baumannii	CDC CarbaNP-5	$bla_{OXA-24-like}$	+	−	−	−	>8	>16	>2
A. baumannii	CDC CarbaNP-14	$bla_{OXA-23-like}$	+	−	−	−	>8	>16	>2
A. baumannii	CDC CarbaNP-21	$bla_{OXA-58-like}$	+	−	−	−	>8	16	>2
A. baumannii	CDC CarbaNP-25	$bla_{OXA-23-like}$	+	−	−	−	>8	>16	>2
A. baumannii	CDC CarbaNP-32	$bla_{OXA-23-like}$ and $bla_{OXA-24-like}$	+	−	−	−	>8	>16	>2
A. baumannii	CDC CarbaNP-39	$bla_{OXA-58-like}$	+	−	−	−	8	8	>2
K. pneumoniae	CDC CarbaNP-8	bla_{OXA-48}	+	−	$bla_{OXA-48/245}$	bla_{OXA-48}	2	2	>2
K. pneumoniae	CDC CarbaNP-20	bla_{OXA-48}	+	−	$bla_{OXA-48/245}$	bla_{OXA-48}	4	8	>2
K. pneumoniae	CDC CarbaNP-35	$bla_{OXA-232}$	+	−	$bla_{OXA-232}$	−	8	>16	>2
K. pneumoniae	CDC CarbaNP-44	bla_{OXA-48}	+	−	$bla_{OXA-48/245}$	bla_{OXA-48}	4	>16	>2
Enterobacter aerogenes	CDC CarbaNP-43	bla_{OXA-48}	+	−	$bla_{OXA-48/245}$	bla_{OXA-48}	8	2	>2
Genomic DNA prepared from isolates harboring bla_{OXA-48}-like genes									
Sample 1		$bla_{OXA-162}$			$bla_{OXA-162}$	bla_{OXA-48}			
Sample 2		$bla_{OXA-204}$			$bla_{OXA-181/204}$	−			
Sample 3		$bla_{OXA-244}$			$bla_{OXA-244}$	bla_{OXA-48}			
Sample 4		$bla_{OXA-245}$			$bla_{OXA-48/245}$	bla_{OXA-48}			

[a] MHT, modified Hodge test; HRMA, high-resolution melt analysis; CRE, carbapenem-resistant *Enterobacteriaceae*.
[b] IPM, imipenem; MEM, meropenem; ETP, ertapenem.
[c] Isolate identified initially, by whole-genome shotgun sequencing, as harboring $bla_{OXA-232}$.

several carbapenemase genes, including $bla_{OXA-48-like}$ (22). However, this PCR assay yielded negative results for a *K. pneumoniae* strain identified by whole-genome shotgun (WGS) sequencing as harboring $bla_{OXA-232}$ (unpublished data). It was later determined that this false-negative PCR result was due to a low level of homology of the $bla_{OXA-48-like}$ forward primer for $bla_{OXA-232}$. In a previous report, we identified several CRE isolates that tested negative with this multiplex carbapenemase gene PCR assay, spanning back to 2011 (22). There was thus a desire to determine the first occurrence of $bla_{OXA-232}$ at our institution and its prevalence among our CRE isolates. This report describes a real-time PCR assay with LunaProbe-enhanced high-resolution melt analysis (HRMA) that was developed for specific identification of $bla_{OXA-232}$ in clinical isolates of CRE and differentiation between $bla_{OXA-48-like}$ genes.

MATERIALS AND METHODS

Clinical isolates used for $bla_{OXA-48-like}$ PCR assay validation. Previously characterized clinical *Enterobacteriaceae* isolates carrying different carbapenemase genes (bla_{KPC}, bla_{SME}, bla_{IMP}, bla_{NDM-1}, bla_{VIM}, bla_{OXA-48}, $bla_{OXA-162}$, $bla_{OXA-181}$, and $bla_{OXA-232}$) were used to verify the performance of the LunaProbe HRMA assay. In addition, seven *Acinetobacter baumannii* isolates with known OXA genes were evaluated (Table 1). Genomic DNA prepared from *Enterobacteriaceae* isolates harboring $bla_{OXA-162}$, $bla_{OXA-204}$, $bla_{OXA-244}$, and $bla_{OXA-245}$ were kindly provided by Corinne Jay and Maryse Touchard (bioMérieux, Marcy l'Etoile, France); these isolates were previously characterized by Sanger sequencing of the OXA genes.

All CRE strains that had been isolated between January 2011 and September 2014 and were negative for the presence of six carbapenemase genes (bla_{KPC}, bla_{SME}, bla_{IMP}, bla_{NDM-1}, bla_{VIM}, and bla_{OXA-48}) detectable with our previously reported multiplex real-time PCR assay (22) were

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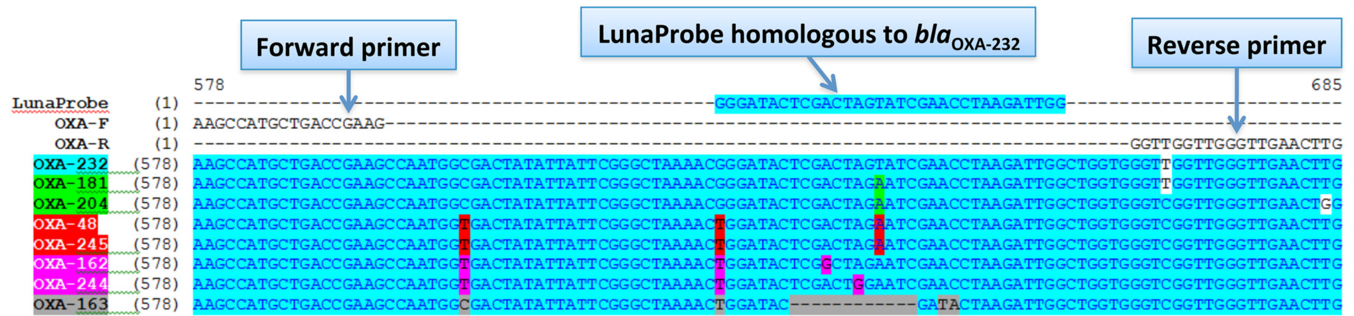

FIG 1 Nucleic acid alignment of $bla_{\text{OXA-48-like}}$ genes with PCR primer and LunaProbe binding regions.

evaluated. In this study, CRE isolates were defined as isolates with nonsusceptible MICs (i.e., MICs of >1 μg/ml) for imipenem and/or meropenem, irrespective of the MICs for third-generation cephalosporins. Susceptibility testing was performed on all isolates by the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution method (23), using panels prepared in-house. The antimicrobials tested included meropenem, imipenem, and ertapenem. The modified Hodge test (MHT) was performed using a meropenem disk, as described elsewhere (23, 24). The Carba NP test (25) was performed according to procedures described by the CLSI (23) to detect carbapenemase activity against imipenem *in vitro*.

***bla*$_{\text{OXA-48-like}}$ β-lactamase gene detection and differentiation by PCR and LunaProbe HRMA assay.** Genomic DNA was extracted from CRE isolates using the EZ1 tissue kit with an EZ1 Biorobot (Qiagen, Valencia, CA), according to the manufacturer's instructions. Forward (OXA-F, 5′-AAGCCATGCTGACCGAAG-3′) and reverse (OXA-R, 5′-CAAGTTCAACCCAACCAACC-3′) PCR primers amplifying a discriminatory region in $bla_{\text{OXA-48-like}}$ genes were designed using Primer3Plus (26). An unlabeled 3′-phosphorylated oligonucleotide probe (LunaProbe) (27) complementary to the reverse strand of the $bla_{\text{OXA-232}}$ β-lactamase gene amplicon (OXA-P, 5′-GGGATACTCGACTAGTATCGAACCTAAGATTGG-3′) was designed to increase the discriminatory power of the assay (Fig. 1). Asymmetric PCR with a forward/reverse primer concentration ratio of 1:5 was performed to generate excess reverse-strand single-stranded DNA (ssDNA) that would bind to the LunaProbe, resulting in enhanced ssDNA-LunaProbe duplex melt signals. Each 20-μl PCR mixture included 2× LightCycler 480 high-resolution melting master mix (Roche Diagnostics, Indianapolis, IN) and 10 to 50 ng of genomic DNA prepared from clinical isolates, as the template, along with OXA-F, OXA-R, OXA-P, and $MgCl_2$ at final concentrations of 200 nM, 1 μM, 200 nM, and 2.5 mM, respectively. PCR cycling parameters were as follows: preincubation at 95°C for 10 min followed by 35 cycles of 95°C for 10 s, 58°C for 15 s, and 72°C for 10 s.

High-resolution melting was performed after PCR as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN). Briefly, the PCR product was denatured at 95°C for 1 min and then allowed to anneal with its own complementary strand or the LunaProbe at 40°C for 1 min. Melting was subsequently performed from 50°C to 95°C, with a ramp rate of 0.02°C/s. Data analysis was performed using the gene scanning module in the LightCycler 480 software (version 1.5.0 SP3; Roche Diagnostics, Indianapolis, IN). Analytical parameters included a temperature shift of 5°C, premelt slider settings of 61.59 to 63.25°C, and postmelt slider settings of 83.35 to 85.07°C to cover the predicted melting temperatures of the ssDNA-LunaProbe duplex and the PCR product (approximately 68°C and 80°C, respectively). A normalized and temperature-shifted difference plot for each PCR was created using melt data for $bla_{\text{OXA-48}}$ as the reference. The shapes and peaks of the melt plots were evaluated for both the PCR product and the ssDNA-LunaProbe duplex, if present. Therefore, the assay results were predicted through *in silico* evaluations to discriminate $bla_{\text{OXA-48-like}}$ genes into the following groups, based on evaluations of both the PCR product and ssDNA-LunaProbe duplex melt curves: $bla_{\text{OXA-48}}$/$bla_{\text{OXA-245}}$, $bla_{\text{OXA-181}}$/$bla_{\text{OXA-204}}$, $bla_{\text{OXA-162}}$, $bla_{\text{OXA-244}}$, or $bla_{\text{OXA-232}}$ (28).

OXA-48-like β-lactamase gene sequencing. To confirm all PCR results, a discriminatory region of the putative $bla_{\text{OXA-48-like}}$ gene was amplified from each isolate through PCR in a 20-μl PCR mixture, which included AmpliTaq Gold Fast PCR master mix (Life Technologies, Grand Island, NY) along with forward (OXA-SeqF, 5′-CCTCGATTTGGGCGTGGTTA-3′) and reverse (OXA-SeqR, 5′-AAACCATCCGATGTGGGCAT-3′) PCR primers, with final concentrations of 500 nM for each primer. PCR cycling parameters were as follows: preincubation at 95°C for 10 min, 35 cycles of 96°C for 10 s, 58°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 1 min. PCR products were purified using an UltraClean PCR clean-up kit (MO BIO, Carlsbad, CA) and sequenced on a 3130xl genetic analyzer (Life Technologies, Grand Island, NY). Sequencing results were evaluated with BLAST (29).

Multiplex real-time PCR assay for detection of common carbapenemase genes. A multiplex TaqMan real-time PCR assay for the detection of bla_{KPC}, bla_{SME}, bla_{IMP}, $bla_{\text{NDM-1}}$, bla_{VIM}, and $bla_{\text{OXA-48}}$ was performed as described previously (22) with the following minor modification: AmpliTaq Gold Fast PCR master mix (Life Technologies, Grand Island, NY) was used instead of AmpliTaq DNA polymerase and supplemental magnesium.

RESULTS

Antimicrobial susceptibility test, MHT, Carba NP assay, and CRE PCR assay results for the validation set of organisms are presented in Table 1. The LunaProbe HRMA assay correctly identified the presence of and differentiated among the $bla_{\text{OXA-48-like}}$ β-lactamase genes in these control organisms. In contrast, none of the isolates harboring a non-OXA-48-like carbapenemase gene yielded a signal in the LunaProbe HRMA assay (data not shown). Of note, there was no PCR amplification for the *A. baumannii* isolates carrying non-$bla_{\text{OXA-48-like}}$ β-lactamase genes (Table 1). As expected, isolates positive for $bla_{\text{OXA-181}}$, $bla_{\text{OXA-204}}$, and $bla_{\text{OXA-232}}$, all of which contained 5 nucleotide mismatches in the forward primer binding region (data not shown), yielded no signals in the multiplex CRE PCR assay (Table 1), whereas both $bla_{\text{OXA-48-like}}$ and $bla_{\text{OXA-162}}$ genes were detectable as $bla_{\text{OXA-48}}$.

Differences in the peaks and shapes of the normalized and temperature-shifted melt curves were observed between $bla_{\text{OXA-48}}$ and its variants in both the PCR product and ssDNA-LunaProbe duplex melt regions; $bla_{\text{OXA-48}}$/$bla_{\text{OXA-245}}$, $bla_{\text{OXA-181}}$/$bla_{\text{OXA-204}}$/$bla_{\text{OXA-232}}$, $bla_{\text{OXA-162}}$, and $bla_{\text{OXA-244}}$ were differentiated by their respective melting profiles in the PCR product region (Fig. 2A). Further discrimination of $bla_{\text{OXA-181}}$/$bla_{\text{OXA-204}}$ and $bla_{\text{OXA-232}}$, which differ by just one base pair (A to T) in the PCR amplicon,

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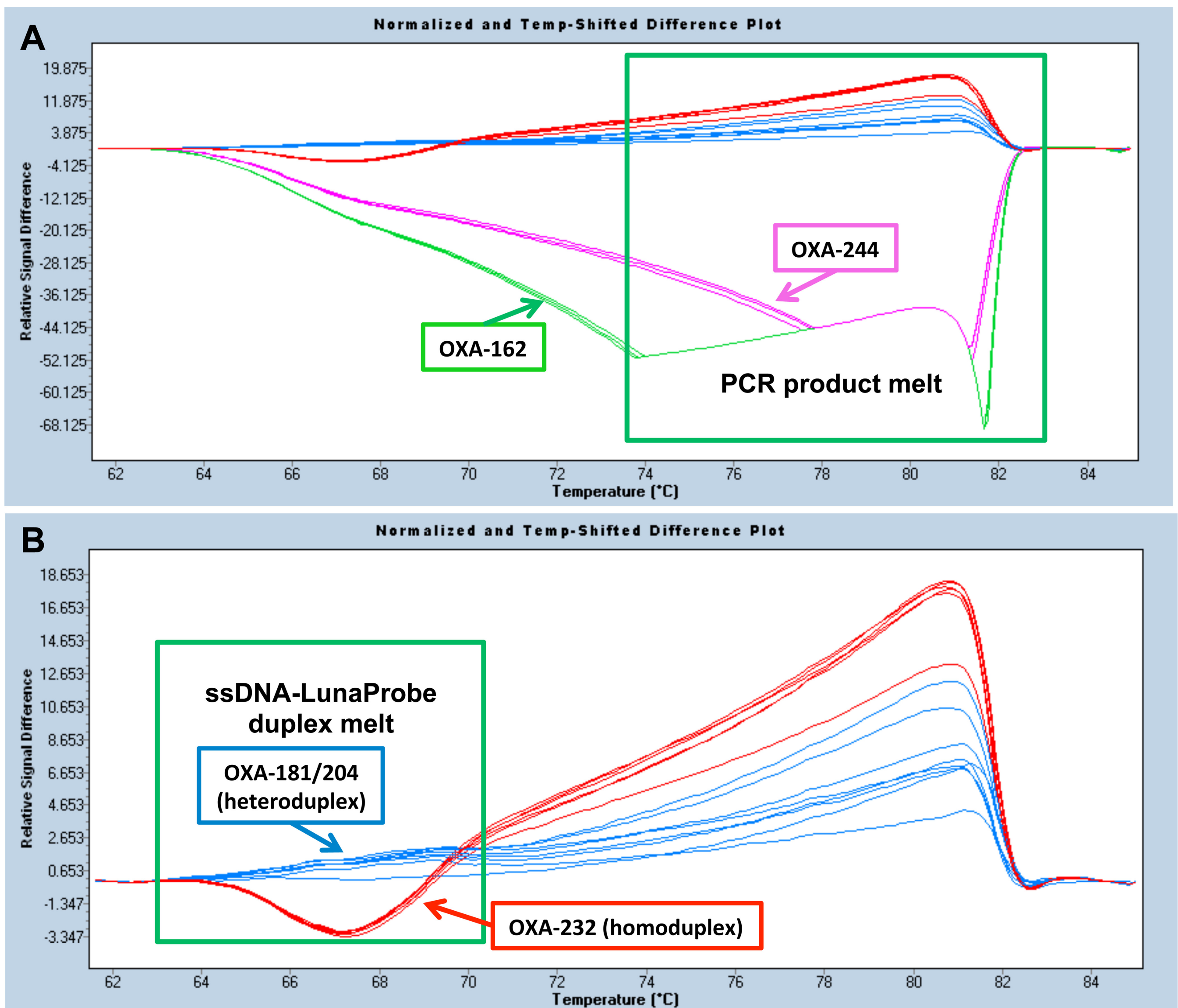

FIG 2 Normalized temperature-shifted difference plots, demonstrating different PCR product and ssDNA-LunaProbe duplex melting profiles for replicates of $bla_{\text{OXA-48-like}}$ genes. (A) Differentiation of $bla_{\text{OXA-162}}$ and $bla_{\text{OXA-244}}$ based on PCR product melting profiles. (B) Differentiation of $bla_{\text{OXA-181/204}}$ and $bla_{\text{OXA-232}}$ based on ssDNA-LunaProbe duplex melting profiles. All plots were calculated using $bla_{\text{OXA-48}}$ as a reference.

was possible through evaluation of the melting profiles of the ssDNA-LunaProbe duplexes for these genes. Figure 2B demonstrates how the homoduplex formed by the $bla_{\text{OXA-232}}$-homologous LunaProbe and the $bla_{\text{OXA-232}}$ amplicon generated a different melting peak from that of the heteroduplex formed by the LunaProbe and the $bla_{\text{OXA-181}}$ or $bla_{\text{OXA-204}}$ amplicon. This differentiation could not have been accomplished using conventional HRMA of the PCR amplicon alone. Results from the LunaProbe HRMA assay were all confirmed by Sanger sequencing (Table 1). These observations suggested that our real-time PCR assay was effective in identifying and differentiating $bla_{\text{OXA-48-like}}$ β-lactamase genes in CRE isolates.

To demonstrate the application of our assay for surveillance of CRE in our institution, we tested clinical isolates from our recent study (22) that were negative for carbapenemase genes detectable by our multiplex real-time PCR assay (Table 2). All CRE isolates collected prior to September 2014 were negative for $bla_{\text{OXA-48-like}}$ β-lactamase genes. In contrast, *K. pneumoniae* isolates obtained from 2 patients in October 2014 were positive for $bla_{\text{OXA-232}}$, including the first isolate identified by WGS analysis (Tables 1 and 2). One patient had received health care in India recently, whereas the second patient had no travel history to suggest acquisition of the $bla_{\text{OXA-232}}$-carrying *K. pneumoniae* strain abroad. No epidemiological link between these two patients could be identified, despite exhaustive evaluation. Together, these data suggest recent emergence of $bla_{\text{OXA-232}}$ in our institution and/or region.

DISCUSSION

Rapid intercontinental spread of class D β-lactamases has been described (30). The first OXA-48-like β-lactamase was identified

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TABLE 2 LunaProbe HRMA results for strains isolated at UCLA between January 2011 and September 2014 that were negative for carbapenemase genes by real-time multiplex PCR assay

Organism	Strain	Assay result			MIC (μg/ml)[a]		
		MHT	Carba NP	LunaProbe HRMA	IPM	MEM	ETP
Enterobacter cloacae	UCLA-MR6141	+	−	−	1	0.5	2
Enterobacter aerogenes	UCLA-MR6143	+	−	−	4	2	1
Klebsiella pneumoniae	UCLA-MR6161	−	−	−	>8	>16	>4
E. cloacae	UCLA-MR6167	−	−	−	1	1	4
Citrobacter freundii	UCLA-MR6185	−	−	−	2	0.5	4
Enterobacter spp.	UCLA-MR6241	−	−	−	8	4	>4
E. aerogenes	UCLA-MR6272	−	−	−	8	4	>4
E. aerogenes	UCLA-MR6285	−	−	−	8	2	>4
K. pneumoniae	UCLA-MR6287	+	−	−	>8	>16	>4
E. aerogenes	UCLA-MR6293	+	−	−	8	4	>4
K. pneumoniae	UCLA-MR6325	−	−	−	1	2	>4
K. pneumoniae	UCLA-MR6352	−	−	−	8	1	>4
E. cloacae	UCLA-MR6356	+	−	−	4	4	>4
E. aerogenes	UCLA-MR6379	−	−	−	4	1	>4
Escherichia coli	UCLA-MR6411	−	−	−	>8	>16	>4
E. aerogenes	UCLA-MR6414	+	−	−	2	2	>4
K. pneumoniae	UCLA-MR6417	+	−	−	8	4	>4
E. aerogenes	UCLA-MR6466	−	−	−	8	2	>4
E. aerogenes	UCLA-MR6467	+	−	−	4	2	>4
K. pneumoniae	UCLA-14-36-14	+	−	$bla_{OXA-232}$	>8	>16	>2

[a] IPM, imipenem; MEM, meropenem; ETP, ertapenem.

in 2001 from a *K. pneumoniae* isolate obtained from a urine specimen collected in Istanbul, Turkey (16). Over the following decade, however, organisms carrying $bla_{OXA-48-like}$ genes quickly spread to India and several countries in Europe, the Middle East, and North Africa, rendering these countries reservoirs for isolates producing these enzymes (15, 30); $bla_{OXA-232}$ was isolated first in France and now is common in India (31), and a *K. pneumoniae* isolate harboring the gene was identified in the United States in 2013 (21). Considering how quickly KPC-producing strains have become endemic in the United States (32), there is a real threat for the dissemination of OXA-48-like β-lactamases across the country in coming years. Although OXA-48 β-lactamase and its variants typically have low-level hydrolytic activity against many carbapenems, they can contribute to high-level carbapenem resistance in combination with other mechanisms (17). With this imminent threat, recognition of OXA-48 and its variants is vital in order to control their dissemination at the local, regional, and national levels.

Early recognition of these isolates can be difficult, however. As shown here, $bla_{OXA-232}$ was not identified by our laboratory-developed CRE PCR assay, and none of the isolates harboring OXA-48-like genes was positive by the phenotypic Carba NP assay, a tool that is commercially available and may be adopted by clinical laboratories as a means to screen for the presence of carbapenemases. While many OXA-48-like carbapenemase-producing CRE either are susceptible to broad-spectrum cephalosporins or have only low-level carbapenem resistance (15), these differences do not reliably distinguish OXA-48-like from the more commonly encountered KPCs (21). Moreover, the cooccurrence of various other β-lactamases in the same clinical isolates could affect the overall susceptibility of the organisms to β-lactams, making phenotypic differentiation of bla_{OXA-48} variants difficult or impossible (31). Indeed, the two $bla_{OXA-232}$ isolates encountered at our institution were resistant to all cephalosporins tested (ceftriaxone, ceftazidime, and cefepime) (data not shown) and were resistant to ertapenem, imipenem, and meropenem (Tables 1 and 2). In contrast, because the level of carbapenem resistance in these isolates can be low (33), it may be overlooked in routine antimicrobial susceptibility testing. PCR screening assays for bla_{OXA-48} and its variants in CRE have been reported in several studies, as single-target PCR assays (16, 34, 35) or as part of multiplex assays that also detected other carbapenemase genes (22, 36–38). The DNA microarray technique has also been used to identify bla_{OXA-48} and other commonly encountered carbapenemases (39). However, no molecular assay has been developed for rapid detection and accurate differentiation of highly homologous $bla_{OXA-48-like}$ genes.

Our $bla_{OXA-48-like}$ PCR not only detects the presence of $bla_{OXA-48-like}$ carbapenemase genes but also distinguishes between $bla_{OXA-48/245}$, $bla_{OXA-162}$, $bla_{OXA-244}$, $bla_{OXA-181/204}$, and $bla_{OXA-232}$ without the need to perform sequencing. HRMA has been successfully used for genotyping and for determination of single-nucleotide polymorphisms (SNPs) in both research and clinical settings (40–42), with exceptional sensitivity and specificity (43). However, only a few studies have utilized HRMA in bacterial genotyping to distinguish rapidly among sequences with high levels of homology (44–48), and only one other study used HRMA to detect single-nucleotide mutations (A to G) in bacteria (44). For our study, in order to determine confidently the presence of $bla_{OXA-232}$-carrying CRE in our institution, it was crucial that the assay be able to discriminate between $bla_{OXA-232}$ and other bla_{OXA-48} variants. The nucleic acid sequences of $bla_{OXA-181/204}$ and $bla_{OXA-232}$ differ by only 1 bp (A to T at position 642), causing an amino acid mutation (R214S) (31). This mutation results in decreased hydrolytic activity of the enzyme against carbapenems but increased activity against penicillins. This single-base-pair substitution is exceptionally challenging to detect by PCR, due to

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the subtle difference in the melting temperatures of amplicons from the two alleles. The use of HRMA coupled with LunaProbe analysis, along with our PCR design to generate a small amplicon, contributed to the success of the assay in determining the subtle difference between the nucleic acid sequences of these two variants. This LunaProbe assay, with our multiplex real-time PCR panel for carbapenemase genes, allows comprehensive screening of CRE isolates and rapid identification of $bla_{\text{OXA-48-like}}$ genes at our institution.

In conclusion, we have successfully developed an assay that is capable of identifying and distinguishing different variants of $bla_{\text{OXA-48-like}}$ β-lactamase genes. Our PCR assay has the potential for use in epidemiological investigations and continuous surveillance to help control the spread of CRE carrying $bla_{\text{OXA-232}}$.

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