

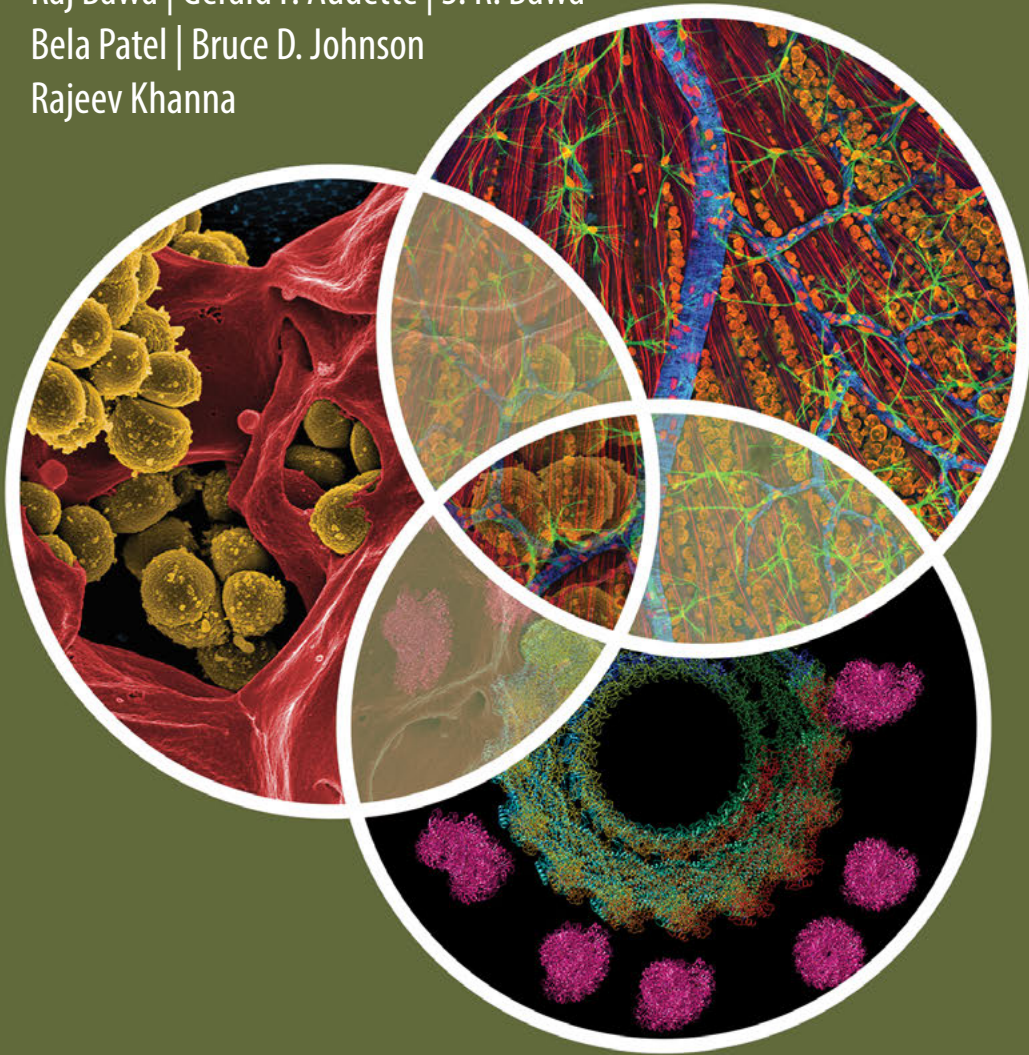
# ADVANCES IN MEDICAL IMAGING, DETECTION, AND DIAGNOSIS

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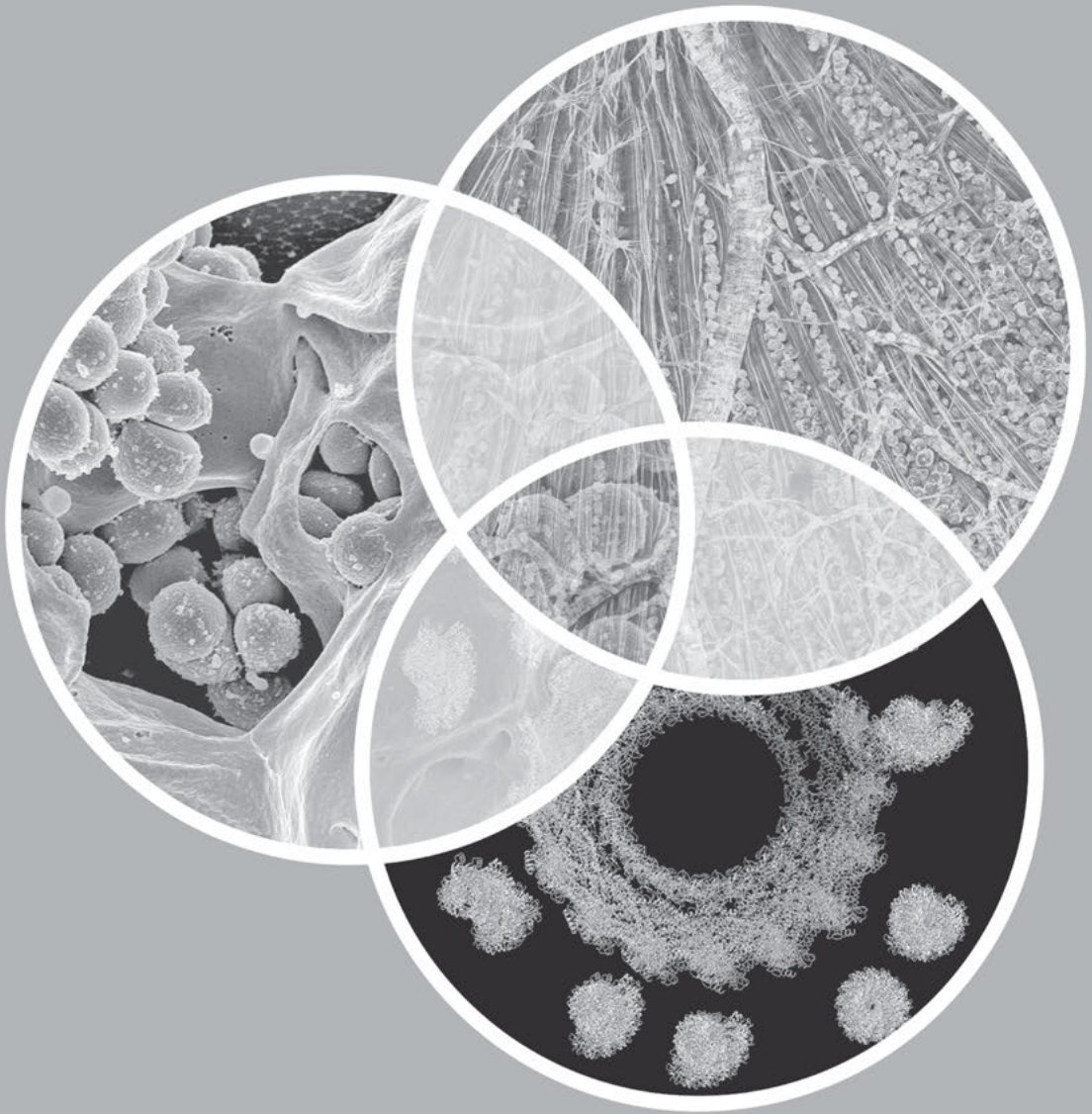
Raj Bawa | Gerald F. Audette | S. R. Bawa

Bela Patel | Bruce D. Johnson

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# ADVANCES IN MEDICAL IMAGING, DETECTION, AND DIAGNOSIS



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Current Issues in Medicine Vol. 4

# **Advances in Medical Imaging, Detection, and Diagnosis**

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Extensive efforts have been made to make the information provided herein as accurate and up-to-date as possible. It is important to note that knowledge and best practices in the various fields represented in this book (pathology, clinical microbiology, vaccines, nutrition, surgical tools and procedures, stem cell research, biochemistry, drug delivery, nanomedicine, precision medicine, genomics, tissue engineering, pharmaceutical sciences, etc.) are constantly evolving. This book is no substitute for individual patient assessment based on health care professionals' examination of each patient and consideration of specific factors unique to that patient. These include, but are not limited to, the following: age, weight, height, gender, current and past medical history, family medical data, laboratory data, etc. Therefore, it is imperative that the reader not rely solely on the information presented herein. The reader should always consult: (i) appropriate medical professionals (physicians, dentists, pharmacists, licensed healthcare professionals, etc.), (ii) federal agencies (FDA, CDC, NIH, NIOSH, CPSC, etc.), and/or (iii) product manufacturer, including drug/device product labels (regarding use, warnings, directions, etc.) before consuming any drug product, using any medical device, selecting any diagnostic procedure, or undergoing any surgery. To the fullest extent of the law, the publisher, the editors, and the authors make no representations or warranties, express or implied, with respect to the information presented in this book, for its use or misuse, or interpretation thereof. In this regard, they assume no liability for any injury and/or damage to persons or property as a matter of product liability, negligence, or otherwise.

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### **Advances in Medical Imaging, Detection, and Diagnosis**

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# Dedication

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It is an honor and privilege to dedicate this volume to our friend, colleague, mentor, and collaborator

## **Prof. Howard E. Gendelman, MD**

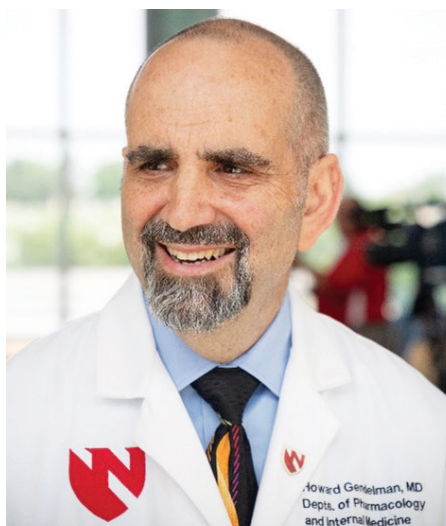
For demonstrating the determination and ingenuity to not only revolutionize the field of neuroimmunology but to develop the cross section between pharmacology and neuroscience in the novel therapies for neurodegenerative disorders.

For his pioneering leadership and ceaseless energy as a physician-scientist, and as a mentor to countless young professionals dedicated to building the next generation of scientific leaders.

For his innovative research in therapeutics for Parkinson's and Alzheimer's diseases that serve to slow or improve clinical disease outcomes.

For the co-development of ultra-long-acting antiretroviral and CRISPR excision therapies for treatment, prevention, and elimination of viral infections.

And for serving as a tenacious advocate for building collaborations, and being an inspirational role-model of the power of never accepting 'no' as the final answer.  
Today but not tomorrow.



*Margaret R. Larson Professor of Internal Medicine and Infectious Diseases  
Professor and Chair, Department of Pharmacology and Experimental Neuroscience  
Head, Carol Swarts MD Laboratory of Innovative Neuroscience  
Co-Director of the Center for Neurodegenerative Disorders  
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## The Editors



**Raj Bawa, PhD, MD**, is president of Bawa Biotech LLC (founded in 2002), a biotech/pharma consultancy and patent law firm based in Ashburn, Virginia, USA. Trained as a microbiologist and biochemist, he is an inventor, author, entrepreneur, professor, and registered patent agent (since 2002) licensed to practice before the US Patent & Trademark Office. He is currently a scientific advisor to Teva Pharmaceutical Industries, Israel (since 2010), and full professor (adjunct) at Northern Virginia Community College, Annandale, Virginia (since 2004). He is vice president and chief IP officer at Guanine, Inc., Rensselaer, New York (since 2017), a company focused on rapid, accurate detection of infective pathogens. He has served as a principal investigator of various National Cancer Institute (NCI) research grants; most recently as a principal investigator of a Centers for Disease Control and Prevention (CDC) grant to develop an assay for *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria. He was an adjunct professor at Rensselaer Polytechnic Institute, Troy, New York, from 1998 to 2018. After earning a BSc (Honors School) in microbiology, he earned an MS in cancer biology, a PhD in biophysics/biochemistry, and an MD. In the 1990s, Dr. Bawa held various positions at the US Patent & Trademark Office, including primary examiner from 1996–2002. Currently, he is a life member of Sigma Xi, cochair of the nanotech and precision medicine committees of the American Bar Association, and founding director of the American Society for Nanomedicine (established in 2008). He has authored over 100 publications, edited 10 texts, and serves on the editorial boards of numerous peer-reviewed journals, including serving as an associate editor of *Nanomedicine* (Elsevier).



**Gerald F. Audette, PhD**, is associate dean, Faculty of Science, professor of chemistry, and member of the Centre for Research on Biomolecular Interactions at York University, Toronto, Canada. His research focuses on the correlation between protein structure and biological activity of proteins involved in bacterial conjugation, in particular, the type 4 secretion system from the conjugative F-plasmid of *Escherichia coli*. In addition, his research targets the type IV pilins and associated assembly systems from multiple bacterial pathogens and is exploring the adaptation of these protein systems for applications in bionanotechnology and nanomedicine. Dr. Audette is the co-editor of volumes 1–4 of the *Jenny Stanford Series on Nanomedicine* and is a subject editor of structural chemistry and crystallography for the journal *FACETS*.



**S. R. Bawa, MSc, PhD**, is currently scientific advisor at Bawa Biotech LLC, a biotechnology and patent law firm founded in 2002 and based in Ashburn, Virginia. Previously, he was Founding Chairman and Professor of Biophysics at Panjab University, Chandigarh, India (1964–1992). At Panjab University, he also served as Dean of Foreign Students (1986–1988) and Coordinator of the Biotechnology Center (1986–1988). He was president of the

Electron Microscopy Society of India (1986–1992), Secretary of the Indian Biophysical Society (1986–1988), and Founding Secretary of the Northern India Science Association (1966–1992). Dr. Bawa received his BSc (University Gold Medal), MSc (University Gold Medal), and PhD degrees in 1949, 1951, and 1954, respectively, from Panjab University. He was a Fulbright Fellow and Instructor (1958–1960) and a Boese Postdoctoral Research Fellow (1959–1960), both at Columbia University. He was an Instructor (1961–1963) in the Department of Anatomy at Cornell University Medical College. In 1964, at the age of 34, he assumed the position of Founding Head and Reader of the newly established Biophysics Department at Panjab University, Chandigarh, India. He was promoted to Professor and Head in 1969. After retiring from Panjab University in 1992, Dr. Bawa joined the David Axelrod Institute of the New York State Department of Health in Albany, New York, from where he retired in 1999. Dr. Bawa has published over 150 scientific papers in peer-reviewed journals, books, and conference proceedings. His numerous accolades include Alexander von Humboldt Fellowship, Germany (five times); Fulbright Fellowship, US; US Alumni Research Travel Grant, US; Boese Postdoctoral Fellowship, Columbia University, US; British Council Invitee, UK; Diatome Award of the Electron Microscope Society of America; PL-480 Research Project and Appreciation Award, US Department of Agriculture; Kazato Research Award, Japan; and National Lectureship, India. He is an elected member or life member of various professional societies and organizations. He has served on various international scientific committees, advisory boards, government expert panels, and held visiting professorships in the US, Canada, and Europe. He has been a member of various peer-reviewed international journal editorial boards, including *Ultramicroscopy* (1986–1995, Elsevier), *Andrologia* (1993–1995, Blackwell/Wiley), *Acta Anatomica* (1974–1977, Karger), *Journal of Ultrastructure Research* (1969–1985, Elsevier) and *Journal of Submicroscopic Cytology* (1970–1977, Università di Bologna). Since 2004, the *Dr. S. R. Bawa Merit Scholarship* is awarded by Panjab University to a student standing first in the BSc (Honors School) class in biophysics. In 2022, Panjab University presented Dr. Bawa the *Distinguished Alumnus Award*.





**Bela Patel, MD**, is a professor of medicine, vice dean of Healthcare Quality, and division director for Pulmonary, Critical Care and Sleep Medicine at the McGovern Medical School at the University of Texas Health Science Center in Houston. She is regional chief medical officer and executive medical director of Critical Care for Memorial Hermann Hospital Texas Medical Center. Dr. Patel attended the University of Texas in Austin and the University of Texas McGovern Medical School in Houston. She completed her training in internal medicine in 1996 and subsequently a fellowship in pulmonary, critical care and sleep medicine in 1999 also at the University of Texas Health Science Center in Houston. Dr. Patel was appointed as the chief of Pulmonary, Critical Care and Sleep Medicine in 2002 and later appointed the chief of Medicine at Lyndon B. Johnson General Hospital and vice chair of the Department of Medicine in 2007. In 2009, she became the assistant chief medical officer and subsequently regional chief medical officer in 2017 for Memorial Hermann Hospital Texas Medical Center. Through her work in quality and patient safety, she was inducted as a fellow in Clinical Safety and Effectiveness by the University of Texas System. Dr. Patel was appointed as the assistant dean of Healthcare Quality in 2011 and the vice dean in 2017 and supports the 18 vice chairs of quality in Medicine at McGovern Medical School. Dr. Patel's research interest includes sepsis, ARDS, pulmonary hypertension, cognitive complexity and error reduction in critical care, and quality improvement implementation.



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**Rajeev Khanna, MD**, is an internal medicine consultant practicing in a group practice in Northern Virginia, as a part of Loudoun Medical Group. He is an internist with interest in long-term management of chronic health problems such as diabetes, high blood pressure, heart disease, and heart failure. He obtained his MBBS and MD in internal medicine from Dayanand Medical College, Ludhiana, Punjab, India, Dr. Khanna was in training for neurology postgraduation (DM) at the Postgraduate Institute of Medical Education and Research, Chandigarh, India before moving to the United States. He completed his residency in internal medicine at PG Hospital Center, Maryland, USA, in 1994. He has been published in multiple peer-reviewed journals.

## Note from the Series Editor

A hallmark of medicine is that it is continuously evolving, its knowledge base continuously expanding. Clearly, the pace and sophistication of advances in medicine in the past two decades have been truly breathtaking. This has necessitated a growing need for a comprehensive reference that highlights the current issues in specific sectors of medicine. Keeping this in mind, each volume in the *Current Issues in Medicine series* is a stand-alone text that provides a broad survey of various critical topics in a focused area of medicine—all accomplished in a user-friendly yet interconnected format. The series not only highlights current issues and advances but also explores related topics such as translational medicine, precision medicine, nanomedicine, regulatory science, neglected global diseases, emerging pandemics, FDA and patent law, immunotoxicology, theranostics, big data, artificial intelligence, novel medical instrumentation, clinical procedures, combination drug products, and novel therapies. While bridging the gap between basic research and clinical medicine, this series provides a thorough understanding of medicine's potential to address health problems from both the patient's and the provider's perspectives in a healthcare setting. Each volume is an excellent resource for medical practitioners, medical students, nurses, fellows, residents, undergraduate and graduate students, educators, venture capitalists, policymakers, and biomedical researchers. The multidisciplinary approach of the series makes it a valuable reference for health care systems, the pharmaceutical and device industry, academia, and governments. However, unlike other series on medicine or medical texts, this series focuses on current trends, perspectives, and critical issues in medicine that are central to healthcare delivery in the 21<sup>st</sup> century.

The first two volumes in this series focus on the current issues in basic medical sciences, subjects that are fundamental to the practice of medicine. These subjects, traditionally taught in the first two years of medical school that precede clinical instruction, provide a core of basic knowledge crucial for the success in clinical medicine during rotations, training, and medical practice. The subsequent volumes are dedicated to clinical topics or specialties in medicine. A separate volume on medical history and another on perspectives/editorials are part of the series.

Medical imaging, detection, and diagnosis have all aided in treatment and prevention of disease throughout human history. Technological innovations in these critical sectors of medicine continue to provide for safer, more accurate and faster diagnosis for patients. They have revolutionized medicine. Hence, it is critical that practitioners stay current with these latest advances to provide the best care for nursing and clinical practices. Given this backdrop, the current volume focuses on the tools, technologies, techniques, and testing protocols related to diagnostics and imaging that currently impact medicine. Fundamental coverage

on the use of technology in clinical practice is highlighted. The range of topics covered here and the expertise of the contributing authors accurately reflect the rapidly evolving areas within medical diagnostics and imaging.



**Raj Bawa, PhD, MD**  
*Series Editor*

## Chapter 26

# Carbapenem-Resistant *Enterobacteriaceae* Testing in 45 Minutes Using Oligonucleotide Detection Tags

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**Keywords:** 8-oxoguanine,  $\beta$ -lactam antibiotics, amperometry, antibiotic resistance, carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE), DNA biosensor, electrochemical detection, electrochemistry, guanine, imipenemase (IMP), isolation culture, *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo- $\beta$ -lactamase (NDM), oligonucleotide detection tags, oxacillinase-48 (OXA-48), point of care (POC), quadruplex, Verona integron-encoded metallo- $\beta$ -lactamase (VIM)

## 26.1 Introduction

Antibiotic-resistant bacterial infections are becoming a public health crisis in the United States and worldwide. In the United States alone, antibiotic-resistant bacteria are responsible for at least 2.8 million infections and 35,000 deaths annually [1]. The Centers for Disease Control and Prevention (CDC) has led a series

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of actions involving education, surveillance, and antibiotic stewardship that have reduced antibiotic-resistant infections and deaths by 18% in communities and 28% in hospitals. The breakdown of infections and deaths is supplied in the CDC report for seven antibiotic-resistant bacteria for 2012 and 2017 and is summarized in Table 26.1. The data show that the CDC actions succeeded in reducing illnesses and deaths across all antibiotic-resistant bacteria, except for *Enterobacteriaceae* such as *Klebsiella pneumoniae* and *Escherichia coli*. Of particular concern are infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE) since carbapenems are considered the drug of last resort with limited new treatments on the horizon [2].

**Table 26.1** Changes in infections and deaths from antibiotic resistant bacteria in 2012 and 2017

Antibiotic-resistant bacteria	2012 infections	2017 infections	% Variance	2012 deaths	2017 deaths	% Variance
<i>Enterobacteriaceae</i> increases in infections and deaths						
ESBL-producing <i>Enterobacteriaceae</i>	131,900	197,000	49%	6,300	9,100	44%
Carbapenem-resistant <i>Enterobacteriaceae</i>	11,800	13,100	11%	1,000	1,100	10%
<i>Non-Enterobacteriaceae</i> decreases in infections and deaths						
Methicillin-resistant <i>Staphylococcus aureus</i>	401,000	323,700	-19%	13,600	10,600	-22%
Drug-resistant <i>Candida</i>	44,800	34,800	-22%	2,200	1,700	-23%
Carbapenem-resistant <i>Acinetobacter</i>	11,700	8,500	-27%	1,000	700	-30%
Drug-resistant <i>Neisseria gonorrhoeae</i>	46,000	32,600	-29%	3,900	2,700	-31%
Vancomycin-resistant <i>Enterococcus</i>	84,800	54,500	-36%	8,500	5,400	-36%

Note: Data based on the CDC Report [1].

Abbreviation: ESBL, extended-spectrum beta-lactamases.

Carbapenems are highly effective and are often the only treatment option for severe bacterial infections. Some CREs possess genes that produce carbapenemase enzymes including KPC, New Delhi metallo- $\beta$ -lactamase (NDM), oxacillinase-48 (OXA-48), Verona integron-encoded metallo- $\beta$ -lactamase (VIM), and imipenemase (IMP) [3]. These carbapenemase enzymes allow CREs to survive carbapenem treatments by hydrolyzing the carbapenem's  $\beta$ -lactam ring thus deactivating the molecule's antibacterial properties. Some CREs have developed additional resistance mechanisms including redundant  $\beta$ -lactamases, genes conferring resistance to other antimicrobial classes, chromosomal porin mutations that prevent accumulation of  $\beta$ -lactam agents, and over-expression of efflux pumps that



extrude  $\beta$ -lactam agents [4–6]. Carbapenemases are commonly expressed from mobile genetic elements such as plasmids or transposons, which can be acquired and passed on through horizontal gene transfer, making CP-CREs extremely virulent and highly resistant to any therapy.

Timely treatment of CP-CRE infections is critical and is most successful when administered at the early stage of the infection when the bacteria are at a low concentration. A study of over 50,000 patients hospitalized with complicated urinary tract infections, complicated intra-abdominal infection, hospital-associated pneumonias, or bloodstream infections determined that CRE patients whose treatment is delayed by 2 or more days have double the hospital costs and a fourfold increased risk of mortality compared with timely treatment (Table 26.2) [7–9].

**Table 26.2** Impact of delayed therapy on CRE infection

Infection	Timely appropriate therapy	Delayed appropriate therapy by 2+ days
<b>Hospital cost/patient</b>		
CRE infection	\$9,875	\$25,506
Non-CRE infection	\$11,539	\$21,828
<b>Risk of mortality or discharge to hospice</b>		
CRE infection	0.9%	3.7%
<b>Hospital stay</b>		
CRE infection	5.1 days	8.5 days

Note: Data based on [7, 8].

Abbreviation: CRE, carbapenem-resistant *Enterobacteriaceae*.

Despite the need for timely treatment of CP-CRE infections, the current turnaround time for detecting CP-CRE, including all approved antimicrobial susceptibility testing (AST) assays for CRE, is 2 to 4 days as noted on Table 26.3. These include broth microdilution, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, real-time polymerase chain reaction (RT-PCR), microarray, multidisc mechanism testing, gradient minimum inhibitory concentration (MIC) strip (Etest), and Carba NP. Virtually all CP-CRE detection methods require a time-intensive bacteria culture to isolate and produce a measurable quantity of bacteria for testing as the minimum threshold of detection is well above concentrations found in human samples. CP-CRE detection tests also vary by their availability in clinical labs, accuracy, ease of use, cost, and need for specialized equipment [10–12]. Based on the increasing prevalence of antibiotic-resistant CRE infections [1], the rise of new resistance strains and the lack of new antimicrobials planned for the market, the best defense against antibiotic-resistant CRE would be a rapid, simple, inexpensive, and accurate test that could be used in essentially any healthcare setting.

**Table 26.3** Antimicrobial susceptibility testing methods for detecting CP-CRE

CP-CRE detection method	Time from sample to result		
	Isolation culture	Test process	Total
<b>Quadruplex hybridization assay</b>	<b>None</b>	<b>45 min</b>	<b>45 min</b>
Broth microdilution	1–2 days	1–2 days	2–4 days
MALDI-TOF	1–2 days	< 1 day	2–3 days
Real-time PCR	1–2 days	hours	2–3 days
Microarray	1–2 days	< 1 day	2–3 days
Multidisc mechanism testing	1–2 days	2 days	3–4 days
Gradient MIC strip (E-test)	1–2 days	1–2 days	2–4 days
Carba NP	1–2 days	<2 h	2–3 days

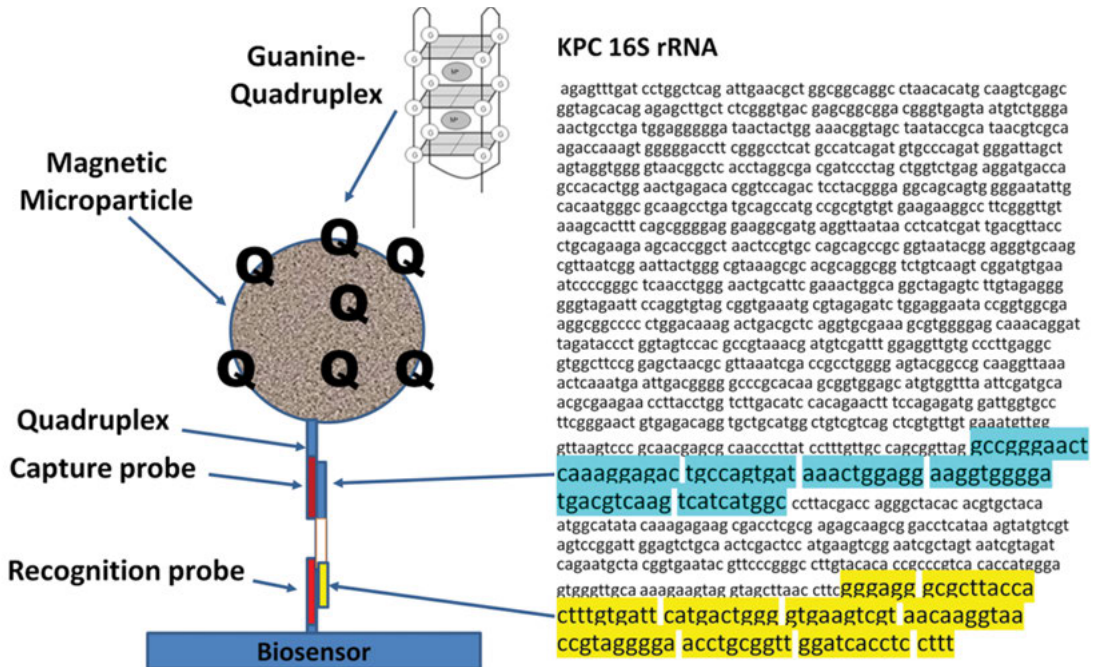
Note: Data based on [10–12].

Abbreviations: CP-CRE, carbapenemase-producing carbapenem-resistant *Enterobacteriaceae*; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction.

### 26.1.1 Approach

CP-CRE detection was performed using a sandwich hybridization assay. Nucleic acid targets were captured using a magnetic microparticle containing oligonucleotides with guanine detection tags and capture probes complementary to a segment of the target. A second portion of the target hybridizes to a recognition layer of oligonucleotides on a biosensor electrode (Fig. 26.1). A voltammetry technique produces an electrical signal from the redox properties of the detection tags bound to the particle. In order to overcome the poor detection limits currently encountered with nucleic acid biosensors, guanine nucleotides are employed as 20-mer polyguanine oligonucleotide detection tags and millions of detection tags are conjugated to the microparticle to amplify the detection signal. The 20-mer polyguanine oligonucleotides are pre-fabricated into guanine-quadruplexes which generate 8-oxoguanine oxidization signals at  $\sim 0.47$  V for improved signal-to-noise resolution over guanine oxidation signals. The microparticle has a magnetic core which allows the nucleic acid targets and detection tags to be magnetically separated from nonspecific materials that cause false detection signals and degrade the signal-to-noise resolution.

The detection method used in the experiments provides a new paradigm for electrochemical detection of nucleic acids [13–17]. Electrochemical detection is an appealing technique because of its rapid, simple, and inexpensive applications in measuring redox chemicals such as glucose. Electrochemical biosensors for detecting redox nucleotides such as guanine have not attained widespread appeal because of their inability to achieve low detection limits necessary for clinical applications.



**Figure 26.1** Quadruplex sandwich hybridization assay components [13, 14] with KPC 16S rRNA sequence used in hybridization assay.

## 26.2 Materials and Methods

### 26.2.1 Isolates

Study isolates included three quality control (QC) strains of CP-CRE that are available to clinical laboratories: *Klebsiella pneumoniae* ATCC BAA 1705 (KPC-2), *Klebsiella pneumoniae* ATCC BAA-2814 (KPC-3), and *E. coli* ATCC BAA 2340. Non-carbapenemase-producing *Klebsiella pneumoniae* ATCC 13883 was also evaluated. The study tested 28 samples comprising 22 carbapenemase-producing sample, 4 non-carbapenemase-producing samples, and 2 samples with no bacteria.

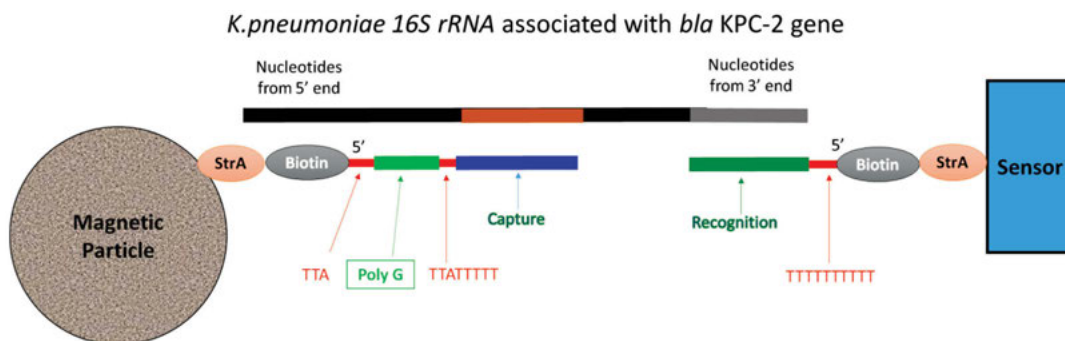
**Table 26.4** Identification of CP-CRE carbapenemase tested

Bacteria	Carbapenemase	Identification
<i>K. pneumoniae</i>	KPC-2	ATCC BAA-1705
<i>K. pneumoniae</i>	KPC-3	ATCC BAA-2814
<i>E. Coli</i>	KPC	ATCC BAA-2340
<i>K. pneumoniae</i>	None	ATCC 13883

**Abbreviations:** CP-CRE, carbapenemase-producing carbapenem-resistant *Enterobacteriaceae*; KPC, *Klebsiella pneumoniae* carbapenemase; KPC-2 *Klebsiella pneumoniae* ATCC BAA 1705; KPC-3, *Klebsiella pneumoniae* ATCC BAA-2814.

## 26.2.2 Probe Design

Detection probes were designed to hybridize with targets associated with the *bla*<sub>KPC</sub> gene. Suitable targets were identified as the 16S rRNA gene (GenBank: X87276.1) associated with strain ATCC BAA-1705 and corresponding to *bla*<sub>KPC-2</sub> and with strain ATCC BAA-2814 corresponding to *bla*<sub>KPC-3</sub> (ATCC, Manassas, VA). As illustrated in Fig. 26.2, nucleotide sequences of ~70 bases were used for capture and recognition. The recognition sequence was selected at the 3' end and the capture sequence was selected a few hundred bases from the 3' end. This configuration produced a larger oxidation signal peak than binding at the 5' end because the quadruplexes were closer to the biosensor. The capture probe also contained a 20-mer polyguanine sequence on the same oligonucleotide (Table 26.5). Polyguanine was transformed into guanine-quadruplexes by heating the oligonucleotides in a sodium acetate-formamide solution.



**Figure 26.2** A *K. pneumoniae* 16S rRNA associated with *bla*<sub>KPC-2</sub> forms a sandwich hybridization assay with a magnetic particle conjugated with a polyguanine capture probes and a biosensor conjugated with a recognition probe.

**Table 26.5** Sequences for detecting *bla*<sub>KPC-2</sub> gene from *K. pneumoniae* 16S rRNA

Target	Sequence ID.	RNA sequence	ssOligo Probe	
KPC-2 16s rRNA (ATCC BAA 1705)	GenBank: X87276.1	GCCGGGA ACT CAAAGGAGAC TGCCAGTGAT AACTGGAGG AAGGTGGGGA TGACGTCAAG TCATCATGGC	5' Biotin-TTA GGGGGGGGGGGGGGGGGGGG TTATTTTT CGGCCCTGA GTTTCCTCTG ACGGTCACTA TTTGACCTCC TTCCACCCT ACTGCAGTTC AGTAGTACCG 3'	Capture
		GGGAGG GCGTTACCA CTTTGTGATT CATGACTGGG GTGAAGTCGT AACAAGGTAA CCGTAGGGGA ACCTGCGGTT GGATCACCTC CTTT	5' Biotin-TT TTT TTT TT AAAG GAGGTGATCC AACCGCAGGT TCCCCTACGG TTACCTTGTT ACGACTTCAC CCCAGTCATG AATCACAAAG TGGTAAGCGC CCTCCC 3'	Recognition

Abbreviation: KPC-2 *Klebsiella pneumoniae* ATCC BAA 1705.

The recognition and capture probes are biotinylated. The capture probes were conjugated to streptavidin-coated magnetic microparticles (Bangs Laboratories, Fishers, IN) and the recognition probes were conjugated to unique working electrodes on a 96-well streptavidin-coated carbon biosensor microtiter (DropSens, Llanera, Spain) using streptavidin–biotin interactions.

### 26.2.3 Filter Concentration and Lysis

Bacteria were prepared in tryptic soy agar medium (Becton, Dickinson (BD), Franklin Lakes, NJ) and serially diluted in broth medium and commercial urine (Sigma-Aldrich, St. Louis, MO) to  $10^4$  cfu/mL. Samples were concentrated with a 0.45  $\mu\text{m}$  filter (Pall, Port Washington, NY) and re-suspended in biology-grade water and incubated at room temperature for 15 min. The  $\beta$ -lactam antibiotic meropenem was added to the resuspension to assess if meropenem caused a signal increase from the KPC-producing bacteria. Meropenem doses ranged from 0  $\mu\text{g/mL}$  to 2048  $\mu\text{g/mL}$ . Bacteria were then lysed in 200  $\mu\text{L}$  lysis buffer consisting of 2 M guanidinium thiocyanate (GTC), 80 mM beta-mercaptoethanol (BME), 25 mM sodium citrate, 20  $\mu\text{g/mL}$  of glycogen (pH 6) with 5  $\mu\text{L}$  dimethyl sulfoxide (1%) then incubated at room temperature (RT) for 5 min.

### 26.2.4 Magnetic Separation

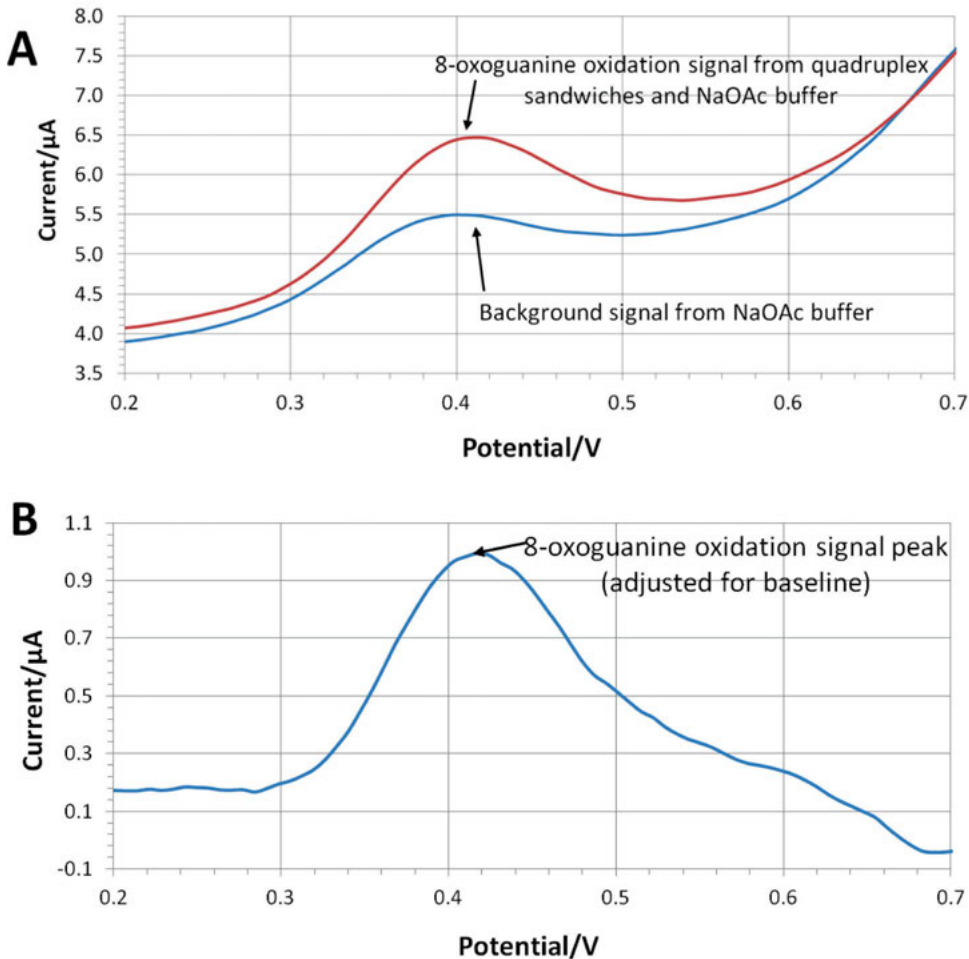
The solution was mixed for 5 min with 7  $\mu\text{L}$  of 1.5  $\mu\text{m}$  streptavidin-coated magnetic particles conjugated with quadruplex tags and DNA detection probes (Integrated DNA Technologies (IDT), San Jose, CA), then incubated at RT for 10 min. Samples were placed in a magnetic separation microtiter (Epigentek, Farmingdale, NY) and a magnet was applied for 2 min, then the supernatant was discarded. The magnet was removed and the magnetic particle complexes were washed with 100  $\mu\text{L}$  80 mM sodium acetate (pH 9). The magnetic particle complexes were then re-suspended in sodium acetate and allowed to hybridize for 10 min at RT on a streptavidin-coated carbon working electrode conjugated with capture probes and enable sandwich structures to form as illustrated in Fig. 26.1.

Prior to the experiments, different sized particles were evaluated for their corresponding 8-oxoguanine signals. The 100 nm and 350 nm magnetic particles (Micromod, Rostock, Germany) did not provide measurable 8-oxoguanine oxidation signals and the particles appeared to have decomposed during the test process. The 500 nm, 750 nm and 1.5  $\mu\text{m}$  streptavidin-coated magnetic microparticles (Bangs Laboratories, Fishers, IN) were all successfully able to measure 8-oxoguanine oxidation signals. The 1.5  $\mu\text{m}$  particles generated the strongest signals and were thus used in the experiments.

### 26.2.5 Amperometric Voltammetry

The biosensor microtiter was connected to a potentiostat (PalmSens). A square wave voltammetry scan produces a peak electrical current at  $\sim 0.47$  V from

8-oxoguanine oxidation in sodium acetate (pH 9) (Fig. 26.3A). A voltammetry scan was made with sodium acetate (pH 9) before the magnetic particle-RNA-biosensor sandwiches were formed to provide a baseline signal. The net 8-oxoguanine oxidation signal was determined to be the difference between the 8-oxoguanine oxidation signal in sodium acetate and sodium acetate signal (Fig. 26.3B).



**Figure 26.3** (A) Voltammetry scan of quadruplex 8-oxoguanine oxidation current peak in sodium acetate (pH 9) with a peak current signal at  $\sim 0.47$  V and the background signal from sodium acetate (pH 9) on the same sensor before the sandwiches were formed, and (B) the net signal calculated from the difference of the two scans in A.

The assay was initially developed with the 96-well carbon microtiter sensors and was coated in the lab with streptavidin. Each sensor was evaluated for a baseline signal with 80 mM sodium acetate (pH 9) to ensure proper sensor functionality. The 8-oxoguanine oxidation signal generated for the same sample varied by 2–10% between sensors. When the development work switched to



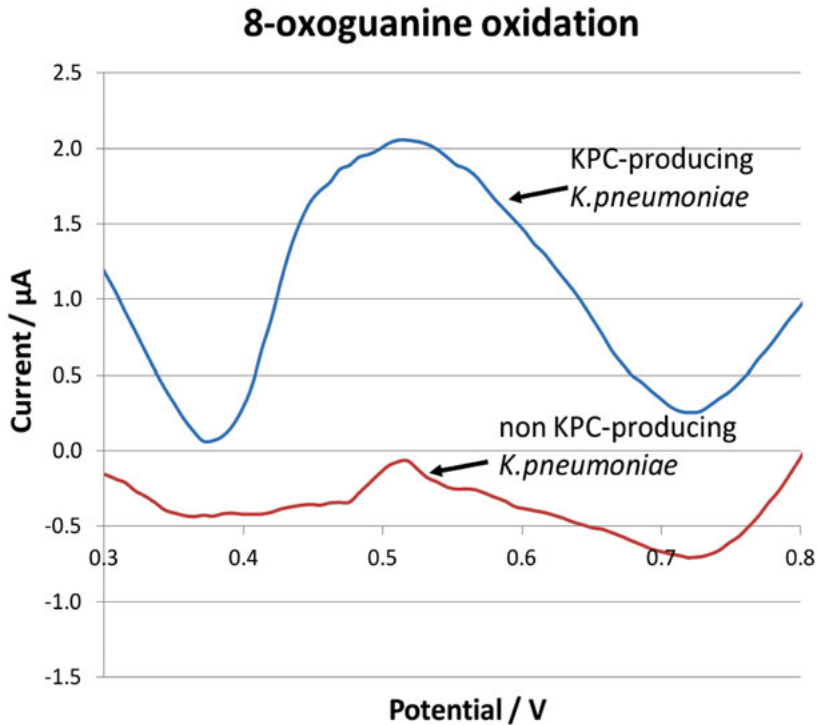
microtiter sensors pre-coated with streptavidin, the variability increased by up to 40%. Variability was reduced by subtracting the baseline sodium acetate buffer signal measured prior to hybridization, from the 8-oxoguanine oxidation signal measured on the same sensor. This reduced the same sample variation to 3–16% between sensors. The pre-coated microtiter sensor displayed a new peak at 0.15 V, which did not occur when streptavidin was coated in the lab. This new peak was likely due to oxidation from residual chemicals from the streptavidin coating process employed by DropSens. It was observed that the 0.15 V peak varied in amplitude on different sensors and in some cases 10 or more baseline scans were needed to remove the 0.15 V peak before the sensor could be used for hybridization.

Square wave voltammetry (SWV) scan parameters were optimized for 8-oxoguanine in advance of the KPC experiments by adsorbing quadruplex oligonucleotides on the sensor surface and applying SWV scans. Variations of SWV settings were evaluated to find the setting that produced the greatest 8-oxoguanine oxidation signal peak. This setting was then used for KPC assay development.

### 26.3 Results

Twenty-eight samples were tested for the presence of KPC enzymes from CP-CRE and non-CP-CRE. The results are summarized in Table 26.6. All 22 samples containing CP-CRE generated a positive signal which was greater than the 0.2  $\mu\text{A}$  background baseline. Of the 6 samples containing the susceptible *K. pneumoniae* or no bacteria, 5 reported true negative with the signal below the threshold. The normalized 8-oxoguanine oxidation signal peak at  $\sim 0.47$  V was used to detect the presence of carbapenemase KPC enzymes as illustrated in Fig. 26.4. One false positive occurred with a non-KPC-producing *K. pneumoniae* and was likely caused by poor sensor calibration.

The magnitude of the KPC signal was measured for each CP-CRE species. As shown in Table 26.7, all three species reported an average signal greater than the threshold but with different average signal magnitudes: 1.2  $\mu\text{A}$  for the KPC-2 species, 1.1  $\mu\text{A}$  for the KPC-3 species, and 0.4  $\mu\text{A}$  for the *E. coli* KPC species. The strain that did not produce KPC had an average signal of 0.1  $\mu\text{A}$ , which was below the threshold detection value. The magnitude of the KPC signal was also measured for samples resuspended in meropenem for the ATCC BAA 1705 (KPC-2) and ATCC BAA-2814 (KPC-3) species. High dose exposure between 512  $\mu\text{g/mL}$  and 2048  $\mu\text{g/mL}$  meropenem produced an average signal of 1.2  $\mu\text{A}$ , while low dose exposure between 0  $\mu\text{g/mL}$  and 128  $\mu\text{g/mL}$  (Table 26.8) meropenem produced an average signal of 1.1  $\mu\text{A}$ . Based on the test results, there appeared to be no relationship between meropenem exposure and the KPC signal. Testing with bacteria samples in broth and urine provided near identical results suggesting that filtered urine followed by magnetic separation had no impact on hybridization or 8-oxoguanine oxidation signal generation.



**Figure 26.4** Voltammetry scan of quadruplex 8-oxoguanine oxidation signal from KPC-producing and non-KPC-producing *K. pneumoniae* after baseline scan subtraction.

**Table 26.6** Summary of samples tested for KPC

	True outcome	False outcome	
<b>Positive test prediction</b>	22	1	PPV = 22/23
<b>Negative test prediction</b>	0	5	NPV = 5/5
	Sen. = 22/22	Spec. = 5/6	

Abbreviation: KPC, *Klebsiella pneumoniae* carbapenemase.

**Table 26.7** KPC signal amplitude by CP-CRE species

CP-CRE species	Average signal
ATCC BAA 1705 (KPC-2) (8)	1.2 µA
ATCC BAA-2814 (KPC-3) (12)	1.1 µA
ATCC BAA-2340 ( <i>E. coli</i> KPC) (2)	0.4 µA
ATCC 13883 (Non-KPC) (4)	0.1 µA
No Bacteria (2)	0.1 µA

Abbreviations: CP-CRE, carbapenemase-producing carbapenem-resistant *Enterobacteriaceae*; KPC, *Klebsiella pneumoniae* carbapenemase.

**Table 26.8** KPC signal amplitude by meropenem dose

Meropenem dose	Average signal
512–2048 µg/mL Meropenem (13)	1.2 µA
0–128 µg/mL Meropenem (7)	1.1 µA

Abbreviation: KPC, *Klebsiella pneumoniae* carbapenemase.

## 26.4 Discussion

The 2014 US National Strategy for Combating Antibiotic-Resistant Bacteria [18] identified a critical goal for improved diagnostics to detect antibiotic resistance profiles in 30 min or less for 18 bacteria of highest concern. This was intended to allow physicians to make optimal treatment decisions, and help public health officials prevent the transmission of disease and slow the development of resistance. Despite this goal most effective antibiotic-resistant diagnostic tests still take several days from specimen collection to results and can be extended by days or weeks if cultures are required. As a consequence treatment decisions are typically made before laboratory results are available. Patients may be initially treated with antibiotics when none are needed, prescribed an inappropriate antibiotic, or treated with multiple antibiotics when a single antibiotic would have been effective [18].

The objective of this study was to detect carbapenem-resistant *Enterobacteriaceae* directly from a sample without using a culture. The approach employed a modified lateral flow test protocol that captured nucleic acid targets from a KPC RNA sequence and formed a hybridization sandwich structure on a biosensor electrode. The novelty of the approach was to replace optical labels with a microparticle conjugated with millions of electrical quadruplex tags. The study investigated if rapid direct detection of KPC using quadruplex tags was able to produce sufficient sensitivity and specificity and avoid the need for a time-intensive culture.

The study demonstrated that three different CP-CRE bacteria tested positive in all 22 samples, and non CP-CRE samples tested negative in 5 of 6 samples. This study was the first use of quadruplex tags in a hybridization assay and as a consequence significant effort was spent optimizing the assay which prevented more samples from being tested.

Hybridization time and additional process steps for filtration, meropenem incubation, lysing and magnetic separation extended the test time to 45 min. Meropenem incubation was found to have no impact on carbapenemase production and could be removed from the protocol. The assay did not employ PCR so hybridization components were not limited in length to 20 mer sequences. It was found that probes with 70 mer sequences substantially reduced hybridization time and improved detection accuracy over shorter sequences.

The study employed bacteria concentrations of  $10^4$  cfu/mL. A further reduction in detection limit would be needed for clinical use. Lower concentration

samples containing 100 cfu/mL were successfully detected but not included in the study results. Further work will increase the magnetic particle size beyond 1.5  $\mu\text{m}$  diameter. A larger microparticle will deliver more quadruplex tags per KPC RNA complex and provide a measurable signal at lower concentrations. Further work will also employ a sample volume greater than 1 mL. Filter concentrating a larger sample volume will capture more bacteria for detection and achieve lower detection limits.

The study was limited to KPC and did not evaluate other carbapenemase enzymes. One of the advantages of electrochemical detection is the ability to detect a number of unique analytes from the same sample using multiple working electrodes. A commercial example is the Abbott i-STAT. Future work will detect KPC, NDM, OXA-48, VIM and IMP separately on individual electrodes to indicate which carbapenemase is present.

The quadruplex CP-CRE assay is intended to be used in a point-of-care cartridge without requiring the sample to be prepared with a culture. A culture-free CP-CRE test will allow appropriate treatment to be provided days earlier than with current tests. Not only will this improve patient outcomes, but the associated healthcare costs can be reduced. A POC test will provide the opportunity to fast track testing for CP-CREs by identifying patients who are at high risk of encountering a CP-CRE infection. A POC test can also be administered at a physician's office.

Another unmet need is rapid culture-free testing of extended-spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae*. ESBL-producing *Enterobacteriaceae* have over 10 times higher incidences than carbapenemase-producing *Enterobacteriaceae*, and incurred a 49% increase in infections and 44% increase in deaths *between 2012 and 2017*. A culture-free ESBL-producing *Enterobacteriaceae* test can complement the CP-CRE test to determine if carbapenem or ESBL treatment is more appropriate.

The study encountered some limitations which will be addressed in further development activities. The assay was tested with spiked broth and urine samples instead of patient specimens. Future work will test a wider range of KPC strains and CP-CRE organisms from actual patient specimens.

## 26.5 Conclusion

The study demonstrated that KPC from CP-CRE is accurately detected using quadruplex tags in 45 min without bacteria isolation, culture, or PCR. While the experiments were done with  $10^4$  cfu/mL spikes, high sensitivity and specificity will be required at lower detection limits for clinical use. All process steps are conducted at room temperature which will enable the test to be conducted in a point-of-care cartridge with a portable potentiostat.

The study also demonstrated that signal amplification using quadruplex tags could be an alternative to PCR and cultures for detecting bacteria and viruses since it is faster, easier, and less expensive to attach millions of tags than to replicate millions of copies.

The core components of the quadruplex detection approach are mostly generic. A new assay can be rapidly adapted to detect a nucleic acid target from a genome sequence of a RNA, DNA, or gene. Sensor electrodes and magnetic microparticle conjugates can be customized for new targets by conjugating the required probes. This allows a baseline assay to be rapidly developed and subsequently optimized for the required sample type and assay performance. Protein targets can use aptamers or antibodies in a sandwich immunoassay along with the quadruplex tags on the magnetic particle conjugates.

Many applications can benefit by detecting nucleic acid targets with quadruplex hybridization assays at the POC or in a resource-limited setting where PCR equipment, laboratory, and skilled operators are not available. Quadruplex hybridization assays conducted at the POC can avoid false-negative test outcomes caused by nucleic acid degradation from transporting samples to a laboratory, and from freezing and thawing processes when temporarily stored before sample preparation. Quadruplex tags with magnetic separation can also reduce the incidence of false-positive test outcomes by avoiding complex sample process steps and removing materials that interfere with detection.

Another limitation of nucleic acid amplification tests, including PCR, is the inability for the test to distinguish viable organisms from dead organisms. Pathogenic nucleic acids can remain in the body for weeks after the organisms are dead and produce a positive test outcome. This can incorrectly indicate that the patient requires treatment, can infect others or needs to be quarantined. In many cases a culture is the recommended test to determine if microorganisms are viable. However cultures can take several days or weeks for microorganisms to be isolated and cultured to produce a measurable optical signal or color change. The quadruplex test is quantitative and generates an electrical signal that is proportional to the number of analytes in the sample. A portion of the sample can undergo a rapid test to determine if the target microorganism is present. A new sample can be measured at a later time or a second portion of the original sample can be incubated at an elevated temperature with nutrients to allow a few reproduction cycles to produce a higher signal than the initial test if the target microorganisms are viable. Because of the sensitivity of the tags, as few as 4–6 reproduction cycles could be needed which is a fraction of the time of a traditional culture using insensitive dye or optical labels.

## Abbreviations

AST:	antimicrobial susceptibility testing
BME:	beta-mercaptoethanol
BSL3:	Biosafety Level 3
CDC:	Centers for Disease Control and Prevention
CLIA:	Clinical Laboratory Improvement Amendments
COVID-19:	Coronavirus disease 2019
CP-CRE:	carbapenemase-producing carbapenem-resistant <i>Enterobacteriaceae</i>
CRE:	Carbapenem-resistant <i>Enterobacteriaceae</i>

ESBL:	extended-spectrum beta-lactamases
GTC:	guanidinium thiocyanate
IMP:	imipenemase
KPC:	<i>Klebsiella pneumoniae</i> carbapenemase
KPC-2:	<i>Klebsiella pneumoniae</i> ATCC BAA 1705
KPC-3:	<i>Klebsiella pneumoniae</i> ATCC BAA-2814
MALDI-TOF:	matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MIC:	minimum inhibitory concentration
NDM:	New Delhi metallo- $\beta$ -lactamase
OXA-48:	oxacillinase-48
PCR:	polymerase chain reaction
POC:	point of care
QC:	quality control
RT:	room temperature
RT-PCR:	real-time polymerase chain reaction
SWV:	square wave voltammetry
VIM:	Verona integron-encoded metallo- $\beta$ -lactamase

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Medical care is the most critical issue of our time and will be so for the foreseeable future. In this regard, the pace and sophistication of advances in medicine in the past two decades have been truly breathtaking. This has necessitated a growing need for comprehensive reference resources that highlight current issues in specific sectors of medicine. Keeping this in mind, each volume in the *Current Issues in Medicine* series is a stand-alone text that provides a broad survey of various important topics in a focused area of medicine—all accomplished in a user-friendly yet interconnected format. This volume addresses advances in medical imaging, detection, and diagnostic technologies. Technological innovations in these sectors of medicine continue to provide for safer, more accurate, and faster diagnosis for patients. This translates into superior prognosis and better patient compliance, while reducing morbidity and mortality. Hence, it is imperative that practitioners stay current with these latest advances to provide the best care for nursing and clinical practices. While recognizing how expansive and multifaceted these areas of medicine are, *Advances in Medical Imaging, Detection, and Diagnosis* addresses crucial recent progress, integrating the knowledge and experience of experts from academia and the clinic. The multidisciplinary approach reflected makes this volume a valuable reference resource for medical practitioners, medical students, nurses, fellows, residents, undergraduate and graduate students, educators, venture capitalists, policymakers, and biomedical researchers. A wide audience will benefit from having this volume on their bookshelf: health care systems, the pharmaceutical industry, academia, and government.

#### About the Series Editor



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