

A METHOD OF DETECTING CARBAPENEM RESISTANT *ACINETOBACTER BAUMANNII*

TECHNICAL FIELD

5 The present disclosure generally relates to a method of detecting the presence of bacteria by DNA amplification. In particular, the present disclosure relates to a method of rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* by multiplex real-time polymerase chain reaction (RT-PCR). The present disclosure also relates to a test kit for rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* by
10 multiplex RT-PCR. The present disclosure further relates to a method of preparing the test kit.

BACKGROUND

15 The emergence of multidrug resistant bacteria has been a challenge to clinicians worldwide. One such multidrug resistant bacterium, carbapenem resistant *Acinetobacter baumannii*, is of great concern as it has showed resistance to almost all available antibacterial agents.

The management of carbapenem resistant *Acinetobacter baumannii* infection is a public
20 health problem in many countries. For example, carbapenem resistant *Acinetobacter baumannii* is often circulated or spread in hospitals resulting in the infection of immune-compromised patients and patients in intensive care units. Carbapenem resistant *Acinetobacter baumannii* is able to readily and rapidly acquire resistant genes from other bacteria genera and integrate the resistant genes into a 86kb resistant island called AbaR1
25 integrated into the ATPase gene. Generally, resistance to carbapenems is mediated by the OXA-type gene, which produces class D β -lactamases that hydrolyze the carbapenems.

Additionally, the current therapy for carbapenem resistant *Acinetobacter baumannii* infection utilizes the antibiotic colistin, which is nephrotoxic.

Currently used methods of detecting Carbapenem resistant *Acinetobacter baumannii* include the Kirby-Bauer disc diffusion test and conventional PCR. However, the Kirby-Bauer disc diffusion test requires two days to grow the bacteria and is prone to cross-contamination. Further, the results of the Kirby-Bauer disc diffusion test can be ambiguous and the detection of carbapenem resistant *Acinetobacter baumannii* can become complicated if the specimen contains a mixed culture.

The use of conventional PCR typically is a better alternative to the Kirby-Bauer disc diffusion test; however, conventional PCR requires post-PCR processing, such as, gel electrophoresis, which is time-consuming and laborious.

Thus, there is a need to provide a method and test kit for rapidly and accurately detecting the presence of carbapenem resistant *Acinetobacter baumannii* that avoid or at least ameliorate one or more of the disadvantages described above.

Additionally, there is a need to develop a method and test kit for rapidly and accurately detecting the presence of carbapenem resistant *Acinetobacter baumannii* for better patient management and to limit the spread of this multidrug resistant bacterium in hospitals.

SUMMARY

A first aspect of the present disclosure provides a method of detecting the presence of carbapenem resistant *Acinetobacter baumannii*, wherein the method can comprise: using a multiplex real-time Polymerase Chain Reaction (RT-PCR) assay to detect the presence of an *adeB* gene of *Acinetobacter baumannii*.

In some embodiments, the assay can be used for detecting the presence of an OXA gene. In some embodiments, the OXA gene can comprise OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.

In some embodiments, the method can further comprise at least one of designing, preparing and using a first primer comprising a nucleotide sequence of GCAATTGTGGCGCCTATTGCCTTAC.

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In some embodiments, the method can further comprise at least one of designing, preparing and using a second primer comprising a nucleotide sequence of GCCGCCAATACCAGCGTAATACCAA.

10 In some embodiments, the method can further comprise: at least one of designing, preparing and using a first TaqMan probe comprising a nucleotide sequence of CCGTGATGTTGCTTGCCGG; wherein the first TaqMan probe further comprises a 5' end and a 3' end.

15 In some embodiments, the method can further comprise: attaching a first fluorescent dye that emits a first wavelength to the 5' end of the first TaqMan probe and attaching a first quencher dye to the 3' end of the first TaqMan probe; wherein the first primer, second primer, and first TaqMan probe are used for detecting the *adeB* gene of *Acinetobacter baumannii*.

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In some embodiments, the method can further comprise: at least one of designing, preparing and using a third primer comprising a nucleotide sequence of GGGSAATGGMTRTARAMCCRCAAGTGG; wherein S is G or C; M is A or C; and R is A or G.

25 In some embodiments, the method can further comprise: at least one of designing, preparing and using a fourth primer comprising a nucleotide sequence of ACARGTAGGYTGGTTRACTGGWTGGGT; wherein R is A or G; Y is C or T; and W is A or T.

In some embodiments, the method can further comprise: at least one of designing,
30 preparing and using a second TaqMan probe comprising a nucleotide sequence of

ACTTTTACCATTTTGATCTGGGCCA; wherein the second TaqMan probe further comprises a 5' end and a 3' end.

In some embodiments, the method can further comprise: attaching a second fluorescent dye that emits a second wavelength to the 5' end of the second TaqMan probe and attaching a second quencher dye to the 3' end of the second TaqMan probe; wherein the third primer, fourth primer, and second TaqMan probe are used for detecting the OXA gene. In some embodiments, the OXA gene can comprise OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.

In some embodiments, the method can further comprise: wherein in close proximity the first fluorescent dye and the first quencher dye form a quenched system and no fluorescence is observed; wherein when the first TaqMan probe hybridizes to its DNA template, a 5'exonuclease activity of a Taq polymerase hydrolyses the first TaqMan probe; wherein the first fluorescent dye is detected.

In some embodiments, the method can further comprise: wherein in close proximity the second fluorescent dye and the second quencher dye form a quenched system and no fluorescence is observed; wherein when the second TaqMan probe hybridizes to its DNA template, a 5'exonuclease activity of a Taq polymerase hydrolyses the second TaqMan probe; wherein the second fluorescent dye is detected.

In some embodiments, the method does not comprise post-PCR processing such as gel electrophoresis. In some embodiments, the method does not comprise the Kirby-Bauer disc diffusion test.

In some embodiments, the detection results can be generated in about 24 hours or less, about 20 hours or less, about 15 hours or less, about 10 hours or less, about 5 hours or less, about 3 hours or less, about 2.5 hours or less, or about 2 hours or less.

In some embodiments, the method can have an accuracy of about 95% or more, about 96% or more, about 97% or more, about 98% or more, about 99% or more, or 100% in detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

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A second aspect of the present disclosure provides a multiplex real-time Polymerase Chain Reaction (RT-PCR) assay for detecting the presence of carbapenem resistant *Acinetobacter baumannii*, wherein the assay can be used to detect the presence of an *adeB* gene of *Acinetobacter baumannii*.

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In some embodiments, the assay can be used for detecting the presence of an OXA gene. In some embodiments, the OXA gene can comprise OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.

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In some embodiments, the assay can further comprise at least one of designing, preparing and using a first primer comprising a nucleotide sequence of GCAATTGTGGCGCCTATTGCCTTAC.

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In some embodiments, the assay can further comprise at least one of designing, preparing and using a second primer comprising a nucleotide sequence of GCCGCCAATACCAGCGTAATACCAA.

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In some embodiments, the assay can further comprise: at least one of designing, preparing and using a first TaqMan probe comprising a nucleotide sequence of CCGTGATGTTGCTTGCCGG; wherein the first TaqMan probe further comprises a 5' end and a 3' end.

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In some embodiments, the assay can further comprise: attaching a first fluorescent dye that emits a first wavelength to the 5' end of the first TaqMan probe and attaching a first

quencher dye to the 3' end of the first TaqMan probe; wherein the first primer, second primer, and first TaqMan probe are used for detecting the *adeB* gene of *Acinetobacter baumannii*.

- 5 In some embodiments, the assay can further comprise: at least one of designing, preparing and using a third primer comprising a nucleotide sequence of GGGSAATGGMTRTARAMCCRCAAGTGG; wherein S is G or C; M is A or C; and R is A or G.

- 10 In some embodiments, the assay can further comprise: at least one of designing, preparing and using a fourth primer comprising a nucleotide sequence of ACARGTAGGYTGGTTRACTGGWTGGGT; wherein R is A or G; Y is C or T; and W is A or T.

- 15 In some embodiments, the assay can further comprise: at least one of designing, preparing and using a second TaqMan probe comprising a nucleotide sequence of ACTTTTACCATTTTGATCTGGGCCA; wherein the second TaqMan probe further comprises a 5' end and a 3' end.

- 20 In some embodiments, the assay can further comprise: attaching a second fluorescent dye that emits a second wavelength to the 5' end of the second TaqMan probe and attaching a second quencher dye to the 3' end of the second TaqMan probe; wherein the third primer, fourth primer, and second TaqMan probe are used for detecting the OXA gene. In some embodiments, the OXA gene can comprise OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.

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- In some embodiments, the assay can further comprise: wherein in close proximity the first fluorescent dye and the first quencher dye form a quenched system and no fluorescence is observed; wherein when the first TaqMan probe hybridizes to its DNA template, a 5'exonuclease activity of a Taq polymerase hydrolyses the first TaqMan probe; wherein the first fluorescent dye is detected.
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In some embodiments, the assay can further comprise: wherein in close proximity the second fluorescent dye and the second quencher dye form a quenched system and no fluorescence is observed; wherein when the second TaqMan probe hybridizes to its DNA
5 template, a 5' exonuclease activity of a Taq polymerase hydrolyses the second TaqMan probe; wherein the second fluorescent dye is detected.

In some embodiments, the assay does not comprise post-PCR processing such as gel electrophoresis. In some embodiments, the assay does not comprise the Kirby-Bauer disc
10 diffusion test.

In some embodiments, the detection results can be generated in about 24 hours or less, about 20 hours or less, about 15 hours or less, about 10 hours or less, about 5 hours or less, about 3 hours or less, about 2.5 hours or less, or about 2 hours or less.
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In some embodiments, the assay can have an accuracy of about 95% or more, about 96% or more, about 97% or more, about 98% or more, about 99% or more, or 100% in detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

20 A third aspect of the present disclosure provides a test kit for detecting the presence of carbapenem resistant *Acinetobacter baumannii*, wherein the test kit can comprise a first primer comprising a nucleotide sequence of GCAATTGTGGCGCCTATTGCCTTAC.

In some embodiments, the test kit can further comprise a second primer comprising a
25 nucleotide sequence of GCCGCCAATACCAGCGTAATACCAA.

In some embodiments, the test kit can further comprise: a first TaqMan probe comprising a nucleotide sequence of CCGTGATGTTGCTTGCCGG; wherein the first TaqMan probe further comprises a 5' end and 3' end.
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In some embodiments, the test kit can further comprise: wherein a first fluorescent dye that emits a first wavelength is attached to the 5' end of the first TaqMan probe; wherein a first quencher dye is attached to the 3' end of the first TaqMan probe; and wherein the first primer, second primer, and first TaqMan probe are used for detecting an *adeB* gene of *Acinetobacter baumannii*.

In some embodiments, the test kit can further comprise: a third primer comprising a nucleotide sequence of GGGSAATGGMTRTARAMCCRCAAGTGG; wherein R is A or G; S is G or C; and M is A or C.

In some embodiments, the test kit can further comprise: a fourth primer comprising a nucleotide sequence of ACARGTAGGYTGGTTRACTGGWTGGGT; wherein Y is C or T; R is A or G; and W is A or T.

In some embodiments, the test kit can further comprise: a second TaqMan probe comprising a nucleotide sequence of ACTTTTACCATTTTGATCTGGGCCA; wherein the second TaqMan probe further comprises a 5' end and a 3' end.

In some embodiments, the test kit can further comprise: wherein a second fluorescent dye that emits a second wavelength is attached to the 5' end of the second TaqMan probe and wherein a second quencher dye is attached to the 3' end of the second TaqMan probe; wherein the third primer, fourth primer, and second TaqMan probe are used for detecting an OXA gene. In some embodiments, the OXA gene can comprise OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.

In some embodiments, the test kit can further comprise: wherein in close proximity the first fluorescent dye and the first quencher dye form a quenched system and no fluorescence is observed; wherein when the first TaqMan probe hybridizes to its DNA template, a

5' exonuclease activity of a Taq polymerase hydrolyses the first TaqMan probe; wherein the first fluorescent dye is detected.

In some embodiments, the test kit can further comprise: wherein in close proximity the second fluorescent dye and the second quencher dye form a quenched system and no fluorescence is observed; wherein when the second TaqMan probe hybridizes to its DNA template, a 5' exonuclease activity of a Taq polymerase hydrolyses the second TaqMan probe; wherein the second fluorescent dye is detected.

10 In some embodiments, the test kit can generate detection results in about 24 hours or less, about 20 hours or less, about 15 hours or less, about 10 hours or less, about 5 hours or less, about 3 hours or less, about 2.5 hours or less, or about 2 hours or less.

In some embodiments, The test kit of any one of claims 18-28, wherein the test kit has an accuracy of about 95% or more, about 96% or more, about 97% or more, about 98% or more, about 99% or more, or 100% in detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

A fourth aspect of the present disclosure provides a method of preparing a test kit for detecting the presence of carbapenem resistant *Acinetobacter baumannii* by multiplex real-time polymerase chain reaction (RT-PCR), wherein the method comprises: at least one of designing and preparing a first primer comprising a nucleotide sequence of GCAATTGTGGCGCCTATTGCCTTAC.

25 In some embodiments, the method can further comprise at least one of designing and preparing a second primer comprising a nucleotide sequence of GCCGCCAATACCAGCGTAATACCAA.

In some embodiments, the method can further comprise: at least one of designing and preparing a first TaqMan probe comprising a nucleotide sequence of

CCGTGATGTTGCTTGCCGG; wherein the first TaqMan probe further comprises a 5' end and a 3' end.

In some embodiments, the method can further comprise: attaching a first fluorescent dye that emits a first wavelength to the 5' end of the first TaqMan probe and attaching a first quencher dye to the 3' end of the first TaqMan probe; wherein the first primer, second primer, and first TaqMan probe are used for detecting an *adeB* gene of *Acinetobacter baumannii*.

In some embodiments, the method can further comprise: at least one of designing and preparing a third primer comprising a nucleotide sequence of GGGSAATGGMTRTARAMCCRCAAGTGG; wherein R is A or G; S is G or C; and M is A or C.

In some embodiments, the method can further comprise: at least one of designing and preparing a fourth primer comprising a nucleotide sequence of ACARGTAGGYTGGTTRACTGGWTGGGT; wherein Y is C or T; R is A or G; and W is A or T.

In some embodiments, the method can further comprise: at least one of designing and preparing a second TaqMan probe comprising a nucleotide sequence of ACTTTTACCATTTTGATCTGGGCCA; wherein the second TaqMan probe further comprises a 5' end and a 3' end.

In some embodiments, the method can further comprise: attaching a second fluorescent dye that emits a second wavelength to the 5' end of the second TaqMan probe and attaching a second quencher dye to the 3' end of the second TaqMan probe; wherein the third primer, fourth primer, and second TaqMan probe are used for detecting of an OXA gene. In some embodiments, the OXA gene can comprise OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.

BRIEF DESCRIPTION OF DRAWINGS

Embodiments of the present disclosure are described herein with reference to the drawings in which:

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FIG. 1 illustrates the concepts of the TaqMan probe assay. When in close proximity, the reporter dye (R) and the quencher dye (Q) form a quenched system and no fluorescence is observed. Upon exonuclease digestion during the PCR reaction, the reporter dye is released from the close proximity of the quencher dye and is hence able to fluoresce and be detected by the fluorimeter.

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FIG. 2 is a graph illustrating the detection of *Acinetobacter baumannii* at a wavelength of 520nm, wherein water is used as a negative control in accordance with an embodiment of the present disclosure.

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FIG. 3 is a graph illustrating the specific detection of an OXA gene variant at a wavelength of 556nm, wherein water is used as a negative control in accordance with an embodiment of the present disclosure.

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DETAILED DESCRIPTION

In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments can be utilized, and other changes can be made, without departing from the spirit or scope of the subject matter presented herein.

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Unless specified otherwise, the terms "comprising" and "comprise" as used herein, and grammatical variants thereof, are intended to represent "open" or "inclusive" language such

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that they include recited elements but also permit inclusion of additional, un-recited elements.

As used herein, the term "about", in the context of concentrations of components, conditions, other measurement values, etc., means +/- 5% of the stated value, or +/- 4% of the stated value, or +/- 3% of the stated value, or +/- 2% of the stated value, or +/- 1% of the stated value, or +/- 0.5% of the stated value, or +/- 0% of the stated value.

Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

As used herein, unless specified otherwise, the term "detection" includes diagnosis. Additionally, as used herein, unless specified otherwise, the term "detecting" includes diagnosing.

The present disclosure relates to a method of rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* in a sample and/or specimen. The method of rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* of the present disclosure allows diagnostic results to be generated in two (2) hours or less, which is significantly faster than presently known methods for detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

In some embodiments, a multiplex real-time polymerase chain reaction (RT-PCR) assay can be used that allows for the detection of individual genes (e.g., the *adeB* gene, OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51 and/or OXA gene variant type-58) based on fluorescence emitted at different wavelengths. In some
5 embodiments, the presence of the *adeB* gene of *Acinetobacter baumannii* and the presence of at least one OXA gene (e.g., OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51 and/or OXA gene variant type-58) can be detected in a single test tube. The generated result confirms the presence or absence of carbapenem resistant *Acinetobacter baumannii*.

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In accordance with the present disclosure, the method of rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* can be 100% efficient and/or 100% accurate in detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

15 The present disclosure also relates to a test kit for rapidly detecting carbapenem resistant *Acinetobacter baumannii* in a sample and/or specimen. In some embodiments, the test kit of the present disclosure allows diagnostic results to be generated in two (2) hours or less, which is significantly faster than presently known methods for detecting carbapenem resistant *Acinetobacter baumannii*. In some embodiments, the test kit can be used to
20 detect the presence of the *adeB* gene of *Acinetobacter baumannii* and the presence of at least one OXA gene (e.g., OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, and/or OXA gene variant type-58) in a single test tube. The generated result confirms the presence or absence of the carbapenem resistant *Acinetobacter baumannii*.

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In accordance with the present disclosure, the test kit for rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* can be 100% efficient and/or 100% accurate in detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

In accordance with the present disclosure, specific primers and fluorescent probes can be designed, prepared and/or used to amplify and detect carbapenem resistant *Acinetobacter baumannii*.

5 In accordance with the present disclosure, a first TaqMan probe and a second TaqMan probe can each be labelled with a different fluorescent dye, wherein each different fluorescent dye emits fluorescence at a different wavelength from the other. This allows for the detection of the *adeB* gene of *Acinetobacter baumannii* in one channel and also the simultaneous detection of at least one OXA gene or OXA gene variant (e.g., OXA gene
10 variant type-23, OXA gene variant type-40, OXA gene variant type-51, and/or OXA gene variant type-58) in another channel. Such a system eliminates spectral crosstalk amongst the detection channels and reads fluorescence from each sample well with high sensitivity. In addition, the results can be interpreted in real-time situations without the need to carry out a post-PCR process, thereby eliminating the risk of cross contamination. The elimination
15 of the risk of cross contamination further enhances the reliability of the method and/or test kit for rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* of the present disclosure.

In accordance with the present disclosure, the method and/or test kit for rapidly detecting
20 the presence of carbapenem resistant *Acinetobacter baumannii* can be a useful tool for conclusively and/or efficiently identifying carbapenem resistant *Acinetobacter baumannii*. The method and kit for rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* of the present disclosure can allow clinicians to make a more rapid and/or accurate diagnosis of carbapenem resistant *Acinetobacter baumannii* infection,
25 which in turn allows for better patient management. The rapid and/or accurate diagnosis of carbapenem resistant *Acinetobacter baumannii* infection can be especially critical if the patient is in an intensive care unit or is immune-compromised.

In accordance with the present disclosure, the chemistry employed in the method and kit
30 for rapidly detecting the presence of carbapenem resistant *Acinetobacter baumannii* uses a

Taq polymerase that has a 5' exonuclease activity. In the TaqMan probe-based approach, the TaqMan probe can contain one fluorophore reporter dye at the 5' terminus of the TaqMan probe and a quencher dye at the 3' terminus of the TaqMan probe.

5 **FIG. 1** illustrates the concepts of a TaqMan probe assay. When in close proximity, a fluorogenic/fluorophore reporter dye (R) and a quencher dye (Q) form a quenched system and no fluorescence is observed. When the TaqMan probe hybridizes to its template, the 5' exonuclease activity of the *Taq* polymerase can hydrolyse the TaqMan probe during PCR amplification. Upon exonuclease digestion during the PCR reaction, the
10 fluorogenic/fluorophore reporter dye (R) is released from the close proximity of the quencher dye (Q) thereby allowing the fluorogenic/fluorophore reporter dye (R) to fluoresce and be detected by a fluorimeter. In other words, once the fluorophore is separated from the quencher, the emissions of the fluorophore are no longer quenched and fluorescence can be detected.

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Further, the sample mentioned herein throughout the specification shall refer to any biological sample collected from a subject suspected to carry or being infected by *Acinetobacter baumannii*. The sample described is preferably subjected to further extraction process to acquire the DNA for the diagnostic identification disclosed. The sample or
20 biological sample can be tissue sample, blood sample, urine sample or other bodily fluid of the subject through blood sample is more preferred.

EXAMPLE

25 Experimental Set-Up

Design of Primers and TaqMan Probes

The sequences of the *adeB* gene and *OXA* gene of carbapenem resistant *Acinetobacter baumannii* were extracted and analyzed from the GenBank (<http://www.ncbi.nlm.nih.gov>).

30 The *adeB* region codes for a subunit of an aspecific drug efflux pump, *adeABC*, that is

common to all multidrug resistant *Acinetobacter baumannii*. The OXA gene codes for oxacillinase, which can hydrolyse carbapenems. To date, several variants of the OXA gene have been isolated.

5 The extracted sequences of the adeB gene and OXA gene of carbapenem resistant *Acinetobacter baumannii* were then loaded in Lasergene 7 software. The Lasergene 7 software was then used to scan for relevant and potential corresponding primer sequences and corresponding TaqMan probe sequences. Corresponding primer sequences and corresponding TaqMan probe sequences were then identified. The specificity of the
10 corresponding primer sequences and corresponding TaqMan probe sequences was analysed and checked using BLAST analysis in the GenBank of the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>). Once the corresponding primer sequences and corresponding TaqMan probe sequences were identified, analysed and designed, the corresponding primers and corresponding TaqMan probes were synthesized or prepared by
15 a commercial provider such as Sigma Aldrich or Integrated DNA Technologies.

The primers and TaqMan probes were designed to ensure optimal sequence length for specificity and sensitivity. Shortening the sequences or introducing mismatches will lower the specificity, whereas lengthening the sequences will not be effective due to secondary
20 structure formations of the primers and/or the TaqMan probes.

In designing the adeB primer and TaqMan probe set, the adeB region targeted was from nucleotide position 1105 to 1352 of the *Acinetobacter baumannii* genome. The adeB primer and TaqMan probe set was designed using Lasergene 7 software to ensure they were
25 effective with no secondary structure formations. The specificity of the adeB primer and probe was also analysed by BLAST analysis in the GenBank of the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>).

The most common OXA gene variants worldwide, OXA gene type-23 and OXA gene type-51,
30 were used in designing the OXA primers and TaqMan probe set. The OXA primers and

TaqMan probe were designed using Lasergene 7 software to ensure they were effective with no secondary structure formations. The specificity of the OXA primers and TaqMan probe was also analysed by BLAST analysis in the GenBank of the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>).

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A pair of ade primers (designated as adeFP and adeRP) and a first TaqMan probe (designated as adeProbe) were designed and synthesized/prepared for specific amplification of the adeB gene of *Acinetobacter baumannii*.

10 A pair of OXA degenerate primers (designated as oxa 23/58 and oxa 40/51) and a second TaqMan probe (designated as oxaProbe) were designed and synthesized/prepared for specific amplification of at least one OXA gene or OXA gene variant (e.g., OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51 and/or OXA gene variant type-58).

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For example, the pair of OXA degenerate primers (designated as oxa 23/58 and oxa 40/51) and the second TaqMan probe (designated as oxaProbe) were designed using data from the attached documents, Primer Design Document 1 and Primer Design Document 2.

20 A first fluorescent reporter dye was selected for the detection of the adeB gene and a different second fluorescent reporter dye was selected for the detection of the at least one OXA gene or OXA gene variant (e.g., OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51 and/or OXA gene variant type-58). The first fluorescent reporter dye was attached to the 5' end of the adeProbe and a quencher dye (e.g., Black Hole Quencher (BHQ 1) was attached to the 3' end of the adeProbe. The second fluorescent reporter dye was attached to the 5' end of the oxaProbe and a quencher dye (e.g., Black Hole Quencher (BHQ 1) was attached to the 3' end of the oxaProbe.

30 The first fluorescent reporter dye comprised 6-carboxy-fluorescein (6-FAM) for the detection of the adeB gene. The second fluorescent reporter dye comprised hexachloro-

fluorescein (HEX) for detection of the at least one OXA gene or OXA gene variant (e.g., OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51 and/or OXA gene variant type-58).

- 5 As such, the first TaqMan probe (adeProbe) and the second TaqMan probe (oxaProbe) were each labelled with a different fluorescent dye that emits fluorescence at a different wavelength from the other. The first TaqMan probe (adeProbe) was labelled with the first fluorescent reporter dye (6-FAM) for detection of the adeB gene, wherein 6-FAM emits fluorescence at 520nm. The second TaqMan probe (oxaProbe) was labelled with the second
10 fluorescent reporter dye (HEX) for detection of the at least one OXA gene or OXA gene variant (e.g., OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51 and/or OXA gene variant type-58), wherein HEX emits fluorescence at 556 nm.

- This allows for the simultaneous detection and differentiation of the two targets (e.g., the
15 adeB gene and the at least one OXA gene or OXA gene variant) in different channels. In accordance with the present disclosure, such as system system eliminates spectral crosstalk amongst the detection channels and reads fluorescence from each sample well with high sensitivity.

- 20 The sequence of the primers and probes are shown in Table 1 below.

Table 1: Nucleotide sequence of the specific primers and TaqMan probes designed for detection of carpapenem resistant *Acinetobacter baumannii* in accordance with the present disclosure.

Primer/Probe	Nucleotide Sequence (5' – 3')	Variants targeted
adeFP	GCAATTGTGGCGCCTATTGCCTTAC	–
adeRP	GCCGCCAATACCAGCGTAATACCAA	–
adeProbe	CCGTGATGTTGCTTGCCGG ^a	–

oxa 23/58	GGGSAATGGMTRTARAMCCRCAAGTGG	Type-23
oxa 40/51	ACARGTAGGYTGGTTRACTGGWTGGGT	Type-51
oxaProbe	ACTTTTACCATTTTGATCTGGGCCA ^b	–

Y is C or T; R is A or G; W is A or T; S is G or C; M is A or C

^a6-FAM (6-carboxy-fluorescein) was labelled at the 5' end of adeProbe

^bHEX (hexachloro-fluorescein) was labelled at the 5' end of oxaProbe

BHQ 1 was labelled at the 3' end of adeProbe and oxaProbe

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Extraction of genomic DNA

Samples containing *Acinetobacter baumannii* were provided by Khoo Teck Puat Hospital. DNA was extracted from the *Acinetobacter baumannii* samples using the Roche MagNA Pure Compact Nucleic Acid Purification system and the MagNA Pure Compact Nucleic Acid Isolation kit I (Roche, Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions and stored at -20°C. All extractions were done in Bio Safety Cabinet Level 2 to prevent any accidental infections from the live bacterium.

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Multiplex Real-time RT-PCR analysis

A multiplex real-time PCR analysis of the extracted DNA was carried out using a CFX96 Real-Time PCR Detection system from Bio-Rad Laboratories, United States, with the components listed in Table 2. The real time PCR reagents were supplied by BioRad Laboratories. The primers and TaqMan probes were identified, analyzed and designed by the inventors. The designed primers and TaqMan probes were then synthesized and supplied by Integrated DNA Technologies.

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Table 2: Components of the multiplex real-time PCR setup

Reagent	Volume
Multiplex reaction mix	25µL
adeFP (10µM)	0.75µL
adeRP (10µM)	0.75µL
oxa 23/58 (10µM)	1.5µL
oxa 40/51 (10µM)	1.5µL
IC FP (1µM)	0.5µL
IC RP (1µM)	0.5µL
adeProbe (10µM)	0.75µL
oxaProbe (10µM)	1.5µL
IC probe (1µM, Texas Red channel)	0.5µL
IC Template (2pg/µl)	0.5 µL
Nuclease-free H ₂ O	13.75µL
DNA Template	2.5µL
Final Reaction Volume	50.0µL
IC – Internal Control	

The following conditions were set up for the real-time PCR reactions. Activation of Taq Polymerase at 95°C was performed for 3 minutes. Thermocycling was performed for 40 cycles of 95°C for 30 seconds and 60°C for 75 seconds in the CFX96 Real-Time PCR Detection system. The thermocycling step was performed over the course of about one (1) hour. The detection results were obtained immediately after completion of the thermocycling step.

- 10 The whole process from the addition of all the real time PCR reagents, primers, TaqMan probes and the DNA sample to obtaining the final detection results took about two hours.

Results

The specificity of the multiplex real-time PCR method and test kit for detection of the presence of carbapenem resistant *Acinetobacter baumannii* is shown in **FIG. 2** and **FIG. 3**.

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Referring to **FIG. 2**, amplification of all the *Acinetobacter baumannii* (e.g., the amplification of the adeB gene of *Acinetobacter baumannii*) under analysis was observed in the fluorescence channel at a wavelength of 520 nm. **FIG. 2** illustrates the specific detection of the adeB gene of *Acinetobacter baumannii* at 520 nm in accordance with an embodiment of
10 the present disclosure. Other strains that were also analysed included *E. coli*, *Klebsiella*, *Pneumonia*, *MRSA*, *Strept. Epidermidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. These non-*Acinetobacter baumannii* organisms were not amplified and showed a negative result. Water was used a negative control.

15 However, as shown in **FIG. 3**, when the fluorescence channel was switched to a wavelength of 556 nm, only fluorescence that was emitted as a result of amplification of an OXA gene of *Acinetobacter baumannii* was observed. **FIG. 3** illustrates the specific detection of an OXA gene of *Acinetobacter baumannii* at 556 nm in accordance with an embodiment of the present disclosure. In this particular example, the OXA gene comprised OXA gene variant
20 type-23. The non-*Acinetobacter baumannii* organisms were not amplified and showed a negative result. As mentioned above, the non-*Acinetobacter baumannii* organisms included *E. coli*, *Klebsiella*, *Pneumonia*, *MRSA*, *Strept. Epidermidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Water was used a negative control.

25 While various aspects and embodiments have been disclosed herein, it will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims. The
30 various aspects and embodiments disclosed herein are for purposes of illustration and

are not intended to be limiting, with the true scope and spirit of the invention being indicated by the appended claims.

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CLAIMS

1. A method of detecting the presence of carbapenem resistant *Acinetobacter*
5 *baumannii*, wherein the method comprises:
using a multiplex real-time Polymerase Chain Reaction (RT-PCR) assay to detect the
presence of an adeB gene of *Acinetobacter baumannii*.
2. The method of claim 1, further comprising wherein the assay is used for detecting
10 the presence of an OXA gene.
3. The method of claim 2, wherein the OXA gene comprises OXA gene variant type-23,
OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a
combination of two or more thereof.
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4. The method of any one of claims 1-3, further comprising:
using a first primer comprising a nucleotide sequence of
GCAATTGTGGCGCCTATTGCCTTAC.
- 20 5. The method of any one of claims 1-4, further comprising:
using a second primer comprising a nucleotide sequence of
GCCGCCAATACCAGCGTAATACCAA.
6. The method of any one of claims 1-5, further comprising:
25 using a first TaqMan probe comprising a nucleotide sequence of
CCGTGATGTTGCTTGCCGG;
wherein the first TaqMan probe further comprises a 5' end and a 3' end.

7. The method of claim 6, comprising:
attaching a first fluorescent dye that emits a first wavelength to the 5' end of the
5 first TaqMan probe and attaching a first quencher dye to the 3' end of the first TaqMan
probe;
wherein the first primer, second primer, and first TaqMan probe are used for
detecting the adeB gene of *Acinetobacter baumannii*.
- 10 8. The method of any one of claims 3-7, further comprising:
using a third primer comprising a nucleotide sequence of
GGGSAATGGMTRTARAMCCRCAAGTGG;
wherein S is G or C; M is A or C; and R is A or G.
- 15 9. The method of any one of claims 3-8, further comprising:
using a fourth primer comprising a nucleotide sequence of
ACARGTAGGYTGGTTRACTGGWTGGGT;
wherein R is A or G; Y is C or T; and W is A or T.
- 20 10. The method of any one of claims 3-9, further comprising:
using a second TaqMan probe comprising a nucleotide sequence of
ACTTTTACCATTTTGATCTGGGCCA;
wherein the second TaqMan probe further comprises a 5' end and a 3' end.
- 25 11. The method of claim 10, further comprising:
attaching a second fluorescent dye that emits a second wavelength to the 5' end of
the second TaqMan probe and attaching a second quencher dye to the 3' end of the second
TaqMan probe;
wherein the third primer, fourth primer, and second TaqMan probe are used for
30 detecting the OXA gene.

12. The method of any one of claims 7-11,
5 wherein in close proximity the first fluorescent dye and the first quencher dye form a quenched system and no fluorescence is observed;
wherein when the first TaqMan probe hybridizes to its DNA template, a 5' exonuclease activity of a Taq polymerase hydrolyses the first TaqMan probe;
wherein the first fluorescent dye is detected.
- 10 13. The method of claim 11,
wherein in close proximity the second fluorescent dye and the second quencher dye form a quenched system and no fluorescence is observed;
wherein when the second TaqMan probe hybridizes to its DNA template, a
15 5' exonuclease activity of a Taq polymerase hydrolyses the second TaqMan probe;
wherein the second fluorescent dye is detected.
14. The method of any one of claims 1-13, wherein the method does not comprise post-PCR processing such as gel electrophoresis.
- 20 15. The method of any one of claims 1-14, wherein the method does not comprise the Kirby-Bauer disc diffusion test.
16. The method of any one of claims 1-15, wherein the detection results are generated
25 in 24 hours or less, 20 hours or less, 15 hours or less, 10 hours or less, 5 hours or less, 3 hours or less, 2.5 hours or less, or 2 hours or less.
17. The method of any one of claims 1-16, wherein the method has an accuracy of 95% or more, 96% or more, 97% or more, 98% or more, 99% or more, or 100% in detecting the
30 presence of carbapenem resistant *Acinetobacter baumannii*.

18. A test kit for detecting the presence of carbapenem resistant *Acinetobacter*
5 *baumannii*, wherein the test kit comprises:
a first primer comprising a nucleotide sequence of GCAATTGTGGCGCCTATTGCCTTAC.
19. The test kit of claim 18, further comprising:
a second primer comprising a nucleotide sequence of
10 GCCGCCAATACCAGCGTAATACCAA.
20. The test kit of claim 18 or 19, further comprising:
a first TaqMan probe comprising a nucleotide sequence of
CCGTGATGTTGCTTGCCGG;
15 wherein the first TaqMan probe further comprises a 5' end and 3' end.
21. The test kit of claim 20,
wherein a first fluorescent dye that emits a first wavelength is attached to the 5' end
of the first TaqMan probe;
20 wherein a first quencher dye is attached to the 3' end of the first TaqMan probe; and
wherein the first primer, second primer, and first TaqMan probe are used for
detecting an adeB gene of *Acinetobacter baumannii*.
22. The test kit of any one claims 18-21, further comprising:
25 a third primer comprising a nucleotide sequence of
GGGSAATGGMTRTARAMCCRCAAGTGG;
wherein R is A or G; S is G or C; and M is A or C.
23. The test kit of any one claims 18-22, further comprising:

a fourth primer comprising a nucleotide sequence of
ACARGTAGGYTGGTTRACTGGWTGGGT;

wherein Y is C or T; R is A or G; and W is A or T.

5 24. The test kit of any one claims 18-23, further comprising:

a second TaqMan probe comprising a nucleotide sequence of
ACTTTTACCATTTTGATCTGGGCCA;

wherein the second TaqMan probe further comprises a 5' end and a 3' end.

10 25. The test kit of claim 24,

wherein a second fluorescent dye that emits a second wavelength is attached to the
5' end of the second TaqMan probe and wherein a second quencher dye is attached to the
3' end of the second TaqMan probe;

wherein the third primer, fourth primer, and second TaqMan probe are used for
15 detecting an OXA gene.

26. The test kit of claim 25, wherein the OXA gene comprises OXA gene variant type-23,
OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a
combination of two or more thereof.

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27. The test kit of any one of claims 21-26,

wherein in close proximity the first fluorescent dye and the first quencher dye form a
quenched system and no fluorescence is observed;

wherein when the first TaqMan probe hybridizes to its DNA template, a
25 5'exonuclease activity of a Taq polymerase hydrolyses the first TaqMan probe;
wherein the first fluorescent dye is detected.

28. The test kit of claim 25 or 26,

wherein in close proximity the second fluorescent dye and the second quencher dye
30 form a quenched system and no fluorescence is observed;

wherein when the second TaqMan probe hybridizes to its DNA template, a 5' exonuclease activity of a Taq polymerase hydrolyses the second TaqMan probe; wherein the second fluorescent dye is detected.

5 29. The test kit of any one of claims 18-28, wherein the test kit generates detection results in 24 hours or less, 20 hours or less, 15 hours or less, 10 hours or less, 5 hours or less, 3 hours or less, 2.5 hours or less, or 2 hours or less.

30. The test kit of any one of claims 18-29, wherein the test kit has an accuracy of 95%
10 or more, 96% or more, 97% or more, 98% or more, 99% or more, or 100% in detecting the presence of carbapenem resistant *Acinetobacter baumannii*.

31. A method of preparing a test kit for detecting the presence of carbapenem resistant *Acinetobacter baumannii* by multiplex real-time polymerase chain reaction (RT-PCR),
15 wherein the method comprises:

at least one of designing and preparing a first primer comprising a nucleotide sequence of GCAATTGTGGCGCCTATTGCCTTAC.

32. The method of claim 31, further comprising:
20 at least one of designing and preparing a second primer comprising a nucleotide sequence of GCCGCCAATACCAGCGTAATACCAA.

33. The method of claim 31 or 32, further comprising:
at least one of designing and preparing a first TaqMan probe comprising a nucleotide
25 sequence of CCGTGATGTTGCTTGCCGG;
wherein the first TaqMan probe further comprises a 5' end and a 3' end.

34. The method of claim 33, further comprising:

attaching a first fluorescent dye that emits a first wavelength to the 5' end of the first TaqMan probe and attaching a first quencher dye to the 3' end of the first TaqMan probe;

wherein the first primer, second primer, and first TaqMan probe are used for
5 detecting an adeB gene of *Acinetobacter baumannii*.

35. The method of any one of claims 31-34, further comprising:

at least one of designing and preparing a third primer comprising a nucleotide sequence of GGGSAATGGMTRTARAMCCRCAAGTGG;

10 wherein R is A or G; S is G or C; and M is A or C.

36. The method of any one of claims 31-35, further comprising:

at least one of designing and preparing a fourth primer comprising a nucleotide sequence of ACARGTAGGYTGGTTRACTGGWTGGGT;

15 wherein Y is C or T; R is A or G; and W is A or T.

37. The method of any one of claims 31-36, further comprising:

at least one of designing and preparing a second TaqMan probe comprising a nucleotide sequence of ACTTTTACCATTTTGATCTGGGCCA;

20 wherein the second TaqMan probe further comprises a 5' end and a 3' end.

38. The method of claim 37, further comprising:

attaching a second fluorescent dye that emits a second wavelength to the 5' end of the second TaqMan probe and attaching a second quencher dye to the 3' end of the second
25 TaqMan probe;

wherein the third primer, fourth primer, and second TaqMan probe are used for detecting of an OXA gene, wherein the OXA gene comprises OXA gene variant type-23, OXA gene variant type-40, OXA gene variant type-51, OXA gene variant type-58 or a combination of two or more thereof.