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(54) Title: METHODS OF DETECTION OF MULTIDRUG RESISTANT BACTERIA

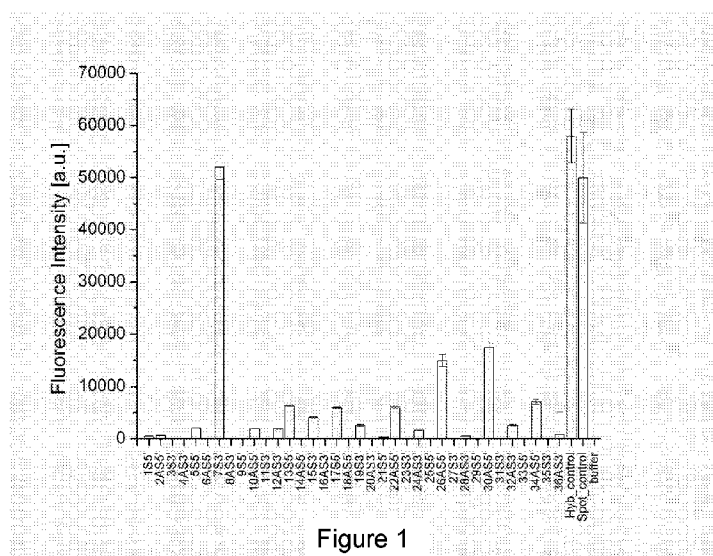


Figure 1

(57) Abstract: There is provided a biochemical probe for the detection of the *bla*_{NDM} gene, the probe comprising a nucleic acid recognition sequence operable to hybridise under stringent conditions with a specific DNA sequence of the *bla*_{NDM} gene complementary to the nucleic acid recognition sequence. In addition, there is provided a kit comprising the biochemical probe, a method of detection using the biochemical probe, and an electrochemical sensor comprising the biochemical sensor.

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Methods of Detection of Multidrug Resistant Bacteria

The invention relates to the field of multidrug resistant bacteria and methods of detecting the same.

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Background of the Invention

In the EU over 25,000 patients die annually from infection due to antibiotic resistance.¹ While until recently resistance in gram positive bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococcus* (VRE) have been the primary focus of clinicians, the increasing prevalence of multidrug resistant gram negative pathogens in combination with the lack of antimicrobials in development have become the biggest and most pressing concern in human healthcare.² Beta-lactam antibiotics conventionally used to target such infections at present make up more than half of antibiotics used in the world and these multi-resistant gram negative strains pose a severe public health threat.³

Organisms harbouring the gene *bla_{NDM}* tend to be multidrug resistant and some are only sensitive *in vitro* to agents of uncertain efficacy such as tigecycline and colistin, leaving few treatment options.² The *bla_{NDM}* gene was first isolated from a Swedish patient previously hospitalised in India in 2008, and has disseminated to broad geographical locations,⁴ predominantly linked to treatment in the Indian region, though also through independent routes.⁵

Originally identified in *E. coli* and *Klebsiella* strains, the *bla_{NDM}* gene is now found in many *Enterobacteriaceae* responsible for a spectrum of infections such as sepsis and urosepsis. The *bla_{NDM}* gene is carried on plasmids of various sizes, easily transferred laterally between gram negative genera. As a result, prompt identification of New Delhi metallo beta-lactamase (NDM) strains is of paramount importance to prevent transmission and dissemination of resistant strains.

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Many diagnostic methods currently in use for detection of extended-spectrum beta-lactamase (ESBL) and carbapenemase producing bacteria are culture based, with a time-to-result (TTR) incompatible with a rapid treatment decision (e.g. ChromID ESBL^{6,7} Etest ESBL⁸, Vitek (bioMerieux)⁹. etc.) Some molecular diagnostic approaches for detection of beta-lactam resistance in gram negative bacteria, such as Check-MDR (Checkpoints)¹⁰, Evigene (AdvanDx)¹¹, Hyplex SuperBug ID (Amplex)¹² etc, are not suited for true point of care detection

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as they are based on sophisticated optical detection systems and require demanding sample preparation and preanalytics.

Electrochemical impedance spectroscopy (EIS) is a technique that detects changes in the resistance to charge transfer (R_{ct}) between two electrodes via a redox mediator. Binding events at the electrode surface changes this R_{ct} value and therefore allows the detection of analytes such as DNA^{13,14}, and has been described in WO 2013/076143 (ITI Scotland Limited), for example, the contents of which are hereby incorporated by way of reference. Whilst EIS has been successfully used to detect the presence of a limited number of pathogens, each potential pathogen requires a separate probe at the electrode surface to bind to a specific DNA sequence of that pathogen. Accordingly, in order to determine whether a multidrug resistant gram negative bacteria is present (for example), probes for every known such bacteria would need to be present at the electrode surface, and even then, such a method of detection would not be able to detect bacteria that have newly acquired multidrug resistance.

Therefore, there is a need for a method of detecting multidrug resistant gram negative bacteria that is suitable for point of care detection by having a sufficiently fast TTR to allow a rapid treatment decision to be made, and that do not require demanding sample preparation or preanalytics.

Accordingly, it is one object of the present invention to provide a probe to allow detection of multidrug resistant gram negative bacteria.

It is a further object of the present invention to provide a method of detecting multidrug resistant gram negative bacteria that is at least more suitable for true point of care detection than existing methods.

Statements of the Invention

According to a first aspect of the invention, there is provided a biochemical probe for the detection of the *bla_{NDM}* gene, the probe comprising a nucleic acid recognition sequence operable to hybridise under stringent conditions with a specific DNA sequence of the *bla_{NDM}* gene complementary to the nucleic acid recognition sequence.

By the term "*bla_{NDM}* gene" we refer to the gene for encoding the New Delhi metallo beta-lactamase, including all known variants of the *bla_{NDM}* gene, for example *bla_{NDM-1}* gene, *bla_{NDM-}*

2 gene, *bla_{NDM-3}* gene, *bla_{NDM-4}* gene, *bla_{NDM-5}* gene, and *bla_{NDM-6}* gene (sequences of which are given below), and variants identified in the future.

By the term “specific DNA sequence of the *bla_{NDM}* gene”, or “target sequence” we refer to a DNA sequence that is unique to the *bla_{NDM}* gene, such that a nucleic acid recognition sequence that is operable to hybridise with a specific DNA sequence of the *bla_{NDM}* gene and will only substantially hybridise with DNA when the *bla_{NDM}* gene is present. This may be a specific DNA sequence that is unique to the *bla_{NDM}* gene generally. This may be a specific DNA sequence that is unique to an individual variant of the *bla_{NDM}* gene, including *bla_{NDM-1}* gene, *bla_{NDM-2}* gene, *bla_{NDM-3}* gene, *bla_{NDM-4}* gene, *bla_{NDM-5}* gene, and *bla_{NDM-6}* gene and any variant identified in the future.

The nucleic acid recognition sequence may be operable to hybridise with a specific DNA sequence adjacent to the *bla_{NDM}* gene and which is closely genetically associated with the *bla_{NDM}* gene. The nucleic acid recognition sequence may be operable to hybridise with a specific DNA sequence adjacent to the *bla_{NDM}* gene and a specific DNA sequence of the *bla_{NDM}* gene. Thus the nucleic acid recognition sequence may be operable to overlap the *bla_{NDM}* gene and an adjacent DNA sequence. For example, the nucleic acid recognition sequence may be operable to hybridise with a consecutive specific DNA sequence comprising a DNA sequence of the *bla_{NDM}* gene and a DNA sequence adjacent to that DNA sequence of the *bla_{NDM}* gene. For example, the promoter regions or after the transcription termination signal sequence. By the term “adjacent” we refer to a sequence of DNA that is non-coding and within 500 bases of the *bla_{NDM}* gene, 200 bases of the *bla_{NDM}* gene or 50 bases of the *bla_{NDM}* gene, for example.

By the term “probe” we refer to a species comprising a nucleic acid or derivative thereof of known sequence to which nucleic acids from a sample (e.g. biological samples such as wound fluid) can hybridise if the nucleic acids from the sample (or derivatives thereof) are of complementary or substantially complementary sequence (target sequences) through one or more types of chemical bonds. Typically, probes comprise nucleic acid recognition sequences of 10 to 100 bases in length, preferably, 10 to 50 bases in length, more preferably, 10 to 30 bases in length. The hybridisation of the probe to the sample nucleic acid is typically detected in an assay to thereby indicate the presence and/or the concentration of the target sequence.

By the term “nucleic acid” we refer to a deoxyribonucleotide polymer, a ribonucleotide polymer, or a derivative thereof, in either single- or double- stranded form, and unless otherwise stated, encompass known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. The terms encompass nucleic acid-like structures with

synthetic backbones (such as, for example, peptide nucleic acids or Morpholinos), as well as amplification products. In some embodiments, a nucleic acid is obtained from a larger nucleic acid molecule, e.g., by fragmentation (whether chemical, physical, enzymatic, or any combination thereof and whether artificially or naturally or both).

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By the terms "hybridise" and "hybridisation" we refer to a process where oligonucleotides and their analogs hybridise by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid molecules consist of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the pyrimidine to the purine is referred to as "base pairing." More specifically, A will hydrogen bond to T or U, and G will bond to C. "Complementary" refers to nucleic acid sequences that base-pair according to the standard Watson-Crick complementary rules outlined above, or that are capable of hybridising to a particular nucleic acid segment under relatively stringent conditions. Nucleic acid polymers may be complementary across only portions of their entire sequences.

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Hybridisation conditions resulting in particular degrees of stringency will vary depending upon the nature of the chosen hybridisation method and the composition and length of the hybridising nucleic acid sequences. Generally, the temperature of hybridisation and the ionic strength (especially the Na⁺ and/or Mg²⁺ concentration) of the hybridisation buffer will contribute to the stringency of hybridisation, though wash times also influence stringency. Calculations regarding hybridisation conditions required for attaining particular degrees of stringency are discussed in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, chs. 9 and 11.

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By the phrase "stringent conditions" we encompass conditions under which hybridisation will only occur if there is less than 50% mismatch between the hybridisation molecule and the DNA target. "Stringent conditions" include further particular levels of stringency. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 50% sequence mismatch will not hybridise; conditions of "high stringency" are those under which sequences with more than 20% mismatch will not hybridise; and conditions of "very high stringency" are those under which sequences with more than 10% mismatch will not hybridise.

In particular embodiments, stringent conditions can include hybridisation at 65 °C, followed by washes at 65 °C with 0.1 x SSC / 0.1% SDS for 40 minutes.

The following are representative, non-limiting hybridisation conditions:

- Very High Stringency: Hybridisation in 5x SSC buffer at 65 °C for 16 hours; wash twice in 2x SSC buffer at room temperature for 15 minutes each; and wash twice in 0.5x SSC buffer at 65 °C for 20 minutes each.
- High Stringency: Hybridisation in 5x-6x SSC buffer at 65-70 °C for 16-20 hours; wash twice in 2x SSC buffer at room temperature for 5-20 minutes each; and wash twice in 1x SSC buffer at 55-70 °C for 30 minutes each.
- Moderate Stringency: Hybridisation in 6x SSC buffer at room temperature to 55 °C for 16-20 hours; wash at least twice in 2x-3x SSC buffer at room temperature to 55 °C for 20-30 minutes each.

In particular embodiments, the recognition sequence and the specific DNA sequence of the *bla_{NDM}* gene complementary to the recognition sequence can remain bound under very high stringency hybridisation conditions. In these and further embodiments, the recognition sequence and the specific DNA sequence of the *bla_{NDM}* gene complementary to the recognition sequence can remain bound under high stringency hybridisation conditions. In these and further embodiments, the recognition sequence and the specific DNA sequence of the *bla_{NDM}* gene complementary to the recognition sequence can remain bound under moderate stringency hybridisation conditions.

Methods of detecting pathogens known in the art typically rely on the detection of analytes that are specific to that pathogen or culturing the microorganism. However, it is often necessary to be able to quickly determine whether a patient has a bacterial infection and whether that bacterial infection is multidrug resistant. For example, if a patient has a multidrug resistant gram negative bacterial infection, administering typical antibiotics may be ineffective and may do more harm than good.

Multidrug resistant gram negative bacteria typically comprise the *bla_{NDM}* gene. Therefore, a biochemical probe that is operable to detect the presence of the *bla_{NDM}* gene within a biological sample via hybridisation of the probe with a sequence of the DNA specific to the *bla_{NDM}* gene (or adjacent sequences) allows the detection of multidrug resistant gram negative bacteria within the biological sample, without necessarily determining the identity of that bacteria. Accordingly, if the *bla_{NDM}* gene is detected, the appropriate treatment can be prescribed immediately, without first subjecting the patient to standard antibiotics to which the bacteria are resistant.

The probe may be operable to detect the presence of the *bla_{NDM}* gene by hybridising with a specific sequence of DNA within the intact plasmid containing the *bla_{NDM}* gene. The probe may hybridise with a sequence of the *bla_{NDM}* gene (or adjacent sequences) that is located on the exterior of the *bla_{NDM}* gene in its folded state. The probe may hybridise with a sequence of the *bla_{NDM}* gene (or adjacent sequences) that is within the interior of the *bla_{NDM}* gene in its folded state and which only becomes available for hybridisation under specific conditions. For example, the sequence may only become available when the *bla_{NDM}* gene has been denatured and has at least partially become unfolded. The probe may hybridise with a sequence of the *bla_{NDM}* gene (or adjacent sequences) that is on the interior of the *bla_{NDM}* gene in its folded state, and the sequence may only become available for hybridisation after the *bla_{NDM}* gene has been fragmented. Therefore, it may be that the probe detects a specific fragment of the *bla_{NDM}* gene. The DNA fragments of the *bla_{NDM}* gene may be formed by any or any combination of methods, including, but not limited to, enzymatic digestion (such as, for example, restriction enzyme digestion) and/or physical fragmentation (e.g., sonication, acoustic shearing, nebulising, point-sink shearing, needle shearing, passing through a French press, etc.). In some embodiments, DNA fragments of the *bla_{NDM}* gene are formed by restriction enzyme digestion. In some embodiments, the DNA fragments of the *bla_{NDM}* gene are formed by sonication. The fragmentation method or methods can be performed on, e.g., plasmid DNA comprising the *bla_{NDM}* gene, larger fragments containing the *bla_{NDM}* gene, and/or polymerase chain reaction (PCR) products containing the *bla_{NDM}* gene. The DNA fragments of the *bla_{NDM}* gene may be polymerase chain reaction (PCR) products.

Preferably, the probe is operable to be used in an assay where a hybridisation event of the recognition sequence of the probe with the specific target DNA sequence is detected. The probe may be adapted for any of a number of assays that rely on such hybridisation events. In some embodiments, the probe is used in solution. Examples of assays in which the probe may be used in solution include, but are not limited to, blot hybridisation assays (including Northern blot and Southern blot assays), *in situ* hybridisation assays (including fluorescence *in situ* hybridisation (FISH)), PCR-based assays (including real-time PCR and PCR clamping), and fluorescence resonance energy transfer (FRET)-based assays.

In some embodiments, the probe is operable to be immobilised on a solid substrate. For example, the probe may be immobilised on a solid substrate during use. In some embodiments, the probe is immobilized on a solid substrate after the probe is synthesised; in some embodiments, the probe is synthesised *in situ* on a solid substrate, so that it is already attached. Any of a variety of different solid substrates can be used, depending on the assay

chosen. As non-limiting examples, suitable solid substrates include substrates made of silica, silicone, glass, plastic (such as, e.g., polypropylene, polyacrylamide, polyamide (nylon), polydimethylsiloxane (PDMS), etc.), cellulose, nitrocellulose, and metals (as further detailed below). The solid substrate can take the form of, for example, slides, chips or microarrays, beads (including magnetic beads), and electrodes.

In some embodiments, the probe is immobilised on an electrode for use in, e.g., an electrochemical assay. Electrodes used in electrochemical assays can be made of any of a variety of materials, including, for example, gold, silver, platinum, carbon, mercury, steel, and graphite.

For example, the probe may be suitable for use in electrochemical impedance spectroscopy (EIS). As another example, the probe may be suitable for use in surface plasmon resonance spectroscopy (SPRS).

The concentration of DNA fragments within the biological sample may be amplified. For example, the concentration of DNA fragments within the biological sample may be amplified using PCR or PCR-based methods.

The recognition sequence may be 7-50 base pairs long. The recognition sequence may be 15-40 base pairs long. Preferably, the recognition sequence is 15-30 base pairs long. For example, the recognition sequence may be 18, 20 or 22 base pairs long.

The recognition sequence may comprise any one of SEQ ID NO 1-36 (Table 1) or variants thereof.

SEQ ID NO	Recognition Sequence (5' to 3')
1	GTC CAT ACC GCC CAT CTT GT
2	ACA AGA TGG GCG GTA TGG AC
3	GTC CAT ACC GCC CAT CTT GT
4	ACA AGA TGG GCG GTA TGG AC
5	GTG CTG CCA GAC ATT CGG TG
6	CAC CGA ATG TCT GGC AGC AC
7	GTG CTG CCA GAC ATT CGG TG
8	CAC CGA ATG TCT GGC AGC ACT
9	GGC GTA GTG CTC AGT GTC GG

10	CCG ACA CTG AGC ACT ACG CC
11	GGC GTA GTG CTC AGT GTC GG
12	CCG ACA CTG AGC ACT ACG CC
13	GTC ATC GGT CCA GGC GGT AT
14	ATA CCG CCT GGA CCG ATG AC
15	GTC ATC GGT CCA GGC GGT AT
16	ATA CCG CCT GGA CCG ATG AC
17	TTG AGG ATC TGG GCG GTC TG
18	CAG ACC GCC CAG ATC CTC AA
19	TTG AGG ATC TGG GCG GTC TGT
20	CAG ACC GCC CAG ATC CTC AAT
21	CGC CGC AAC CAT CCC CTC TT
22	AAG AGG GGA TGG TTG CGG CG
23	CGC CGC AAC CAT CCC CTC TT
24	AAG AGG GGA TGG TTG CGG CG
25	ATC AGG CAG CCA CCA AAA GC
26	GCT TTT GGT GGC TGC CTG AT
27	ATC AGG CAG CCA CCA AAA GC
28	GCT TTT GGT GGC TGC CTG AT
29	GCC AAA GTT GGG CGC GGT TG
30	CAA CCG CGC CCA ACT TTG GC
31	GCC AAA GTT GGG CGC GGT TG
32	CAA CCG CGC CCA ACT TTG GC
33	GAG ATT GCC GAG CGA CTT G
34	CAA GTC GCT CGG CAA TCT C
35	GAG ATT GCC GAG CGA CTT GT
36	CAA GTC GCT CGG CAA TCT CT

Table 1: Probe recognition sequences

Preferably, the recognition sequence comprises SEQ ID NO 7 or SEQ ID NO 25 or variants thereof. Such variants preferably retain the ability to hybridise.

For example, in some embodiments the recognition sequence may comprise at least 75% of any one of SEQ ID NOs 1-36. In some embodiments, the recognition sequence may comprise any one of SEQ ID NOs 1-36 plus one or more additional bases. For example, the recognition

sequence may be thirty bases long and the first twenty bases from the 3' end may comprise SEQ ID NOs 1 or 2, the first twenty bases from the 5' end may comprise SEQ ID NOs 7 or 25, or any other twenty base sequence within the recognition sequence may comprise SEQ ID NOs 7 or 25. Accordingly, the recognition sequence may be longer than any one of SEQ ID NOs 1-36. However, in some embodiments, the recognition sequence may be shorter than any one of SEQ ID NOs 1-36, and may comprise a subset of any one of SEQ ID NOs 1-36.

It may be that two or fewer bases within the sequence of bases of the recognition sequence differs from that of any one of SEQ ID NOs 1-36. However, a sequence of bases within the recognition sequence, which may be the whole recognition sequence or a subset of the recognition sequence, is preferably identical to that of any one of SEQ ID NOs 1-36.

Applicants have found that probes comprising recognition sequences SEQ ID NOs 7 and 25 are surprisingly suitable for use in assays such as EIS to detect the presence of the *bla_{NDM}* gene. Without wishing to be bound by theory, the location of the complementary sequences to SEQ ID NOs 7 and 25 within the *bla_{NDM}* gene may be on the exterior of the *bla_{NDM}* gene and therefore, these complementary sequences may be readily available to hybridise with the probes on the sensing surface.

Probes according to the present aspect of the invention have been found to be particularly suitable for use in EIS-based assays. The impedance of a system is generally determined by applying a voltage perturbation with a small amplitude and detecting the current response.¹³ Faradaic EIS measures the resistance to charge transfer (*R_{ct}*) between two electrodes via a redox mediator that has been added to the biological sample. Within each pair of electrodes, a working electrode is typically covered in a self-assembled monolayer (SAM) comprising probes and a blocking species. When a target analyte, such as DNA, for example, binds to the probes by hybridisation, an increase in *R_{ct}* is observed, due to the combined effects of repulsion of anionic redox mediators at the electrode surface by the bound target analyte (either steric repulsion or electrostatic) and/or the blocking of pinholes in the SAM by the bound target slowing electron transfer.¹⁵

In EIS assays that detect DNA target analytes, probes typically comprise complementary strands of DNA fixed to the surface of the working electrode that hybridise with any target DNA within the biological sample for detection. DNA has a negatively charged phosphate backbone and therefore, the DNA probe sequences on the working electrode repel the anionic redox mediators thereby reducing the concentration of redox mediators at the surface of the working

electrode, leading to a reduction in the sensitivity of the working electrode to a change in the Rct.

The nucleic acid recognition sequence may comprise a naturally occurring nucleic acid, such as DNA, or RNA, for example.

Preferably, the nucleic acid recognition sequence comprises a synthetic nucleic acid. More preferably, the synthetic nucleic acid comprises an uncharged synthetic backbone in ambient conditions. For example, the nucleic acid recognition sequence may be a Morpholino recognition sequence comprising a backbone of morpholine rings linked by phosphorodiamidate groups. In another example, the nucleic acid recognition sequence may be a peptide nucleic acid (PNA) recognition sequence comprising a peptide backbone.

Most preferably, the nucleic acid recognition sequence is a PNA recognition sequence.

The provision of a probe that comprises an uncharged backbone rather than the charged backbone of DNA, for example, to be used on the surface of the working electrode minimises the repulsion of redox mediators from the working electrode surface, and therefore maximises the sensitivity of the working electrode to changes in resistance to charge transfer.

In addition, in embodiments where the recognition sequence is a PNA recognition sequence, PNA has a higher binding strength than DNA and therefore, probes for use in the detection of DNA may have a shorter complementary PNA sequence than equivalent probes comprising DNA. Furthermore, the PNA/DNA helix resulting from the hybridisation of the target DNA sequence to the probe PNA is more sensitive to base mismatches. As a result, probes comprising PNA sequences are less likely to hybridise with DNA other than the target sequence, when compared to an equivalent probe comprising DNA, and therefore, provide a more reliable result.

Preferably, the probe comprises an anchor operable to immobilise the probe to a sensing surface. The anchor may be attached to the 3' end of the recognition sequence. The anchor may be attached to the 5' end of the recognition sequence. Accordingly, the 5' or 3' end of the recognition sequence may be available for hybridisation with the target DNA sequence.

Preferably, the sensing surface comprises a gold surface and the anchor comprises at least one terminal sulphur containing group to bind to the gold surface of the sensing surface. Preferably, the at least one terminal sulphur containing group is a thiol group.

For example, in embodiments where the probe is to be used in an EIS assay, the sensing surface may be a gold electrode and the probe may be immobilised onto the surface of the gold electrode by an anchor comprising a thiol group.

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The anchor may be, for example, a mercapto-alkane group. For example, the anchor may be a mercapto-undecan group, such as C11M.

10 However, the sensing surface may comprise an epoxy-treated glass surface and the anchor may comprise at least one terminal amine group. The sensing surface may comprise glass and the anchor may comprise an alkylchlorosilane, alkylalkoxysilane or alkylaminosilane, for example.

15 In embodiments where the probe is used in EIS or SPRs, a mixed SAM may be immobilised onto the sensing surface, and may comprise a population of probes of the present aspect of the invention and a population of at least one blocking species. The blocking species typically minimises non-specific binding to the sensing surface by analyte, such that at least the majority of binding events at the sensing surface correspond to binding/hybridisation events between the recognition sequence of the probe and the target DNA sequence. Typically, in
20 embodiments where the sensing surface comprises a gold layer, the blocking species comprises a thiol group. The blocking species may be a mercapto alcohol, or a mercapto alkane, for example.

The probe may comprise a linker connecting the anchor to the recognition sequence such that
25 the recognition sequence is available for hybridisation with the target DNA sequence. The linker may space the recognition sequence away from the sensing surface to minimise non-specific interactions of the recognition sequence with the sensing surface. The linker may comprise one or more ether groups. Preferably, the linker is an ethylene glycol such as [2-(2-Amino-ethoxy)-ethoxy]-acetic acid (AEEA) or {2-[2-(2-Amino-ethoxy)-ethoxy]-ethoxy}-acetic
30 acid (AEEEA). The linker may be a polyethylene glycol (PEG), or a functionalised PEG.

The linker may extend between the anchor and the 3' end of the recognition sequence. The linker may extend between the anchor and the 5' end of the recognition sequence. Accordingly, the 5' or 3' end of the recognition sequence may be available for hybridisation
35 with the target DNA sequence.

For example, the probe may have the sequence (5' to 3'): GTGCTGCCAGACATTCGGTG-Lys-AEEEEA-C11M (SEQ ID NO 37). The probe may have the sequence (5' to 3'): C11M-AEEEEA-ATCAGGCAGCCACCAAAAGC (SEQ ID NO 38).

- 5 The probe may be labelled. In some embodiments, a mixture of labelled and unlabelled probes is used. The probes may be labelled in such a way that the detectability of the probe is altered when the probe is bound to the target sequence.

10 The invention extends in a second aspect to a diagnostic kit for the detection of the *bla_{NDM}* gene, the kit comprising at least one biochemical probe according to the first aspect of the invention.

15 The kit may comprise suitable reagents to allow the at least one biochemical probe to bind to a sensing surface. The kit may comprise a solvent, and the at least one biochemical probe according to the first aspect of the invention may be solubilised within the solvent. The kit may comprise one or more suitable buffers for the storage of the at least one biochemical probe. The kit may comprise one or more suitable buffers for the immobilisation of the at least one biochemical probe to a surface, such as a sensing surface.

20 The kit may comprise at least one blocking species. The at least one blocking species may be mixed with the at least one biochemical probe. The mixture of the blocking species and the probe may be operable to form a self-assembled monolayer (SAM) on a sensing surface.

25 The kit may comprise a sensing surface to which the at least one biochemical probe is immobilised. A SAM comprising the at least one probe and the blocking species may be formed on the sensing surface. The sensing surface may comprise a gold electrode and be suitable for use in electrochemical impedance spectroscopy (EIS). The sensing surface may comprise a gold layer and may be suitable for use in surface plasmon resonance spectroscopy (SPRS).

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Preferred and optional features of the first aspect of the invention are preferred and optional aspects of the second aspect of the invention.

35 According to a third aspect of the invention, there is provided a method of detection of the *bla_{NDM}* gene, the method comprising the steps:

- (a) providing a biological sample comprising DNA;

(b) providing a sensing apparatus comprising a sample chamber and a sensing surface comprising at least one probe according to the first aspect of the invention;

(c) treating the biological sample to extract and/or fragment the DNA within the biological sample;

(d) introducing the biological sample into the sample chamber of the apparatus; and

(e) determining whether DNA within the biological sample hybridises with the at least one probe on the sensing surface, wherein hybridisation of DNA to the at least one probe is indicative of the presence of the *bla_{NDM}* gene within the biological sample.

The sensing apparatus may be a surface plasmon resonance spectroscopy (SPRS) apparatus and hybridisation of DNA within the biological sample with the at least one probe on the sensing surface may be determined by a change in the wavelength of light absorbed by the sensing surface, measured as a change in angle of reflection minimum (corresponding to the maximum absorption), for example.

Preferably, the sensing apparatus is an electrochemical impedance spectroscopy (EIS) apparatus comprising at least one working electrode comprising the sensing surface. Hybridisation of DNA within the biological sample with the at least one probe on the sensing surface may be determined by an increase in the resistance to charge transfer (R_{ct}) of a working electrode, wherein an increase in the R_{ct} of the working electrode is indicative of the presence of the *bla_{NDM}* gene.

The increase in the R_{ct} of the working electrode may be determined relative to a reference electrode. The reference electrode may be a Ag/AgCl (silver/silver chloride) reference electrode. The reference electrode may be a platinum electrode or a gold electrode.

The R_{ct} of the working electrode may be measured at room temperature and the biological sample may comprise at least 30% formamide by volume. Preferably, the biological sample comprises at least 50% formamide by volume. For example, the biological sample may comprise 50% formamide by volume. Typically, the amount of formamide by volume is sufficient when the binding of the probe to substrates other than the target sequence is prevented at room temperature.

Alternatively, the R_{ct} of the working electrode may be measured at room temperature and the biological sample may comprise at least 30% by volume of urea, formaldehyde or dimethylsulfoxide (DMSO), for example.

In further alternative embodiments, the Rct of the working electrode may be measured at an elevated temperature, and the biological sample may comprise at least 30% by volume of formamide, urea, formaldehyde or DMSO, for example.

5

In some embodiments of the present aspect of the invention, the method may not comprise the step of amplifying the DNA within the biological sample.

Typically, methods or assays that utilise the detection of DNA to determine the presence of a medically relevant species are not sufficiently sensitive to detect the typical concentrations (e.g. 100 nM) of specific sequences of DNA within biological samples, and therefore, these methods or assays require any DNA within a sample to be analysed to have that DNA amplified, using techniques such as PCR. The amplification process takes time and so increases the time required to determine whether a given sample comprises the specific sequence of DNA of interest. In point of care scenarios where it is necessary to determine whether a certain pathogen is present, for example, the longer it takes to determine whether the DNA (and therefore the biological entity from which the DNA originates) is present delays treatment. Indeed, if the time to result of the assay is too long, the assay may be deemed inappropriate for use at the point of care and therefore, the treatment provided may be inappropriate, ineffective, or at best delayed.

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Therefore, a method of detection of the *bla_{NDM}* gene that does not require the DNA within the biological sample to be amplified will typically have a shorter time to result and therefore be a more appropriate method to be used as a true point of care assay, thereby allowing the appropriate treatment to be provided more rapidly.

25

The step of treating the biological sample to extract and/or fragment the DNA within the biological sample may be carried out after the biological sample is introduced into the sample chamber. Alternatively, the step of treating the biological sample may be carried out before the biological sample is introduced into the sample chamber, or the step of treating the biological sample may be carried out both before and after the biological sample is introduced into the sample chamber. For example, one or more steps in a treatment protocol may be carried out before introducing the biological sample into the sample chamber, and one or more steps may be carried out after introducing the biological sample into the sample chamber.

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The biological sample may be taken from a subject. The biological sample may be a pre-existing sample taken from a subject for an alternative purpose, and/or may be taken for the

purpose of performing a method as described herein. The subject may be a mammal. Preferably, the subject is a human. The biological sample may comprise any solid or fluid (or combination thereof) sample obtained from, excreted by, exuded by, and/or secreted by any living cell or organism or tissue. The biological sample may comprise cells. In certain
5 embodiments, the biological sample comprises nucleic acids and/or proteins. In some such embodiments, at least some of the nucleic acids and/or proteins contained in the biological sample are contained within cells.

For example, the biological sample may comprise a bodily fluid. The biological sample may
10 include, for example, wound fluid, blood, plasma, serum, urine, stool, saliva, cord blood, chorionic villus samples, amniotic fluid, transcervical lavage fluid, or any combination thereof. For example, in some embodiments, the biological sample is or comprises, a blood sample. The biological sample may be obtained from a subject before, during, and/or after a course of treatment. The biological sample itself may be treated (e.g., subject to a process) or it may
15 be untreated. For example, the biological sample may be a treated blood sample. The biological sample may be a swab sample. Typically, to obtain a swab sample, a body part is swabbed, the swab is then incubated in a solution for a period of time, and then the solution is used as a sample. Any kind of swab sample can be used, including, but not limited to cheek swabs, oral swabs, nasal swabs, armpit swabs, perineal swabs, wound site swabs, skin
20 lesions swabs, and other skin swabs. Similar "swab"-like samples can also be used, e.g., samples from used medical devices such as wound dressings, which may contain wound exudates. The biological sample may be a tissue sample, or the biological sample may comprise DNA extracted from cells obtained from the subject. The biological sample may be obtained directly from the subject; however, embodiments in which the biological sample is
25 obtained indirectly from the subject are also contemplated. For example, the biological sample may be obtained by culturing a sample obtained from the subject. For example, cell cultures and/or supernatants or other fluids obtained from cultures can also be used as biological samples.

30 In embodiments where the biological sample comprises cells, the step of treating the biological sample may include the step of lysing cells in the biological sample to extract the DNA from the cells within the biological sample. The extracted DNA may be treated to fragment the DNA by the application of an enzyme, such as DNase, or by sonication, or by the application of heat, for example.

Preferably, in embodiments where the biological sample comprises cells, such as blood samples, the biological sample is treated to extract the DNA from cells within the biological sample, and then treated to fragment the extracted DNA.

- 5 In embodiments where the biological sample comprises extracted DNA, the biological sample may be treated to fragment the extracted DNA. The DNA within the biological sample may have been extracted in a prior step. For example, where the biological sample has been previously obtained for an alternative purpose, cells within the biological sample may have already been lysed.

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In embodiments where the biological sample is a tissue sample, the biological sample may be treated to break down at least some of the tissue into cells before the biological sample is lysed to extract DNA from those cells.

- 15 The EIS apparatus may comprise a second working electrode that does not comprise biochemical probes. In this way, the second working electrode may be used to take into account any non-specific binding to the sensing surface by the target analyte, or by other species that may be present in the biological sample, and thereby provide a more accurate measurement of whether the *bla_{NDM}* gene is present in the biological sample, and allow reliable
20 detection of lower minimum concentrations of the *bla_{NDM}* gene (i.e. a lower limit of detection).

- The method may provide a level of detection of less than 500 nM target analyte. The method may provide a level of detection of less than 200 nM target analyte. Preferably, the method may provide a level of detection of less than 100 nM target analyte. That is, it may be possible
25 to detect concentrations of less than 500 nM, 200 nM or preferably, 100 nM *bla_{NDM}* gene DNA within a biological sample using the method of the present aspect.

- For example, the method may provide a level of detection of 20 nM target analyte, 50 nM target analyte, 100 nM target analyte or 200 nM target analyte.

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- The step of fragmenting the DNA within the biological sample may be adapted to provide a population of DNA fragments between 20 and 1000 base pairs in length. For example, the majority of the DNA fragments within the population of DNA fragments may be between 20 and 1000 base pairs in length, between 20 and 750 base pairs in length, or between 20 and
35 500 base pairs in length. For example, incubation of plasmid DNA for 1.5 minutes with 0.8 U/ng DNase may result in a population of DNA fragments that are 25-50 base pairs long.

According to a fourth aspect of the invention, there is provided an electrochemical sensor for the detection of bacteria having the *bla_{NDM}* gene comprising a sensing surface and a sample chamber; the sensing surface defining an interior surface of the sample chamber; the sensing surface comprising a plurality of biochemical probes according to the first aspect of the invention such that the addition of a biological sample comprising *bla_{NDM}* gene DNA to the sample chamber results in the detection of hybridisation between the recognition sequence of the biochemical probes within the plurality of biochemical probes and the *bla_{NDM}* gene DNA.

The electrochemical sensor may be an electrochemical impedance spectroscopy sensor, and the sensing surface may be a surface of a working electrode. Typically, hybridisation of *bla_{NDM}* gene DNA to the recognition sequence of the biochemical probes within the plurality of biochemical probes on the sensing surface of the working electrode results in an increase in the *R_{ct}* determined by the sensor.

The electrochemical sensor may be a surface plasmon resonance spectroscopy (SPRS) sensor, and the sensing surface may be the sensing surface of the SPRS sensor. Typically, hybridisation of *bla_{NDM}* gene DNA to the recognition sequence of the biochemical probes within the plurality of biochemical probes on the sensing surface results in a change in the wavelength of light absorbed by the sensing surface, measured as a change in angle of reflection minimum (corresponding to the maximum absorption), for example.

Preferred and optional features of the first to third aspects of the invention are preferred and optional features of the fourth aspect of the invention.

Embodiments of the present invention will now be described, by way of non-limiting examples, with reference to the accompanying drawings.

Brief Description of the Figures

Figure 1 shows fluorescence microarray data showing mean fluorescence for each probe developed *in silico*, upon hybridisation of 4 ng/μL fluorescently labelled, 620 bp long *bla_{NDM}* PCR product (amplicon 2); n=5. Excluding controls *bla_{NDM}* probe 7S3' (P7, comprising SEQ ID NO 7) yields highest detection sensitivity. The nomenclature signifies probe number followed by sense (S)/ anti-sense (AS) of sequence output by UPS software, with 5'/ 3' end immobilised on the microarray;

Figure 2 shows fluorescence microarray data showing mean fluorescence for each probe developed *in silico*, upon hybridisation of 4 ng/ μ L fluorescently labelled, 431 bp long *bla*_{NDM} PCR product (amplicon 4); n=5. Excluding controls *bla*_{NDM} probe 25AS5' (P25, comprising SEQ ID NO 25) yields highest detection sensitivity. The nomenclature signifies probe number
5 followed by sense (S)/ anti-sense (AS) of sequence output by UPS software, with 5'/ 3' end immobilised on the microarray;

Figure 3 is a dose-response curve of EIS detection of short synthetic oligonucleotide using PNA probe P7 construct using Rct value at 60 minutes (52 minutes post sample addition)
10 normalized to baseline Rct values;

Figure 4 is an online EIS detection assay plot showing Rct changes on P7 functionalised electrodes over time post addition of 10 nM PCR product treated with Lambda exonuclease for different incubation times (0-25 minutes) normalized to baseline Rct values. A synthetic
15 ssDNA oligonucleotide non-complementary to the probe was included as a negative control (nc ssDNA), showing response comparable to that of dsDNA without Lambda exonuclease treatment (0 minutes). A 20% enhancement in EIS Rct increase was achieved by increasing the Lambda exonuclease incubation time from 5 to 25 minutes;

Figure 5 is a Dose response curve for 620 bp ssDNA *bla*_{NDM} PCR product detected with the online EIS assay normalized to baseline Rct values (LOD of 100 pM, n=3);
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Figure 6 shows Rct changes over time upon hybridisation with 10 nM ssDNA *bla*_{NDM} PCR products and non-complementary *mecA* PCR products (negative control) under ambient
25 conditions in the presence and absence of 50% formamide in the EIS buffer normalized to baseline Rct values (target addition after 8 minutes). The figure shows the enhancement of specificity of *bla*_{NDM} PCR product detection by addition of formamide during hybridisation;

Figure 7 shows direct detection of NDM plasmid DNA. Online EIS detection assay plot
30 showing Rct change on *bla*_{NDM} specific PNA P7 functionalised electrodes normalised to Rct change on negative control PNA functionalised electrode over time (n $\geq$ 2);

Figure 8 shows an example of a specificity check on *bla*_{NDM} probes on DNA microarray. Hybridisation of 4 ng/ μ L fluorescent *Pseudomonas aeruginosa* 16S rDNA PCR product on
35 DNA microarray functionalized with *bla*_{NDM} specific probes and controls. The only probes producing a fluorescence signal are positive control *P. aeruginosa* probes, hybridisation and spotting controls. This indicates very good specificity with all *bla*_{NDM} probes tested;

Figure 9 shows an example of EIS control experiment (left) whereby 1 μ M synthetic perfect match oligonucleotide was hybridised on P7 functionalised and blocked PNA-free electrodes to verify electrode functionality. Right: As a control on linearisation of plasmid sample an agarose gel was run. Lane 1: 1kb ladder, lane 2: Intact plasmid, lane 3: S1 nuclease treated plasmid; and

Figure 10 is a schematic drawing of the relative position of the selected NDM probes P7 and P25, and length and relative position of the four different tested PCR products.

Specific Description of Embodiments of the Invention

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

Materials and Methods

Reagents

DNA oligonucleotides were purchased from Metabion (Martinsried, Germany). PNA oligonucleotides were ordered via Cambridge Research Biochemicals (Cleveland, UK) from Panagene (Daejeon, South Korea). PCR kit and Qiaspin Miniprep kits were purchased from Qiagen (Crawley, UK). Potassium ferricyanide, potassium ferrocyanide, phosphate buffered saline, monosodium phosphate, disodium phosphate and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Poole, UK). Lambda exonuclease was obtained from EURx (Gdansk, Poland) Deionised water was used throughout the study ($>18 \text{ M}\Omega \text{ cm}$). DNase RQ1 was obtained from Promega (Mannheim, Germany). The clinical isolate used for all

investigations was a NDM-1 producing *Citrobacter freundii* strain isolated at the Edinburgh Royal Infirmary.

In-silico NDM-1 probe design

5 *bla*_{NDM-1} specific probes of 20 nt in length were designed *in silico* with an online tool named UPS Unique Probe Selector (UPS) (<http://array.iis.sinica.edu.tw/ups/>). The UPS algorithm considers percent guanine-cytosine (%GC) content, the secondary structure, melting temperature (T_m), the stability of the probe-target duplex estimated by the thermodynamic model, sequence complexity, similarity of probes to non-target sequences, and other empirical
10 parameters used in the laboratory when selecting probes.

The option to select probes at a 'pangenomic level' was selected and the 826 bp *bla*_{NDM-1} sequence (accession no. FN396876.1, sequence given below) was entered in FASTA format. A salt concentration of 330 mM was specified and subsequently 10 probes, 20 nucleotides in
15 length, were generated. The candidate set of probes were further scrutinized by considering the %GC content, melting temperatures (T_m) and the free energy (ΔG) values of possible secondary structures such as self-dimer and hairpin structures. These values were produced using OligoAnalyzer 3.1 (available at
<http://eu.idtdna.com/analyzer/applications/oligoanalyzer/>). The sodium ion concentration was
20 adjusted from 50 mM to 330 mM but default settings remained otherwise. The results were used to refine the probe selection upon recommendation. All probe candidates were subjected to a further specificity test using BLASTN (available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Probecheck (available at <http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home>).¹⁶ The candidate sequences (shown in Table 3 below)
25 were entered in FASTA format to both servers and checked against the nr/nt database within BLASTN and both the 16S/18S rRNA and the 23S/28S rRNA databases within Probecheck. Default settings were used in both cases.

The selection process allowed ten optimal probe sequences to be selected of which seven
30 were selected. The complete probe set chosen for further experiments consisted of two previously identified primers (probes numbered 29-32¹⁷ and 33-36¹⁸) and seven UPS generated probe sequences (Table 2).

Item	ID	Sequence (5'-3')	Comment	SEQ ID NO
NDM specific probe	P7	GTGCTGCCAGACATTCGGTG	3' modification: C11M-AEEEEA	7
NDM specific probe	P25	ATCAGGCAGCCACCAAAAGC	5' modification: C11M-AEEEEA-	25

Negative control probe	P92	TCAATGAGCAAAGGT	5' modification C11M-AEEEEA	39
Synthetic oligonucleotide P7 complement	P7C	CACCGAATGTCTGGCAGCAC		40
Synthetic oligonucleotide negative control	P25 C	GCTTTTGGTGGCTGCCTGAT		41
NDM Primer (amplicon 2 620 bp, forward)	2F	GGTTTGGCGATCTGGTTTTTC	Published sequence ¹⁹	42
NDM Primer (amplicon 2, 620 bp, reverse)	2R	CGGAATGGCTCATCACGATC	Published sequence ¹⁹	43
NDM Primer (amplicon 1 (264 bp, forward)	1F	ACCGCCTGGACCGATGACCA	Published sequence ²⁰	44
NDM Primer (amplicon 1 (264 bp, reverse)	1R	GCCAAAGTTGGGCGCGGTTG	Published sequence ²⁰	45
NDM Primer (amplicon 3 (497 bp, forward)	3F	GGTTTGGCGATCTGGTTTTTC	Published sequence ²¹	46
NDM Primer (amplicon 3 (497 bp, reverse)	3R	CGAATGTCTGGCAGCACACTT	Published sequence ²¹	47
NDM Primer (amplicon 4 (431 bp, forward)	4F	TTGGCCTTGCTGTCCTTGA	Published sequence ²¹	48
NDM Primer (amplicon 4 (431 bp, reverse)	4R	GTCGCTTCCAACGGTTTGAT	Published sequence ²¹	49

Table 2: PNA Probe and Primer Sequences

The investigation took into account the difference between sense and anti-sense hybridisation as well as the potential variability that may arise from immobilizing the probes at either the 5' or the 3' end of the oligonucleotide probe. In this regard, probes were ordered with amino modification at 5' or 3' of the sequence for immobilisation of both orientations. Sense (the strand corresponding to mRNA sequence) and anti-sense (the strand complementary to the mRNA sequence) sequences were also investigated as variability in hybridisation efficiency also occurs in this respect. This doubled the size of the probe set of nine deduced from the selection process to eighteen. Each of the eighteen probes, both sense and anti-sense, was

designed with a 12-thymine spacer and amino-modification at either the 5' or 3' end. This concluded the *b/a_{NDM-1}* specific probe selection process with a total of thirty six probes deduced from the initial pool of nine probes (Table 3).

Probe sequence	Probe position within GenBank: FN396876.1	Probe No.	SEQ ID NO
5'- TTT TTT TTT TTT GTC CAT ACC GCC CAT CTT GT -3'	nt. 2830-2849	1	50
5'- TTT TTT TTT TTT ACA AGA TGG GCG GTA TGG AC -3'	nt. 2849-2830	2	51
5'- GTC CAT ACC GCC CAT CTT GTT TTT TTT TTT TT -3'	nt. 2830-2849	3	52
5'- ACA AGA TGG GCG GTA TGG ACT TTT TTT TTT TT -3'	nt. 2849-2830	4	53
5'- TTT TTT TTT TTT GTG CTG CCA GAC ATT CGG TG -3'	nt. 3037-3056	5	54
5'- TTT TTT TTT TTT CAC CGA ATG TCT GGC AGC AC -3'	nt. 3056-3037	6	55
5'- GTG CTG CCA GAC ATT CGG TGT TTT TTT TTT TT -3'	nt. 3037-3056	7	56
5'- CAC CGA ATG TCT GGC AGC ACT TTT TTT TTT TT -3'	nt. 3056-3037	8	57
5'- TTT TTT TTT TTT GGC GTA GTG CTC AGT GTC GG -3'	nt. 2530-2549	9	58
5'- TTT TTT TTT TTT CCG ACA CTG AGC ACT ACG CC -3'	nt. 2549-2530	10	59
5'- GGC GTA GTG CTC AGT GTC GGT TTT TTT TTT TT -3'	nt. 2530-2549	11	60
5'- CCG ACA CTG AGC ACT ACG CCT TTT TTT TTT TT -3'	nt. 2549-2530	12	61
5'- TTT TTT TTT TTT GTC ATC GGT CCA GGC GGT AT -3'	nt.2932-2951	13	62
5'- TTT TTT TTT TTT ATA CCG CCT GGA CCG ATG AC -3'	nt. 2951-2932	14	63
5'- GTC ATC GGT CCA GGC GGT ATT TTT TTT TTT TT -3'	nt.2932-2951	15	64
5'- ATA CCG CCT GGA CCG ATG ACT TTT TTT TTT TT -3'	nt. 2951-2932	16	65
5'- TTT TTT TTT TTT TTG AGG ATC TGG GCG GTC TG -3'	nt. 2912-2931	17	66
5'- TTT TTT TTT TTT CAG ACC GCC CAG ATC CTC AA -3'	nt. 2931-2912	18	67
5'- TTG AGG ATC TGG GCG GTC TGT TTT TTT TTT TT -3'	nt. 2912-2931	19	68
5'- CAG ACC GCC CAG ATC CTC AAT TTT TTT TTT TT -3'	nt. 2931-2912	20	69
5'- TTT TTT TTT TTT CGC CGC AAC CAT CCC CTC TT -3'	nt. 2749-2768	21	70
5'- TTT TTT TTT TTT AAG AGG GGA TGG TTG CGG CG -3'	nt. 2768-2749	22	71
5'- CGC CGC AAC CAT CCC CTC TTT TTT TTT TTT TT -3'	nt. 2749-2768	23	72
5'- AAG AGG GGA TGG TTG CGG CGT TTT TTT TTT TT -3'	nt. 2768-2749	24	73
5'- TTT TTT TTT TTT ATC AGG CAG CCA CCA AAA GC -3'	nt. 2591-2610	25	74
5'- TTT TTT TTT TTT GCT TTT GGT GGC TGC CTG AT -3'	nt. 2610-2591	26	75
5'- ATC AGG CAG CCA CCA AAA GCT TTT TTT TTT TT -3'	nt. 2591-2610	27	76
5'- GCT TTT GGT GGC TGC CTG ATT TTT TTT TTT TT -3'	nt. 2610-2591	28	77
5'- TTT TTT TTT TTT GCC AAA GTT GGG CGC GGT TG -3'	nt. 2686-2705	29	78
5'- TTT TTT TTT TTT CAA CCG CGC CCA ACT TTG GC -3'	nt. 2705-2686	30	79
5'- GCC AAA GTT GGG CGC GGT TGT TTT TTT TTT TT -3'	nt. 2686-2705	31	80
5'- CAA CCG CGC CCA ACT TTG GCT TTT TTT TTT TT -3'	nt. 2705-2686	32	81
5'- TTT TTT TTT TTT GAG ATT GCC GAG CGA CTT G -3'	nt. 2557-2575	33	82
5'- TTT TTT TTT TTT CAA GTC GCT CGG CAA TCT C -3'	nt. 2575-2557	34	83
5'- GAG ATT GCC GAG CGA CTT GTT TTT TTT TTT T -3'	nt. 2557-2575	35	84
5'- CAA GTC GCT CGG CAA TCT CTT TTT TTT TTT T -3'	nt. 2575-2557	36	85

5 Table 3: Microarray DNA Probe Sequences

DNA microarray fabrication

Amino-modified oligonucleotides were spotted in 1x Schott Nexterion spot buffer (20µM) in replicates of three within each array on Schott Nexterion Slides E (epoxysilane modified surface; Schott, Jena, Germany) with four 200µm (diameter) split pins and a MicroGrid II (BioRobotics, Cambridge, UK) at 40-50% relative humidity at room temperature. Epoxysilane slides were immediately immobilized at a relative humidity of 75% at room temperature for

one hour followed by storage overnight at room temperature under dry conditions. This generated spots with a diameter of approximately 200 µm. Each oligonucleotide was equipped with a 12-thymidine spacer and an amino modification at the 5' end. The slides were then washed with 0.1% TritonX-100 solution under constant mixing for five minutes at room temperature, with 1 mM HCl solution for four minutes, with 100 mM KCl solution for ten minutes, and with deionized water for one minute. The slides were blocked with 50 mM ethanolamine and 0.1% sodium dodecyl sulfate (SDS) in 0.1M Tris buffer (pH 9) for fifteen minutes at 50°C. After blocking the slides were washed in deionized water for one minute and then dried by centrifugation (two minutes at 800g).

Microarray hybridisation and data acquisition

Hybridisation was done using Agilent 8 gasket slides and hybridisation chambers (Agilent Technologies, Stockport, UK). 50µL hybridisation solution (100nM hybridisation control plus *bla_{NDM-1}* PCR product), 2x SSC (300mM NaCl and 30mM sodium citrate) were added to each of the gaskets. The printed slide which had been washed and blocked was placed array facing down on top of the hybridisation solution. The sandwich slides were then sealed using a hybridisation chamber and rotated in a pre-heated oven at 55°C for two hours. Epoxysilane slides were washed with 2x saline sodium citrate buffer (SSC) and 0.1% SDS for ten minutes, 2x SSC for ten minutes, 0.2x SSC for ten minutes. Each slide was then dipped in distilled water for a few seconds and dried by centrifugation for two minutes at 800g.

Fluorescence images were generated with a Tecan LS Reloaded fluorescence scanner (Tecan, Maennedorf, Switzerland) with excitation at 532nm and emission at 575nm. Target sequences were labeled with the Cy3 fluorophore as described below. Quantification of fluorescence signal intensities was performed with the Quantarray software (Perkin Elmer, Waltham, MA) using the histogram quantification method. For further analysis, the mean signal intensity minus local background intensity was processed with Excel (Microsoft Corp., Redmond, USA) and the mean and standard deviation of all replicates were calculated.

DNA extraction and PCR

Plasmid DNA was extracted from an overnight Luria-Bertani Broth culture of an NDM-1 producing *Citrobacter freundii* clinical isolate using Qiaspin Miniprep Kit (Qiagen Crawley, UK). For PCR amplification 5µL *Citrobacter freundii* NDM-1 plasmid DNA was mixed with 4 µM *bla_{NDM-1}* specific primers, 0.1 mM deoxynucleotide triphosphates (dNTPs), 1x Taq buffer (50 mM KCl, 10 mM Tris HCl (pH 9.0 at 25°C), 1.5 mM MgCl₂ and 0.1% Triton X-100), 1x Q-solution, 1.0 mM MgCl₂ and 0.1 U HotstarTaq polymerase (Qiagen, Hilden, Germany) in a final volume of 25 µL. The *bla_{NDM-1}* specific forward primer was 5' phosphate modified for

Lambda exonuclease treatment to produce single-stranded products. Where fluorescent PCR products were required for microarray dNTPs were substituted with 0.1 mM dATP, dGTP and dTTP were added, in combination with 0.6 mM dCTP and 0.4 mM dCTP-Cy3 (GE healthcare, Buckinghamshire, UK). PCR products used in microarray work, TEM, SHV, KPC and 16S PCR were produced using the same reagent concentrations and conditions and primers. The amplification was performed in a Techne TC-512 thermal cycler (Bibby Scientific Limited, Stone, UK) using the following protocol: 95°C for fifteen minutes; 30 cycles at 95°C for one minute, 50°C for one minute and 72°C for one minute; followed by a final elongation at 72°C for ten minutes. Upon completion of the reaction amplicon was pooled and Lambda exonuclease treated. The length and relative size in the *bla_{NDM}* gene of each amplicon used (1-4) are shown in Figure 10.

DNA enzymatic treatments

To perform exonuclease treatment 200ng PCR product was incubated with 15 U Lambda exonuclease (EURx, Gdansk, Poland) in 1x exonuclease buffer for twenty five minutes at 37°C. In optimisation experiments incubation times of five, fifteen and twenty five minutes were applied at 37°C. The enzyme was then deactivated at 95°C for five minutes followed by cooling on ice. DNase treated plasmid was prepared by incubating the DNA with 0.8 mU DNase I (Promega, Mannheim, Germany) per ng plasmid in 1x DNase buffer for one and a half minutes at room temperature. By adding 3 mM ethylene glycol tetraacetic acid (EGTA) and incubating at 65°C for ten minutes, the fragmentation reaction was stopped. Enzymatic treatments were confirmed by standard electrophoresis or capillary electrophoresis (Bioanalyzer 2100, Agilent, UK) according to the manufacturer's instructions. For plasmid linearization, S1 nuclease 100U per µg of DNA was incubated for twenty minutes at 37°C, in the presence of 1X nuclease buffer. The reaction was stopped with 16 mM EDTA and heating at 70°C as recommended by the manufacturer.

Electrode Preparation

Screen printed dual gold working electrode (1.7mm X 4mm) sensors with an integrated Ag/AgCl reference electrode (Dropsens, Oviedo, Spain) were cleaned using cyclic voltammetry in 100 mM aqueous sulphuric acid and functionalised with 1.5 µM thiol-modified PNA solution + 30 µM mercaptohexanol + 5 mM Tris(2-carboxyethyl)phosphine in 50% (v/v).¹⁵

EIS Measurement

Following functionalisation, EIS measurements were performed on screen printed sensors connected to an Autolab PGSTAT12 potentiostat (Metrohm Autolab, Herisau, Switzerland) at open circuit potential at an amplitude of 10mV rms at fifteen frequencies in the range 100,000

Hz – 0.1 Hz.¹⁵ Hybridisation and measurement were performed in 0.1 mM $K_4[Fe(CN)_6]$ + 0.1 mM $K_3[Fe(CN)_6]$ + pH 7.0 10 mM phosphate buffer + 20 mM NaCl. In the case of DNase treated plasmid DNA, sample was heated to 95°C for five minutes, and transferred to ice for two minutes prior to measurement. PCR products and synthetic DNA were measured without preheating.

Results and Discussion

EIS assay design

Uncharged PNA probes were used in place of polyanionic DNA which allow a low background signal to be achieved resulting in improved resolution upon binding of charged DNA target as described previously.²²⁻²⁵

Probe density within an alkane-thiol monolayer of 5% mole fraction was employed for optimal sensitivity. When dealing with long DNA targets a trade-off exists between the theoretical EIS signal enhancement and the accessibility of the target sequence. Enhanced EIS signal transduction is expected resulting from the abundant negative charges of the phosphate backbone of the target DNA hybridised with the PNA probe at the electrode surface causing a higher degree of repulsion of anionic redox mediators. However, a large DNA target may result in a lower accessibility of the 20 nucleotides (nt) complementary to the immobilised probe. In this regard, achieving an optimal probe density and target length are key components of sensitive detection.

The use of low ionic strength solution during EIS measurement allows greater sensitivity to be achieved resulting from the increase in the electrostatic barrier for the redox mediator to reach the electrode surface upon target hybridisation. While the on-line assay format demonstrated herein implies that both DNA-PNA hybridisation and measurement take place in one solution, optimal ionic strength reported by Keighley et al of 10 mM electrolyte was successfully implemented.²⁶ Within each dual working electrode EIS assay, a PNA-free working electrode was included for SAM quality control and background normalization of non-specific absorption of molecules on the electrode surface which contributed to specificity of the assay (see Figure 9).

In silico design and microarray validation of *bla*_{NDM} probe sequences

To facilitate EIS investigations for development of a *bla*_{NDM} biosensor, it was necessary to develop probe sequences which could specifically and sensitively detect *bla*_{NDM} sequences. This was achieved by *in silico* probe design and validation on glass DNA microarray with fluorescently labelled *bla*_{NDM} PCR products. *bla*_{NDM} specific probes of 20 nt in length were designed *in silico* with an online tool named UPS Unique Probe Selector. Since the hybridisation efficiency of long PCR products with probes immobilised on solid supports is influenced by probe orientation and relative probe/target position both 3' and 5' immobilised sense and antisense probes were tested with regard to their affinity for *bla*_{NDM} PCR product.^{27,28} Probes were contact printed on glass epoxy coated slides immobilised via a 12-thymine spacer and terminal amino-modification. The hybridisation of fluorescence-labelled

PCR products which were generated using known *bla_{NDM}* specific primers resulted in the highest fluorescence intensity for probe 7 (with amplicon 2) and for probe 25 (with amplicon 4), which were consequently selected for the further EIS assay development (see Figures 1 and 2). Despite being selected by *in silico* analysis, many probe sequences proved unsuitable for hybridisation of *bla_{NDM}* PCR products. These observations showed that experimental validation of *in silico* designed probes using DNA microarrays is essential for identification of probes which bind efficiently to long DNA targets of interest when immobilised on a solid support. The use of fluorescence-based DNA microarrays for pre-selection of *in silico* designed probes enabled the test of a large number of probes in parallel which could not have been done on the electrochemical platform in the same time frame. This new approach of combining fluorescence-based DNA microarrays with electrochemical detection platforms for assay development has the potential to significantly enhance the assay performance of nucleic acid based, electrochemical *in vitro* diagnostic tests.

The probes were also tested for their cross reactivity towards other relevant antibiotic resistance gene specific PCR products, including genes encoding the beta-lactamases TEM, SHV and KPC, and a *P. aeruginosa* 16S rDNA PCR product.²⁹⁻³² As can be seen in Figure 8 there was virtually no cross-hybridisation of any of the tested probes to *P. aeruginosa* 16S rDNA PCR product. Similar results were obtained with *bla_{TEM}*, *bla_{SHV}* and *bla_{KPC}* PCR products (data not shown). A thiol-terminated PNA probe of equivalent sequence to probe 7 was obtained for EIS biosensor development. PNA probe P7 was designed to bind the *bla_{NDM}* PCR product target with a short (32 nt) 3' and long (571 nt) 5' overhang. The long overhang was expected to be facing towards the bulk solution, which suits EIS application in terms of accessibility and signal transduction. Alignment of published sequences of all six current *bla_{NDM}* variants (GenBank accession numbers JF826285.1, JF703135.1, JQ734687.1, JQ348841.1, JN104597.1, JQ235754.1, for the individual sequences see the *bla_{NDM}* sequence section below) using alignment software (CodonCode Aligner, CodonCode Corporation, USA) showed that this probe sequence targeted a conserved region within the *bla_{NDM}* gene.

EIS detection of synthetic DNA target

The *bla_{NDM}* specific PNA probe 7, comprising an AEEA linker and C11M anchor, was first tested with a short artificial target directly complementary to the probe sequence. Alternative linkers, or spacers, are well known to the person skilled in the art and are described on pages 10 to 16 of WO 2013/076143 (ITI Scotland Limited), for example. The applied EIS setup enabled the direct, label-free detection of target hybridisation to the immobilized probe. EIS measurements prior to and after target addition allowed the hybridisation to be monitored over the course of ten subsequent EIS spectra, each plotted as Nyquist Plots for capture of charge

transfer resistance values (Rct). As an example, the Rct value increased by more than 1600% (signal increase ratio, dRct = 17) after a fifty minute incubation with 500nM short, synthetic target. Figure 3 shows the standard curve which was established based on the dRct after sixty minutes (fifty two minutes after target addition, respectively the tenth consecutive EIS measurement after target addition). The mean increase in Rct above the baseline was plotted relative to time post sample addition for each synthetic oligonucleotide concentration tested. The limit of detection of hybridisation of a synthetic oligonucleotide to immobilised PNA probes was determined to be 10nM.

EIS detection of PCR product

PCR products were generated using plasmid DNA isolated from an NDM-1 producing *Citrobacter freundii* strain as template and published primers. Initially, investigations into hybridisation of double stranded PCR products on PNA probe 7 functionalised electrode chip were carried out. However, the Rct change yielded upon hybridisation of 10nM *bla*_{NDM-1} PCR product (620 bp, dsDNA) was in the range of non-specific absorption observed on the PNA-negative control electrode and upon hybridisation of non-complementary DNA (see Figure 4).

Higher signals caused by an invasion of PNA into the dsDNA helix displacing the complementary DNA strand or forming a stable triplex structures may be expected.³³ It may be that the ability of immobilised PNA to infiltrate the dsDNA target is hindered by the restricted accessibility of the immobilized PNA probe, the length of the dsDNA target, and the ionic strength of the solution.³⁴

Consequently, a protocol for generation of ssDNA target was applied to improve target accessibility. Lambda exonuclease, which degrades one strand of double-stranded DNA in 5'-3' direction when a phosphate is present at the 5' position was employed for this purpose.³⁵ Upon digestion, PCR products were analyzed by capillary electrophoresis. The ssDNA state of digested product was identified by altered migration in the gel (above the upper marker at >1500 bp), since ssDNA tends to fold into secondary structures. The hybridisation of 10 nM of 620 bp long ssDNA *bla*_{NDM} PCR product produced a specific, significant Rct signal increase. Figure 4 shows the signal change over time caused by target binding to the probe under ambient conditions without mixing of the solution. Optimisation of enzyme digestion time over a range of five to twenty five minutes allowed a further enhancement of this detection, with a 20% increase in sensitivity when twenty five minute incubation time was applied (see Figure 4). This optimized Lambda exonuclease treatment protocol applied to 10nM *bla*_{NDM} PCR product generated a Rct value increase of 170% (dRct 2.7) after twenty eight minute

incubation. Figure 4 shows that there is a significant signal change within the first ten minutes after target addition.

The 620 bp *bla_{NDM}* ssPCR product was tested over a concentration range from 0.1 to 50 x 10⁻⁹ M and an EIS standard curve was constructed using Rct data derived from Nyquist plots of baseline EIS spectra (before target addition) and after ten consecutive EIS measurements following sample addition (Figure 5). A LOD of 100 pM (0.1x10⁻⁹M; 0.05 ng/μL) was achieved. This very sensitive detection of long single-stranded PCR products and a 100X lower LOD compared to the detection of a short complementary target was attributed to the enhancement of EIS signal transduction resulting from the large number of negative charges at the electrode surface repelling anionic redox mediators. These results correlate well with data for the EIS based *mecA* detection (a LOD of 10 nM (10x10⁻⁹M) for an artificial target and even 10 pM (10x10⁻¹²M) LOD for *mecA* PCR products.¹⁵)

NDM PCR products of 100pM can be generated from 10³ gene copies/mL as determined by colony counting. This LOD of 10³ gene copies/mL is similar to the LOD described by a commercial molecular assay for ESBL and carbapenem resistances in Gram negative bacteria. The hyplex SuperBug ID test (Amplex Biosystems, Gars, Germany; <http://www.hypex.de/index.php?id=126&L=2>) which is based on PCR amplification and reverse hybridisation can detect NDM-1 genes together with other resistance genes in 2.5 – 4 h with a LOD of 9.3 x 10³ gene copies/mL. While growth based methods such as chromID ESBL have lower limits of detection³⁶, the drawbacks of a much longer TTR and *in vitro* growth conditions are limiting.

The specificity of direct EIS detection of ssPCR products under ambient conditions was investigated by comparison of *bla_{NDM}* PCR product caused EIS response to 550 bp *mecA* ssPCR product¹⁵ on PNA probe 7 functionalised electrodes (all Lambda exonuclease treated). *MecA* PCR product was generated using known primers and no region of >2 nt sequence complementarily to the *bla_{NDM}* probe was identified *in silico*.³⁷

Initial experiments yielded poor discrimination, with the non-complementary DNA (*mecA*) causing Rct increases higher than for the complementary target (*bla_{NDM}*), as shown in Figure 6. This low specificity was not unexpected considering the fact that the hybridisation was performed under highly non-stringent conditions working at room temperature without mixing of the sample solution.

However, for the *bla_{NDM}* assay to be used at the point of care, the assay preferably works at room temperature and without mixing. The inventors have found that the incorporation of 50% formamide (v/v) during hybridisation at room temperature resulted in a 66% enhanced discrimination between the specific hybridisation of the *bla_{NDM}* ssDNA and the non-specific binding of the *mecA* ssDNA to PNA probe 7 as seen in Figure 6.

Amplification-free EIS detection of *bla_{NDM}* plasmid DNA

Sample preparation has become a bottleneck for molecular diagnostics and a shift toward simpler pre-analytics would advantageous in terms of rapid point of care diagnosis to aid informed treatment decisions. An amplification-free detection of nucleic acid targets avoiding PCR and thermal cycling requirements would significantly reduce the time-to-result and the instrumental requirements of the test. Consequently, we investigated the direct detection of the target gene containing plasmid. The *bla_{NDM-1}* gene of the *Citrobacter freundii* clinical isolate detected in this study was encoded on a 3.5 kb plasmid. The size of the plasmid was determined by S1 nuclease treatment and gel electrophoresis.³⁸

DNase treatment is routinely used in hybridisation assays to improve accessibility of target sequences to probes. Success of DNase treatment in EIS depends on generating a target of a length with which the balance between accessibility of the complementary probe sequence to the target sequence and signal enhancement is optimum. The incubation of the plasmid DNA for one and a half minutes with 0.8 U/ng DNase resulted in 25-50 bp long fragments. These DNase treated plasmid samples could be detected directly by EIS at ambient conditions at the low nanomolar range (Figure 7). Data represented in Figure 7 show the specific signal increase normalised to Rct change on a negative control PNA functionalised electrode (sequence detailed in Table 2).

***bla_{NDM}* Sequences**

Accession no. FN396876.1 (*bla_{NDM-1}*) SEQ ID NO 86

```

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     121 catcaccgtc cgcggagatg ttgcggagaa cctgcagccg ccggatgtct ccaccgcgct
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     241 ggggtcgatt gaggaatccg gcaaggccac ccgggcgatg gtgccgttat ttccgataat
35     301 gatcgccctc acgctgctga tcattatcct gcagggtgct tcgctgtcgg cgatgggtcat
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	1021	agggcataaa	tataggcccg	accgagcagc	acgcctttcg	cgccgagggc	cagcagacgt
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	1261	gcatcgagga	tcctggcacc	gctgggggtta	tctcacacct	ttattactgt	gccggaaagt
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	1741	gccttccaca	ccggcttgcc	aaaccgcgcg	cgcaactggg	ccacgcgttc	gggcgattcc
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	1861	tcattccgcat	cgacgaacaa	accgaccatg	gcgatctggc	cagctgcgcg	cgatgtcaaa
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 301 tcctatctcg acatgccggg tttcggggca gtcgcttcca acggtttgat cgtcagggat
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 481 caggacaaga tgggcggtat ggacgcgctg catgcggcgg ggattgcgac ttatgccaat
 40 541 gcgttgtcga accagcttgc cccgcaagag gggctggttg cggcgcaaca cagcctgact

601 ttgcgcgcca atggctgggt cgaaccagca accgcgcccc acttttgccc gctcaaggta
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 781 gatgccgaca ctgagcacta cgccgcgtca gcgcgcgcgt ttggtgcggc gttccccaag
 5 841 gccagcatga tcgtgatgag ccattccgcc ccgcatagcc gcgcgcgaat cactcatacg
 901 gcccgcattg ccgacaagct gcgtgagcc atggctgacc acgtcacccc caatctgcca
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Accession no. JQ235754.1 (NDM-6) SEQ ID NO 92

10 1 cccaatatta tgcacccggt cgcgaagctg agcaccgcat tagccgctgc attgatgctg
 61 agcgggtgca tgcccgggtga aatccgcccg acgattggcc agcaaatgga aactggcgac
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 181 tatctcgaca tgccggggttt cggggcagtc gcttccaacg gtttgatcgt cagggatggc
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 15 301 tggatcaagc aggagatcaa cctgccggtc gcgctggcgg tggtgactca cgcgcacatcag
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 20 601 gcttttggtg gctgcctgat caaggacagc aaggccaagt cgctcgga tctcggatgat
 661 gccgacactg agcactacgc cgcgtcagtg cgcgcgtttg gtgcggcggt cccaaggcc
 721 agcatgatcg tgatgagcca ttccgcccc gatagccgcg ccgcaatcac tcatacggcc
 781 cgcattggccg aca

25 Accession no. KP265931.1 (NDM-7) SEQ ID NO 93

1 atggaattgc ccaatattat gcacccggtc gcgaagctga gcaccgcatt agccgctgca
 61 ttgatgctga gcgggtgcat gcccggtgaa atccgcccga cgattggcca gcaaatggaa
 121 actggcgacc aacggttttg cgatctggtt ttccgcccag tcgcaccgaa tgtctggcag
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 30 241 agggatggcg gccgcgtgct ggtggctgat accgcctgga ccgatgacca gaccgcccag
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 481 ctgactttcg ccgccaatgg ctgggtcgaa ccagcaaccg cgcccaactt tggcccgtc
 35 541 aaggatatttt acccgggccc cggccacacc agtgacaata tcaccgttgg gatcgacggc
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 661 ctcggtgatg ccgacactga gcaactacgc gcgtcagcgc gcgcgttttg tgccggcgttc
 721 cccaaggcca gcatgatcgt gatgagccat tccgccccg atagccgcgc cgcaatcact
 781 catacggccc gcatggccga caagctgcgc tga

Accession no. AB744718 (NDM-8) SEQ ID NO 94

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 61 ttgatgctga gcgggtgcat gcccggtgaa atccgcccga cgattggcca gcaaattggaa
 5 121 actggcgacc aacggttttg cgatctggtt ttccgccagc tcgcaccgaa tgtctggcag
 181 cacacttcct atctcgacat gccgggtttc ggggcagtcg cttccaacgg tttgatcgtc
 241 agggatggcg gccgcgtgct ggtggtcgat accgcctgga ccgatgacca gaccgccag
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 601 accgacatcg cttttggtgg ctgcctgac aaggacagca aggccaagtc gctcggcaat
 661 ctcggtgatg ccgacactga gcactacgcc gcgtcagcgc gcgcgtttgg tgcggcgttc
 15 721 cccaaggcca gcatgatcgt gatgagccat tccgcccccg atagccgcgc cgcaatcact
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Accession no. NG_041673.1 (NDM-9) SEQ ID NO 95

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 121 actggcgacc aacggttttg cgatctggtt ttccgccagc tcgcaccgaa tgtctggcag
 181 cacacttcct atctcgacat gccgggtttc ggggcagtcg cttccaacgg tttgatcgtc
 241 agggatggcg gccgcgtgct ggtggtcgat accgcctgga ccgatgacca gaccgccag
 25 301 atcctcaact ggatcaagca ggagatcaac ctgccggtcg cgctggcggg ggtgactcac
 361 gcgcatcagg acaagatggg cggatgaggc gcgctgcatg cggcggggat tgcgacttat
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 30 601 accgacatcg cttttggtgg ctgcctgac aaggacagca aggccaagtc gctcggcaat
 661 ctcggtgatg ccgacactga gcactacgcc gcgtcagcgc gcgcgtttgg tgcggcgttc
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35

Accession no. KF361506.1 (NDM-10) SEQ ID NO 96:

1 atggaattgc ccaatattat gcacccggtc gcgaagctga gcaccgcatt agccgctgca
 61 ttgatgctga gcgggtgcat gcccggtgaa atcagcccga cgattgacca gcaaattggaa
 121 actggcgacc aacggttttg cgatctggtt ttccgccagc tcgcaccgaa tgtctggcag
 40 181 cacacttcct atctcgacat gccgagtttc ggggcagtca cttccaacgg tttgatcgtc

241 agggatggcg gccgcgtgct ggtggctgat accgcctgga ccgatgacca gaccgcccag
 301 atcctcaact ggatcaagca ggagatcaac ctgccggtcg cgctggcgggt ggtgactcac
 361 ggcgatcagg acaagatggg cggatatggac gcgctgcatg cggcggggat tgcgacttat
 421 gccaatgcgt tgctgaacca gcttgccccg caagagggga tggttgcggc gcaacacagc
 5 481 ctgactttcg ccgccaatgg ctgggtcgaa ccagcaaccg cgcccaactt tggcccgcctc
 541 aaggatatttt accccggccc cgccacacc agtgacaata tcaccgttgg gatcgaccgc
 601 accgacatcg cttttggtgg ctgcctgatc aaggacagca aggccaagtc gctcggcaat
 661 ctcggtgatg ccgacactga gcactacgcc gcgtcagcgc gcgcgttttg tgcggcgcttc
 721 cccaaggcca gcatgatcgt gatgagccat tccgcccccg atagccgcgc cgcaatcact
 10 781 catacgcccc gcatggccga caagctgcgc tga

Accession no. KP265940.1 (NDM-11) SEQ ID NO 97:

1 atggaattgc ccaatattat gcaccgggtc gcgaagctga gcaccgcatt agccgctgca
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 15 121 actggcgacc aacggttttg cgatctggtt ttccgccagc tcgcaccgaa tgtctggcag
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 241 agggatggcg gccgcgtgct ggtggctgat accgcctgga ccgatgacca gaccgcccag
 301 atcctcaact ggatcaagca ggagatcaac ctgccggtcg cgctggcgggt ggtgactcac
 361 ggcgatcagg acaagatggg cggatatggac gcgctgcatg cggcggggat tgcgacttat
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 661 ctcggtgatg ccgacactga gcactacgcc gcgtcagcgc gcgcgttttg tgcggcgcttc
 25 721 cccaaggcca gcatgatcgt gatgagccat tccgcccccg atagccgcgc cgcaatcact
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Accession no. AB926431.1 (NDM-12) SEQ ID NO 98:

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 181 cacacttcct atctcgacat gccgggtttc ggggcagtcg cttccaacgg tttgatcgtc
 241 agggatggcg gccgcgtgct ggtggctgat accgcctgga ccgatgacca gaccgcccag
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 601 accgacatcg cttttggtgg ctgcctgatc aaggacagca aggccaagtc gctcggcaat
 40 661 ctcgatgatg ccgacactga gcactacgcc gcgtcagcgc gcgcgttttg tgcggcgcttc

721 cccaaggcca gcatgatcgt gatgagccat tccgcccccg atagccgcgc cgcaatcact
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5 Accession no. LC012596.1 (NDM-13) SEQ ID NO 99;

1 atggaattgc ccaatattat gcacccggtc gcgaagctga gcaccgcatt agccgctgca
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 121 actggcgacc aacggtttgg cgatctggtt ttccgccagc tcgcaccgaa tgtctggcag
 10 181 cacacttcct atctcgacat gccgggtttc ggggcagtcg cttccaacgg tttgatcgtc
 241 agggatggcg gccgcgtgct ggtggtcgat accgcctgga ccaatgacca gaccgcccag
 301 atcctcaact ggatcaagca ggagatcaac ctgccggtcg cgctggcggg ggtgactcac
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 721 cccaaggcca gcatgatcgt gatgagccat tccgcccccg atagccgcgc cgcaatcact
 20 781 catacggccc gcatggccga caagctgcgc tga

Reference List

25

1. The bacterial challenge - time to react. *Ejhp Practice* 2009, 15 (5), 15.
2. Kumarasamy, K. K.; Toleman, M. A.; Walsh, T. R.; Bagaria, J.; Butt, F.; Balakrishnan, R.; Chaudhary, U.; Doumith, M.; Giske, C. G.; Irfan, S.; Krishnan, P.; Kumar, A. V.; Maharjan, S.; Mushtaq, S.; Noorie, T.; Paterson, D. L.; Pearson, A.; Perry, C.; Pike, R.; Rao, B.; Ray, U.; Sarma, J. B.; Sharma, M.; Sheridan, E.; Thirunarayan, M. A.; Turton, J.; Upadhyay, S.; Warner, M.; Welfare, W.; Livermore, D. M.; Woodford, N. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infectious Diseases* 2010, 10 (9), 597-602.
- 35 3. Elander R.P. Industrial production of b-lactam antibiotics. *Appl Microbiol Biotechnol* 2003, (61), 385-392.
4. Poirel, L.; Revathi, G.; Bernabeu, S.; Nordmann, P. Detection of NDM-1-Producing *Klebsiella pneumoniae* in Kenya. *Antimicrobial Agents and Chemotherapy* 2011, 55 (2), 934-936.

5. Arpin, C.; Noury, P.; Boraud, D.; Coulange, L.; Manetti, A.; Andre, C.; M'Zali, F.; Quentin, C. NDM-1-Producing *Klebsiella pneumoniae* Resistant to Colistin in a French Community Patient without History of Foreign Travel. *Antimicrobial Agents and Chemotherapy* 2012, 56 (6), 3432-3434.
- 5 6. Reglier-Poupet, H.; Naas, T.; Carrer, A.; Cady, A.; Adam, J. M.; Fortineau, N.; Poyart, C.; Nordmann, P. Performance of chromID ESBL, a chromogenic medium for detection of Enterobacteriaceae producing extended-spectrum beta-lactamases. *Journal of Medical Microbiology* 2008, 57 (3), 310-315.
7. Carrer, A.; Fortineau, N.; Nordmann, P. Use of ChromID Extended-Spectrum beta-Lactamase Medium for Detecting Carbapenemase-Producing Enterobacteriaceae. *Journal of Clinical Microbiology* 2010, 48 (5), 1913-1914.
- 10 8. Farber, J.; Moder, K. A.; Layer, F.; Tammer, I.; Konig, W.; Konig, B. Extended-Spectrum Beta-Lactamase Detection with Different Panels for Automated Susceptibility Testing and with a Chromogenic Medium. *Journal of Clinical Microbiology* 2008, 46 (11), 3721-3727.
9. Linscott, A. J.; Brown, W. J. Evaluation of four commercially available extended-spectrum beta-lactamase phenotypic confirmation tests. *Journal of Clinical Microbiology* 2005, 43 (3), 1081-1085.
10. Kaase, M.; Szabados, F.; Wassill, L.; Gatermann, S. G. Detection of Carbapenemases in Enterobacteriaceae by a Commercial Multiplex PCR. *Journal of Clinical Microbiology* 2012, 50 (9), 3115-3118.
- 20 11. Fosheim, G. E.; Carey, R. B.; Limbago, B. M. Evaluation of the AdvanDx VRE EVIGENE assay for detection of vanA in vancomycin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology* 2007, 45 (5), 1611-1613.
- 25 12. Naas, T.; Cuzon, G.; Bogaerts, P.; Glupczynski, Y.; Nordmann, P. Evaluation of a DNA Microarray (Check-MDR CT102) for Rapid Detection of TEM, SHV, and CTX-M Extended-Spectrum beta-Lactamases and of KPC, OXA-48, VIM, IMP, and NDM-1 Carbapenemases. *Journal of Clinical Microbiology* 2011, 49 (4), 1608-1613.
13. Lisdat, F.; Schafer, D. The use of electrochemical impedance spectroscopy for biosensing. *Analytical and Bioanalytical Chemistry* 2008, 391 (5), 1555-1567.
- 30 14. Park, J. Y.; Park, S. M. DNA Hybridisation Sensors Based on Electrochemical Impedance Spectroscopy as a Detection Tool. *Sensors* 2009, 9 (12), 9513-9532.
15. Corrigan, D. K.; Schulze, H.; Henihan, G.; Ciani, I.; Giraud, G.; Terry, J. G.; Walton, A. J.; Pethig, R.; Ghazal, P.; Crain, J.; Campbell, C. J.; Mount, A. R.; Bachmann, T. T. Impedimetric detection of single-stranded PCR products derived from methicillin resistant *Staphylococcus aureus* (MRSA) isolates. *Biosensors & Bioelectronics* 2012, 34 (1), 178-184.
- 35

16. Loy, A.; Arnold, R.; Tischler, P.; Rattei, T.; Wagner, M.; Horn, M. probeCheck - a central resource for evaluating oligonucleotide probe coverage and specificity. *Environmental Microbiology* 2008, 10 (10), 2894-2898.
17. Zarfel, G.; Hoenigl, M.; Wursth, B.; Leitner, E.; Salzer, H. J. F.; Valentin, T.; Posch, J.; Krause, R.; Grisold, A. J. Emergence of carbapenem-resistant Enterobacteriaceae in Austria, 2001-2010. *Clinical Microbiology and Infection* 2011, 17 (11), E5-E8.
18. Chihara, S.; Okuzumi, K.; Yamamoto, Y.; Oikawa, S.; Hishinuma, A. First Case of New Delhi Metallo-beta-Lactamase 1-Producing Escherichia coli Infection in Japan. *Clinical Infectious Diseases* 2011, 52 (1), 153-U170.
19. Nordmann, P.; Poirel, L.; Carrer, A.; Toleman, M. A.; Walsh, T. R. How To Detect NDM-1 Producers. *Journal of Clinical Microbiology* 2011, 49 (2), 718-721.
20. G. Zarfel, M. Hoenigl EL et al. Emergence of New Delhi Metallo- β -Lactamase, Austria. *Emerging Infectious Diseases*. 2011;17(1):129-130.
21. Chihara S, Okuzumi K, Yamamoto Y, Oikawa S, Hishinuma A. First Case of New Delhi Metallo-{beta}-Lactamase 1-Producing Escherichia coli Infection in Japan. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2011;52(1):153-4.
22. Degefa, T. H.; Kwak, J. Electrochemical impedance sensing of DNA at PNA self assembled monolayer. *Journal of Electroanalytical Chemistry* 2008, 612 (1), 37-41.
23. Keighley, S. D.; Li, P.; Estrela, P.; Mighorato, P. Optimization of DNA immobilization on gold electrodes for label-free detection by electrochemical impedance spectroscopy. *Biosensors & Bioelectronics* 2008, 23 (8), 1291-1297.
24. Liu, J. Y.; Tian, S. J.; Nielsen, P. E.; Knoll, W. In situ hybridisation of PNA/DNA studied label-free by electrochemical impedance spectroscopy. *Chemical Communications* 2005, (23), 2969-2971.
25. Steichen, M.; Decrem, Y.; Godfroid, E.; Buess-Herman, C. Electrochemical DNA hybridisation detection using peptide nucleic acids and [Ru(NH₃)₆](3+) on gold electrodes. *Biosensors & Bioelectronics* 2007, 22 (9-10), 2237-2243.
26. Keighley, S. D.; Estrela, P.; Li, P.; Mighorato, P. Optimization of label-free DNA detection with electrochemical impedance spectroscopy using PNA probes. *Biosensors & Bioelectronics* 2008, 24 (4), 906-911.
27. Brinker, A.; Schulze, H.; Bachmann, T.; Moller, R. Lambda exonuclease pre-treatment for improved DNA-chip performance depends on the relative probe-target position. *Biosensors & Bioelectronics* 2010, 26 (2), 898-902.
28. Peytavi, R.; Tang, L. Y.; Raymond, F. R.; Boissinot, K.; Bissonnette, L.; Boissinot, M.; Picard, F. J.; Huletsky, A.; Ouellette, M.; Bergeron, M. G. Correlation between

microarray DNA hybridisation efficiency and the position of short capture probe on the target nucleic acid. *Biotechniques* 2005, 39 (1), 89-96.

29. Peter, H.; Berggrav, K.; Thomas, P.; Pfeifer, Y.; Witte, W.; Templeton, K.; Bachmann, T. T. Direct Detection and Genotyping of KPC Carbapenemases from Urine using a new DNA Microarray Test. *J. Clin. Microbiol.* 2012.
30. Leinberger, D. M.; Grimm, V.; Rubtsova, M.; Weile, J.; Schroppel, K.; Wichelhaus, T. A.; Knabbe, C.; Schmid, R. D.; Bachmann, T. T. Integrated Detection of Extended-Spectrum-Beta-Lactam Resistance by DNA Microarray-Based Genotyping of TEM, SHV, and CTX-M Genes. *Journal of Clinical Microbiology* 2010, 48 (2), 460-471.
31. Malic, S.; Hill, K. E.; Hayes, A.; Percival, S. L.; Thomas, D. W.; Williams, D. W. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent in situ hybridisation (PNA FISH). *Microbiology-Sgm* 2009, 155, 2603-2611.
32. Perry-O'Keefe, H.; Rigby, S.; Oliveira, K.; Sorensen, D.; Slender, H.; Coull, J.; Hyldig-Nielsen, J. J. Identification of indicator microorganisms using a standardized PNA FISH method. *Journal of Microbiological Methods* 2001, 47 (3), 281-292.
33. Shakeel, S.; Karim, S.; Ali, A. Peptide nucleic acid (PNA) - a review. *Journal of Chemical Technology and Biotechnology* 2006, 81 (6), 892-899.
34. Kurakin, A.; Larsen, H. J.; Nielsen, P. E. Cooperative strand displacement by peptide nucleic acid (PNA). *Chemistry & Biology* 1998, 5 (2), 81-89.
35. Higuchi, R. G.; Ochman, H. Production of Single-Stranded-Dna Templates by Exonuclease Digestion Following the Polymerase Chain-Reaction. *Nucleic Acids Research* 1989, 17 (14), 5865.
36. Gazin, M.; Paasch, F.; Goossens, H.; Malhotra-Kumar, S. Current Trends in Culture-Based and Molecular Detection of Extended-Spectrum-beta-Lactamase-Harboring and Carbapenem-Resistant Enterobacteriaceae. *Journal of Clinical Microbiology* 2012, 50 (4), 1140-1146.
37. Strommenger, B.; Kettlitz, C.; Werner, G.; Witte, W. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *Journal of Clinical Microbiology* 2003, 41 (9), 4089-4094.
38. Tullman, J.; Guntas, G.; Dumont, M.; Ostermeier, M. Protein Switches Identified From Diverse Insertion Libraries Created Using S1 Nuclease Digestion of Supercoiled-Form Plasmid DNA. *Biotechnology and Bioengineering* 2011, 108 (11), 2535-2543.

Claims

1. A biochemical probe for the detection of the *bla_{NDM}* gene, the probe comprising a nucleic acid recognition sequence operable to hybridise under stringent conditions with
5 a specific DNA sequence of the *bla_{NDM}* gene complementary to the nucleic acid recognition sequence.
2. The biochemical probe according to claim 1, wherein the nucleic acid recognition sequence is operable to hybridise with a specific DNA sequence adjacent to the *bla_{NDM}*
10 gene and which is closely genetically associated with the *bla_{NDM}* gene.
3. The biochemical probe according to claim 2, wherein the nucleic acid recognition sequence is operable to hybridise with a specific DNA sequence adjacent to the *bla_{NDM}* gene and a specific DNA sequence of the *bla_{NDM}* gene.
15
4. The biochemical probe according to any one preceding claim, wherein the probe is operable to detect the presence of the *bla_{NDM}* gene by hybridising with a specific sequence of DNA within the intact plasmid containing the *bla_{NDM}* gene.
- 20 5. The biochemical probe according to any one preceding claim, wherein the probe hybridises with a sequence of the *bla_{NDM}* gene (or adjacent sequences) that is located on the exterior of the *bla_{NDM}* gene in its folded state.
6. The biochemical probe according to any one of claims 1 to 4, wherein the probe
25 hybridises with a sequence of the *bla_{NDM}* gene (or adjacent sequences) that is within the interior of the *bla_{NDM}* gene in its folded state and which only becomes available for hybridisation under specific conditions.
7. The biochemical probe according to any one preceding claim, wherein the probe is
30 operable to be used in an assay where a hybridisation event of the recognition sequence of the probe with the specific target DNA sequence is detected.
8. The biochemical probe according to any one preceding claim, wherein the probe is operable to be immobilised on a solid substrate.
- 35 9. The biochemical probe according to claim 8, wherein the probe is immobilised on an electrode.

10. The biochemical probe according to claim 9, wherein the probe is suitable for use in electrochemical impedance spectroscopy.
- 5 11. The biochemical probe according to any one preceding claim, wherein the recognition sequence comprises any one of SEQ ID NO: 1 to SEQ ID NO: 36 or variants thereof.
12. The biochemical probe according to claim 11, wherein the recognition sequence comprises SEQ ID NO:7 or SEQ ID NO: 25, or variants thereof.
- 10 13. The biochemical probe according to any one preceding claim, wherein the nucleic acid recognition sequence comprises a synthetic nucleic acid.
14. The biochemical probe according to claim 13, wherein the synthetic nucleic acid
15 comprises an uncharged synthetic backbone in ambient conditions.
15. The biochemical probe according to claim 14, wherein the nucleic acid recognition sequence is a peptide nucleic acid (PNA) recognition sequence comprising a peptide backbone.
- 20 16. The biochemical probe according to any one preceding claim comprising an anchor operable to immobilise the probe to a sensing surface.
17. The biochemical probe according to claim 16, wherein the sensing surface comprises
25 a gold surface and the anchor comprises at least one terminal sulphur containing group.
18. The biochemical probe according any one preceding claim comprising a linker connecting the anchor to the nucleic acid recognition sequence such that the nucleic
30 acid recognition sequence is available for hybridisation with the target DNA sequence.
19. The biochemical probe according to any one preceding claim, wherein the probe is labelled.
- 35 20. A diagnostic kit for the detection of the *bla_{NDM}* gene, the kit comprising at least one biochemical probe according to any one preceding claim.

21. The kit according to claim 20 comprising at least one blocking species.
22. The kit according to either one of claim 20 or claim 21, wherein the kit comprises a sensing surface to which the at least one biochemical probe is immobilised.
- 5 23. The kit according to claim 22, wherein the sensing surface comprises a gold electrode.
24. A method of detection of the *bla_{NDM}* gene, the method comprising the steps:
- 10 (a) providing a biological sample comprising DNA;
- (b) providing a sensing apparatus comprising a sample chamber and a sensing surface comprising at least one probe according to any one of claims 1 to 18;
- (c) treating the biological sample to extract and/or fragment the DNA within the biological sample;
- (d) introducing the biological sample into the sample chamber of the apparatus;
- 15 and
- (e) determining whether DNA within the biological sample hybridises with the at least one probe on the sensing surface, wherein hybridisation of DNA to the at least one probe is indicative of the presence of the *bla_{NDM}* gene within the biological sample.
- 20 25. The method of detection according to claim 24, wherein the method comprises the step of purifying the extracted DNA before the step of introducing the biological sample into the sample chamber of the apparatus.
26. The method of detection according to either one of claim 24 or claim 25, wherein the sensing apparatus is an electrochemical impedance spectroscopy (EIS) apparatus comprising at least one working electrode comprising the sensing surface.
- 25 27. The method of detection according to claim 26, wherein the biological sample comprises at least 30% formamide by volume.
- 30 28. The method of detection according to claim 27, wherein the biological sample comprises at least 50% formamide by volume.
29. The method of detection according to either one of claim 27 or claim 28, wherein the resistance to charge transfer of the working electrode is measured at room temperature.
- 35

30. An electrochemical sensor for the detection of bacteria having the *bla_{NDM}* gene comprising a sensing surface and a sample chamber; the sensing surface defining an interior surface of the sample chamber; the sensing surface comprising a plurality of biochemical probes according to any one of claims 1 to 19 such that the addition of a biological sample comprising *bla_{NDM}* gene DNA to the sample chamber results in the detection of hybridisation between the recognition sequence of the biochemical probes within the plurality of biochemical probes and the *bla_{NDM}* gene DNA.
31. The electrochemical sensor according to claim 30, wherein the electrochemical sensor is an electrochemical impedance spectroscopy sensor, and the sensing surface is a surface of a working electrode.

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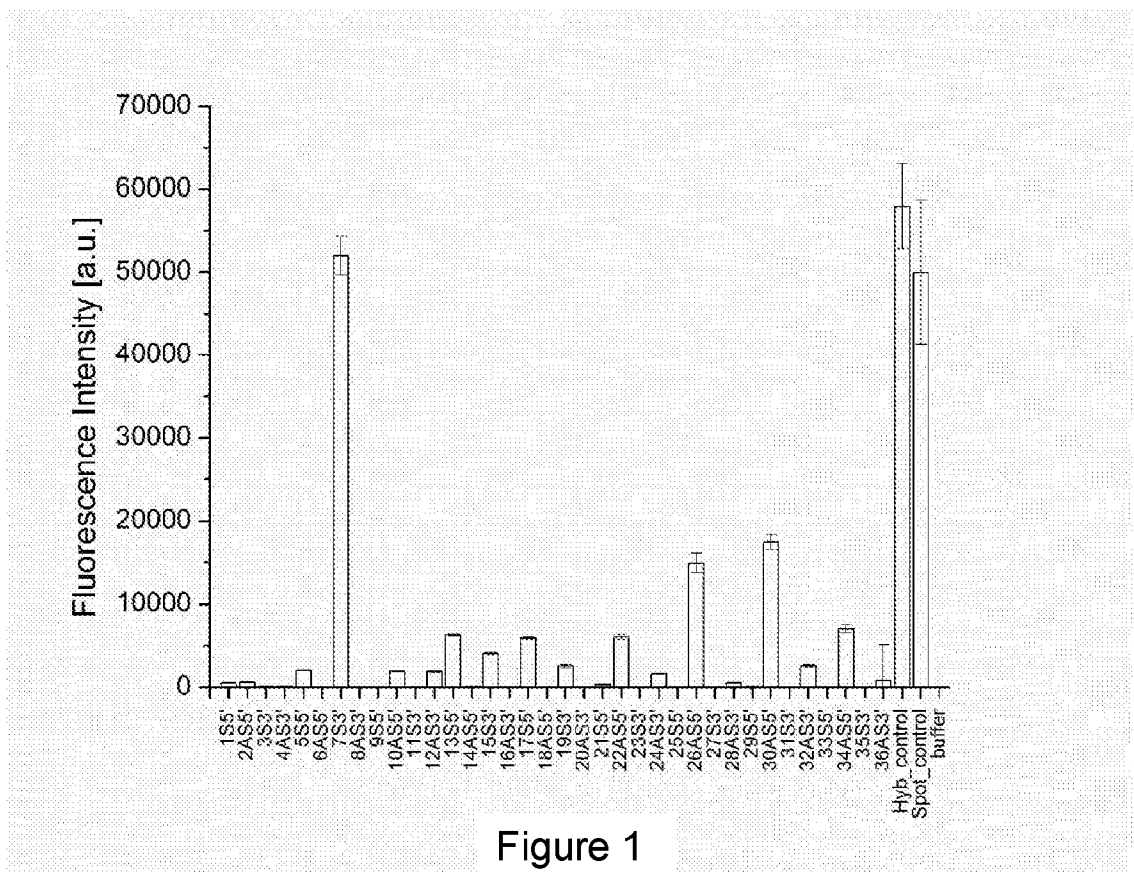


Figure 1

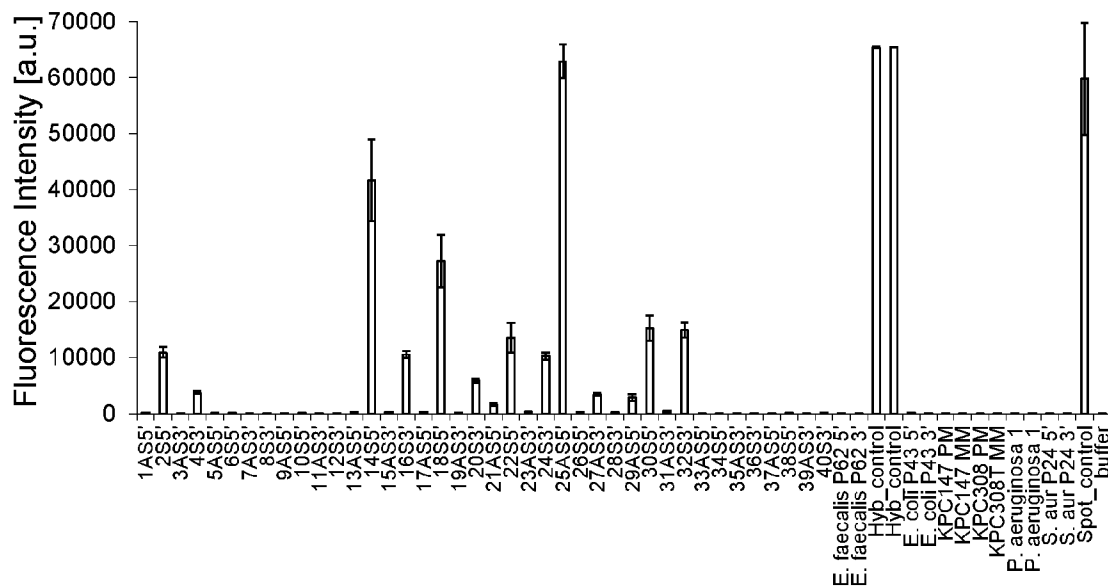


Figure 2

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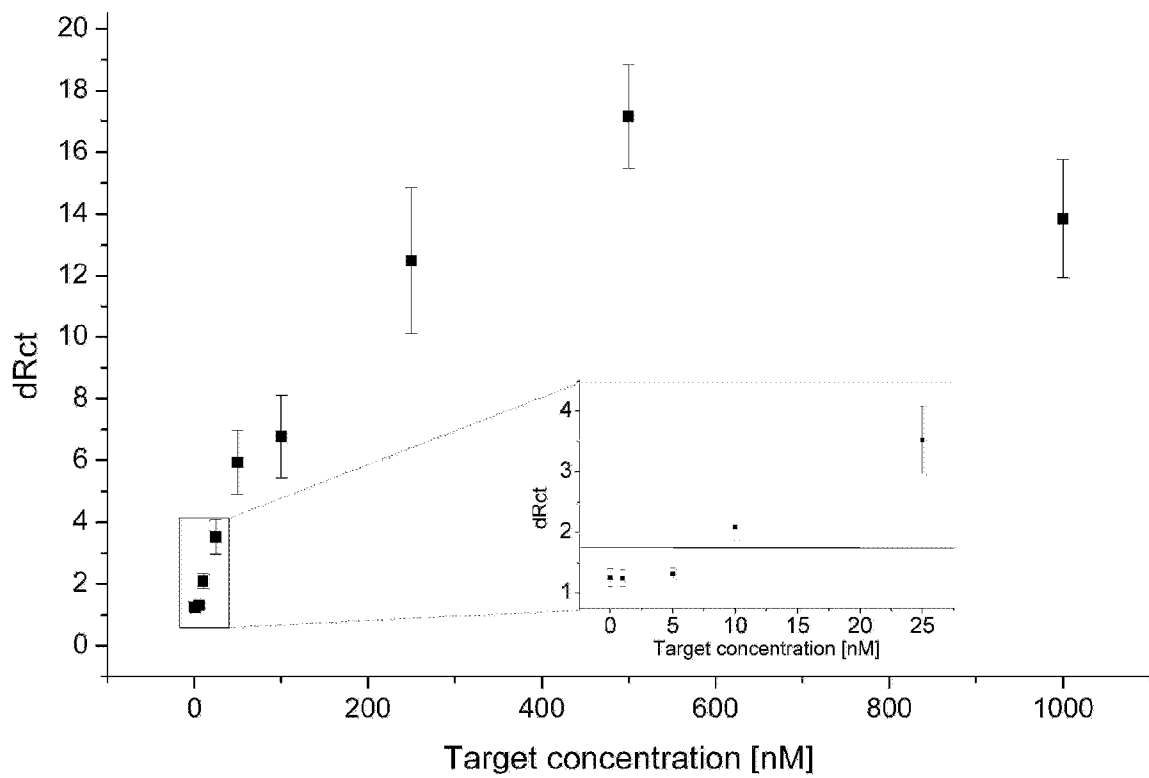


Figure 3

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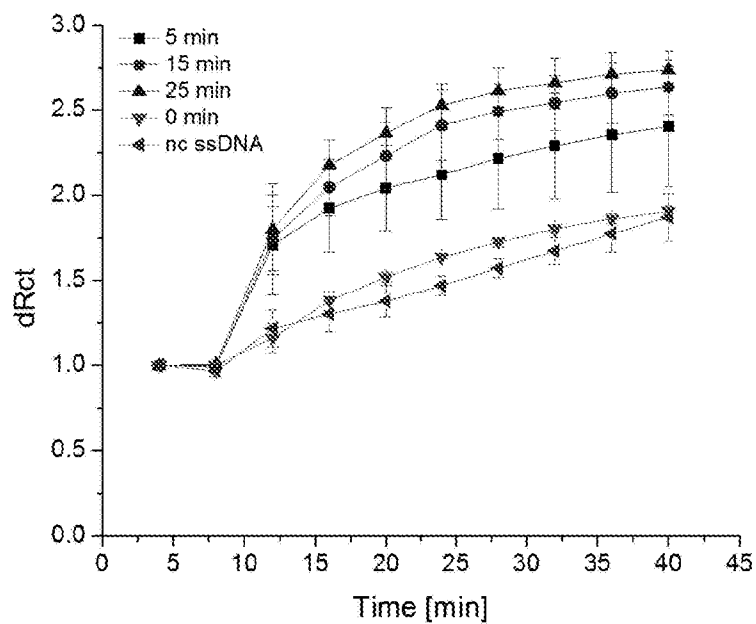


Figure 4

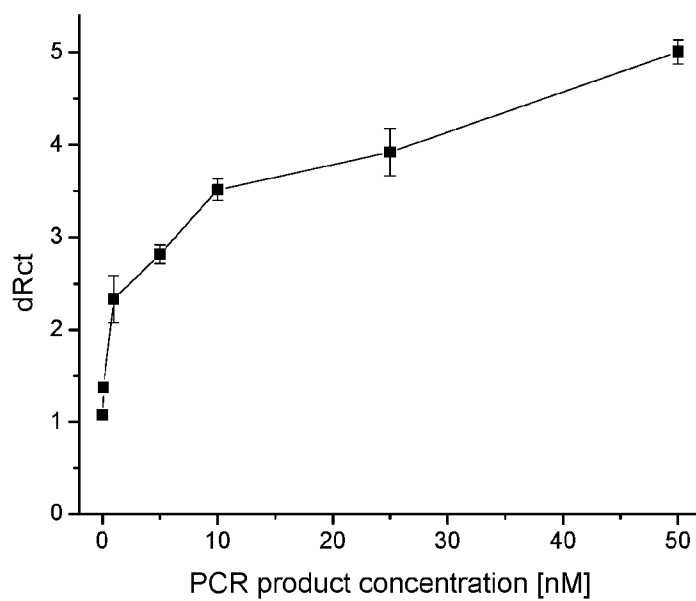


Figure 5

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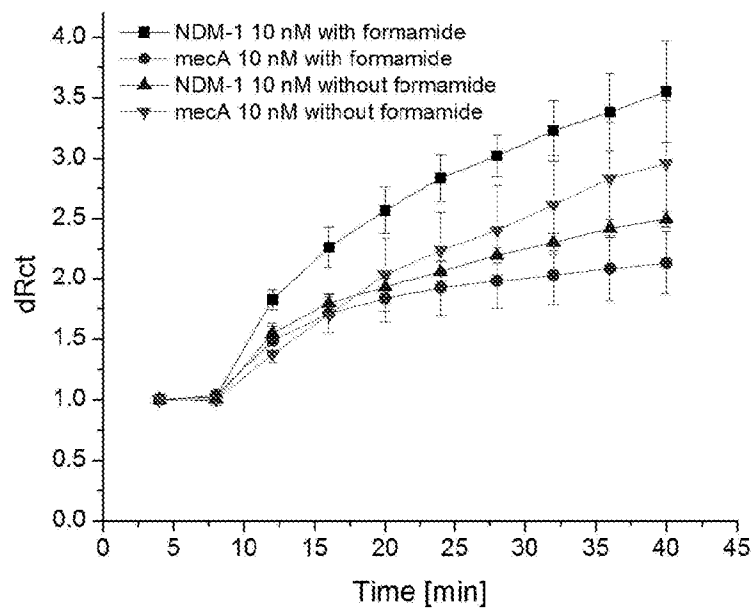


Figure 6

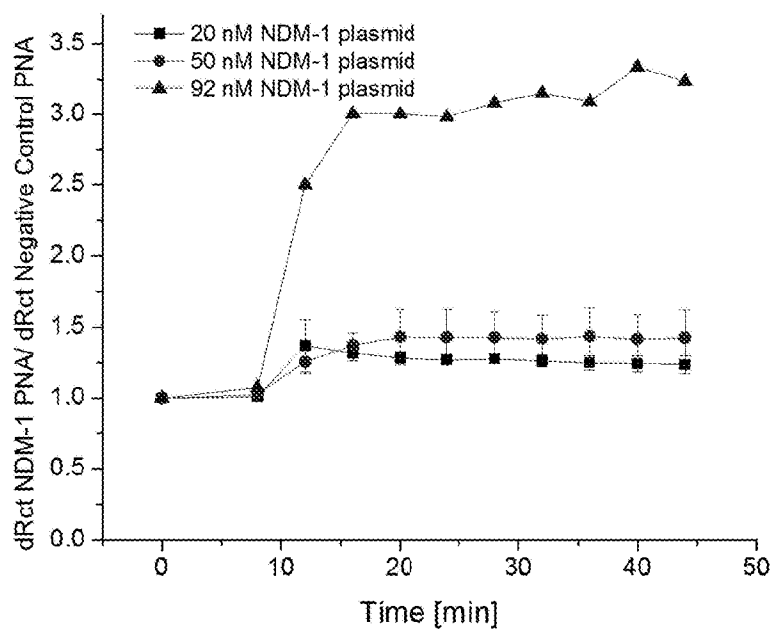


Figure 7

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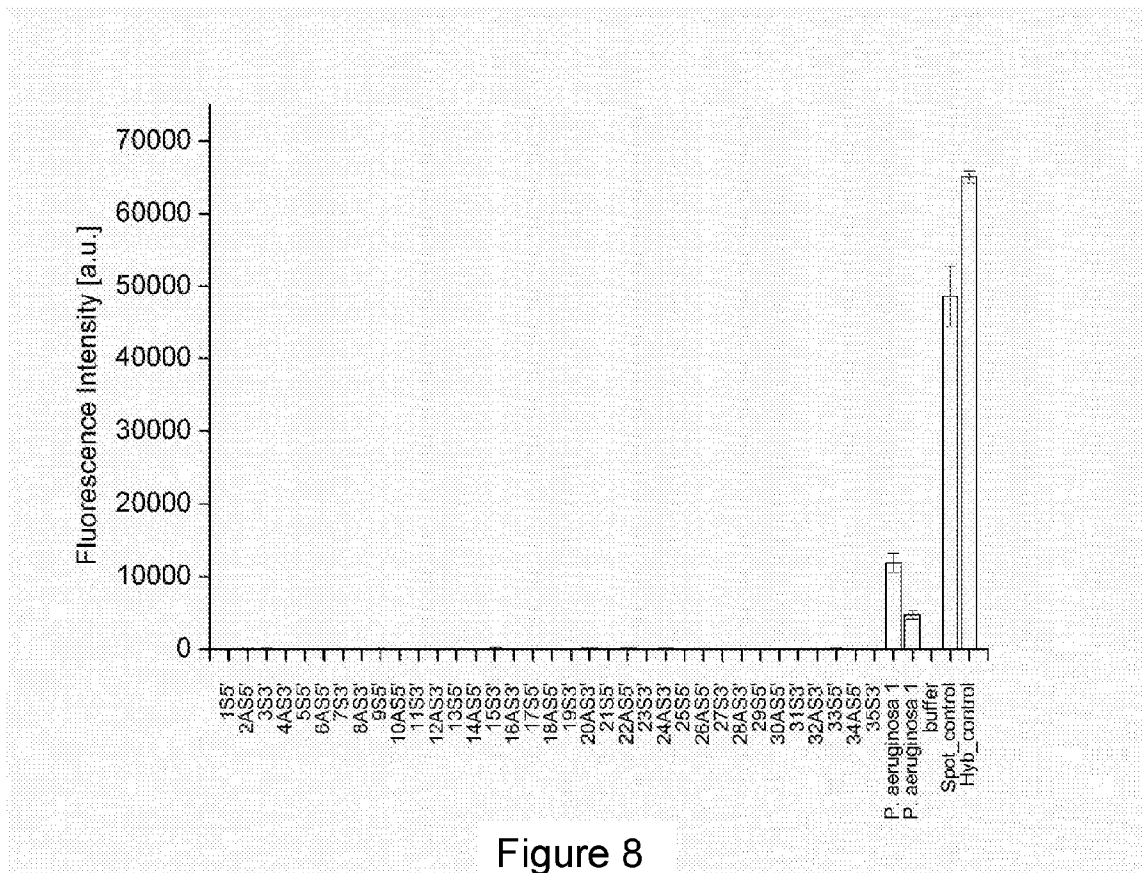


Figure 8

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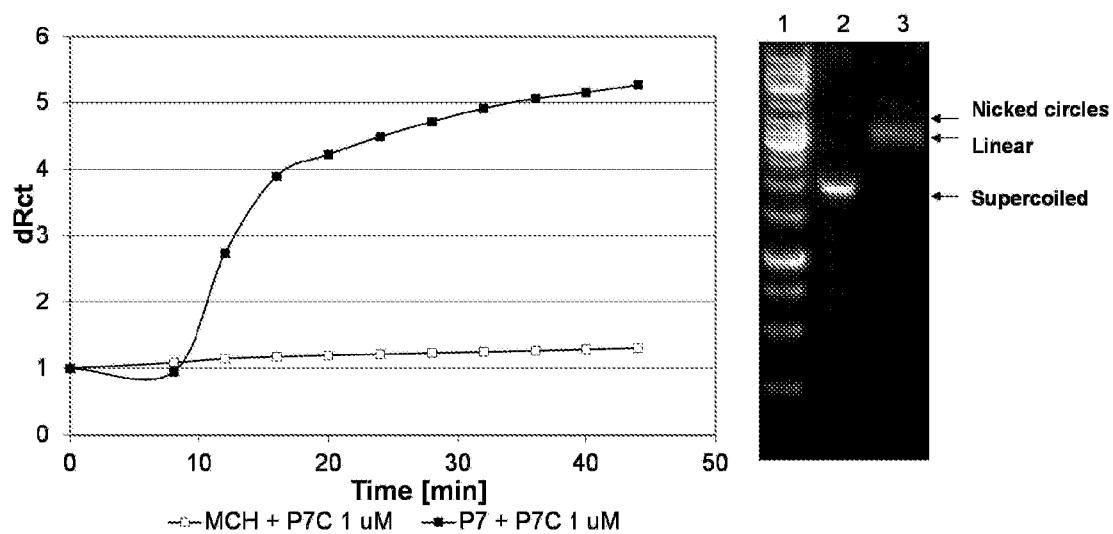


Figure 9

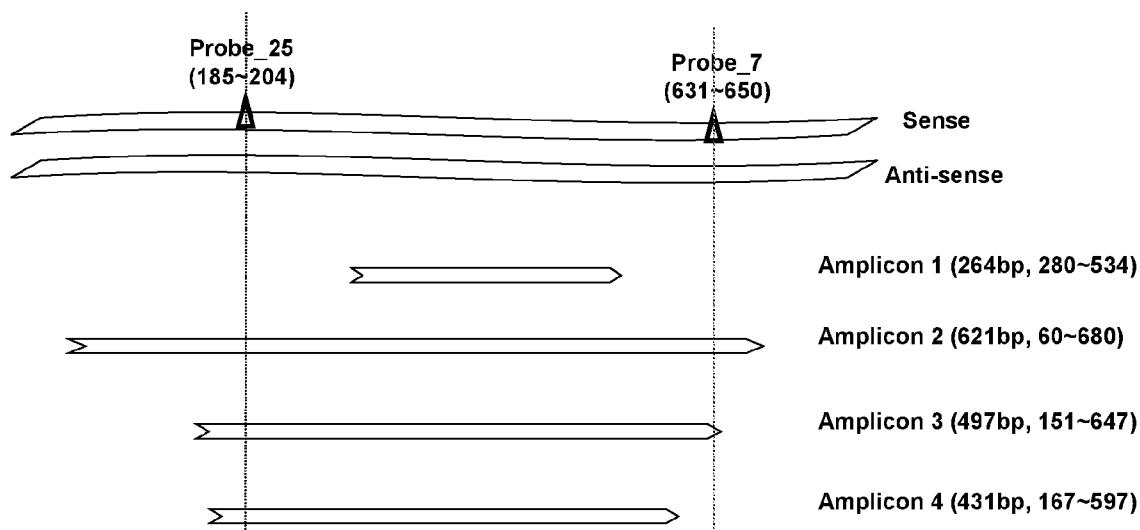


Figure 10

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/052586

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 202 297 608 U (CHINESE ACAD INSP & QUARANTINE) 4 July 2012 (2012-07-04) para. 14 -----	1,4-8, 10-13, 16,18-20
X	CN 102 534 014 A (TRIPLEX INTERNAT BIOSCIENCES CHINA CO LTD) 4 July 2012 (2012-07-04) para. 38; SEQ ID N° 3 -----	1,4-8, 10,11, 13,16, 18-20
X	CN 103 614 465 A (UNIV SOUTHERN MEDICAL) 5 March 2014 (2014-03-05) para. 6; SEQ ID N° 10 ----- -/-	1,4-8, 10-13, 16,18,20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 November 2015

Date of mailing of the international search report

04/12/2015

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Ripaud, Leslie

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2015/052586

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).

☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/052586

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GERNOT ZARFEL ET AL: "Emergence of New Delhi Metallo- [beta]-Lactamase, Austria", EMERGING INFECTIOUS DISEASES, vol. 17, no. 1, 1 January 2011 (2011-01-01), pages 129-130, XP055230818, p. 129, left-hand col., last para., oligonucleotide "NDM-1gr" -----	1,4-8, 10,11, 13,20
X	S. CHIHARA ET AL: "First Case of New Delhi Metallo-beta-Lactamase 1-Producing Escherichia coli Infection in Japan", CLINICAL INFECTIOUS DISEASES, vol. 52, no. 1, 1 January 2011 (2011-01-01), pages 153-154, XP055230822, US ISSN: 1058-4838, DOI: 10.1093/cid/ciq054 cited in the application p. 153, legend of fig. 1, NDM1F1 primer -----	1,4-8, 10,11, 13,20
X	US 2005/202492 A1 (NAM YUN-SUN [KR] ET AL) 15 September 2005 (2005-09-15) SEQ ID N° 90; para. 30-34 -----	1-8,10, 16,20,22
X	Jimmy Ming-Yuan Huang ET AL: "Rapid electrochemical detection of multi-drug resistance NDM-1 producing bacteria", 9 October 2012 (2012-10-09), XP055230430, Retrieved from the Internet: URL: http://www.nrsconference.org.uk/nrsconference2012/documents/PosterWinners2012forwebsite.pdf [retrieved on 2015-11-23] 2nd para. -& Anonymous: "nrs conference 2012 bachmann - Google Search", 23 November 2015 (2015-11-23), XP055230433, Retrieved from the Internet: URL: https://www.google.de/search?q=nrs+conference+2012+bachmann&biw=2001&bih=877&source=Int&tbs=cdr%3A1%2Ccd_min%3A%2Ccd_max%3A9%2F7%2F2014&tbm= [retrieved on 2015-11-23] -----	1,4-10, 13,16, 17,20-31
X	WO 99/37670 A1 (BOSTON PROBES INC [US]; GILDEA BRIAN D [US]; COULL JAMES M [US]) 29 July 1999 (1999-07-29) p. 39-40, ex. 12 and table 1, 2nd oligonucleotide ----- -/--	1,4-8, 10, 13-16, 18-20,22

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/052586

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	JIMMY MING-YUAN HUANG ET AL: "Rapid Electrochemical Detection of New Delhi Metallo-beta-lactamase Genes To Enable Point-of-Care Testing of Carbapenem-Resistant Enterobacteriaceae", ANALYTICAL CHEMISTRY, vol. 87, no. 15, 29 June 2015 (2015-06-29), pages 7738-7745, XP055207712, ISSN: 0003-2700, DOI: 10.1021/acs.analchem.5b01270 the whole document -& Jimmy Ming ET AL: "Supporting Information Rapid electrochemical detection of New Delhi metallo-beta-lactamase genes to enable point of care testing of carbapenem resistant Enterobacteriaceae", 29 June 2015 (2015-06-29), XP055230484, Retrieved from the Internet: URL:http://pubs.acs.org/doi/suppl/10.1021/acs.analchem.5b01270/suppl_file/ac5b01270_si_001.pdf [retrieved on 2015-11-23] the whole document -----	1-31
X,P	CN 104 237 353 A (UNIV PLA 3RD MILITARY MEDICAL) 24 December 2014 (2014-12-24) the whole document -----	1-31
A	D. K. CORRIGAN ET AL: "Development of a PCR-free electrochemical point of care test for clinical detection of methicillin resistant Staphylococcus aureus (MRSA)", THE ANALYST, vol. 138, no. 22, 21 November 2013 (2013-11-21), page 6997, XP055231082, GB ISSN: 0003-2654, DOI: 10.1039/c3an01319g the whole document -----	1-31
A	JIN-YOUNG PARK ET AL: "DNA Hybridization Sensors Based on Electrochemical Impedance Spectroscopy as a Detection Tool", SENSORS, vol. 9, no. 12, 1 January 2009 (2009-01-01), pages 9513-9532, XP055022249, ISSN: 1424-8220, DOI: 10.3390/s91209513 the whole document ----- -/--	1-31

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/052586

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARTIN KAASE ET AL: "Detection of carbapenemases in Enterobacteriaceae by a commercial multiplex PCR", JOURNAL OF CLINICAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US , vol. 50, no. 9 1 September 2012 (2012-09-01), pages 3115-3118, XP002710012, ISSN: 0095-1137, DOI: 10.1128/JCM.00991-12 Retrieved from the Internet: URL:http://jcm.asm.org/content/50/9/3115 [retrieved on 2012-07-11] the whole document -----	1-31

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2015/052586

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
CN 202297608	U	04-07-2012	NONE	
CN 102534014	A	04-07-2012	NONE	
CN 103614465	A	05-03-2014	NONE	
US 2005202492	A1	15-09-2005	KR 20050091548 A US 2005202492 A1	15-09-2005 15-09-2005
WO 9937670	A1	29-07-1999	AT 356827 T AU 2459199 A DE 69935502 T2 DK 1054893 T3 EP 1054893 A1 US 6326479 B1 US 2004102571 A1 WO 9937670 A1	15-04-2007 09-08-1999 13-12-2007 23-07-2007 29-11-2000 04-12-2001 27-05-2004 29-07-1999
CN 104237353	A	24-12-2014	NONE	