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(71) Applicant: **ELITECHGROUP B.V.** [NL/NL]; Van Renssenlaerweg 4, (6956 AV) Spankeren (NL).

(72) Inventors; and
(71) Applicants *(for US only)*: **AFONINA, Irina, A.** [US/US]; 2717 144th Court, S.E., Mill Creek, WA 98012 (US). **BELOUSOV, Yevgeniy, S.** [US/US]; 2717 144th Court. S.E., Mill Creek, WA 98012 (US).

(74) Agents: **BORRELLI, Sara, K.** et al.; Jackson Walker L.L.P., 2323 Ross Avenue, Suite 600, Dallas, TX 75201 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR DETECTING ANTIBIOTIC RESISTANT BACTERIA

(57) Abstract: Primers and probes specific to the genes encoding extended spectrum beta- lactamase that involves CTX-M groups 1 and 9 that cause extended beta-lactamase resistance in bacteria are described herein, with methods and kits for using these primers and probes to detect CTX-M groups 1 and 9 nucleic acids. In the methods described, nucleic acids present in a clinical or test sample obtained from a biological sample or tissue suspected of containing the CTX-M groups 1 and 9 gene are amplified and corresponding sequences for CTX-M groups 1 and 9 are detected. The amplified nucleic acid can be detected by a variety of state of the art methods, including fluorescence resonance energy transfer (FRET), radiolabels, enzyme labels, and the like.

METHODS AND COMPOSITIONS FOR DETECTING ANTIBIOTIC RESISTANT BACTERIA

BACKGROUND

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 62/090,904, entitled "Methods and Compositions for Detecting Antibiotic Resistant Bacteria," filed on December 12, 2014, the entire content of which is hereby incorporated by reference.

[0002] This disclosure relates to bacterial diagnostics, and more particularly to methods and compositions for detecting antibiotic resistant bacteria using preferred primers, probes, and assays directed to genes encoding extended spectrum beta-lactamase.

[0003] β-Lactam antibiotics (beta-lactam antibiotics) are a broad class of antibiotics, consisting of all antibiotic agents that contain a β-lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems.(Holten KB 2000). Most β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. β-Lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms. At first, β-lactam antibiotics were mainly active only against Gram-positive bacteria, yet the recently developed broad-spectrum β-lactam antibiotics are active against various Gram-negative organisms, which has increased their usefulness.

[0004] The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in gram-negative bacteria. The CTX-M beta-lactamases enzymes were named for their greater activity against cefotaxime. Evolution of multidrug resistant (MDR) bacteria under the pressure of excessive antibiotic use with horizontal gene transfer provide the means by which genes such as bla(CTX-M) spread amongst different bacterial species and strains (Hawkey FM 2009, Liebana et al. 2013). More than 80 CTX-M enzymes are currently known. CTX-M1-, and CTX-M9-groups represent five and nine closely related enzymes, respectively, and are particularly clinically relevant (Garrido et al. 2014; Helldal et al. 2013; Onnberg et al. 2011; Dallenne et al. 2010). CTX-M enzymes are observed in different members of the family *Enterobacteriaceae* which include, among others, *Salmonella* and *E.coli*. Typically the blaCTX-M gene is located on transferable plasmids of different sizes and structures, with the group 1 and 9, respectively, 97 and 98% similar (Bonnet 2004). The specific detection of clinical relevant groups 1 and 9 in the background of less clinical relevant CTX-M groups present special challenges.

[0005] There exists a clinical need for the rapid detection of the carriers of antibiotic resistant β-lactamase genes among which the CTX-M1 and CTX-M9 groups have higher clinical prevalence rates.

SUMMARY

[0006] The present disclosure relates to primers and probes specific to the genes encoding extended spectrum beta-lactamase that involves CTX-M groups 1 and 9 that cause extended beta-lactamase resistance in bacteria.

[0007] More specifically, the present disclosure relates to primers and probes for the detection of genes encoding certain beta-lactamases in samples including biological samples (*e.g.*, rectal swabs). The present invention discloses primers and probes to identify the family of specific beta lactamases producers that carry antibiotic resistance genetic markers in clinical isolates of preferably gram negative bacteria. Specific primers and probes to amplify and detect resistance-encoding genes that involve CTX-M groups 1 and 9 are disclosed in the primer and probe sequences herein. In the methods described, nucleic acids present in a clinical or test sample obtained from a biological sample or tissue suspected of containing the CTX-M groups 1 and 9 gene are amplified and corresponding sequences for CTX-M groups 1 and 9 are detected. The amplified nucleic acid can be detected by a variety of state of the art methods, including fluorescence resonance energy transfer (FRET), radiolabels, enzyme labels, and the like. The amplified nucleic acids can also be detected by any combination of detection techniques which may include hybridization detection probes.

[0008] One embodiment pertains to a method for detecting CTX-M groups 1 and 9 in a biological sample from an individual. Other embodiments provide oligonucleotide primers and probes comprising nucleotide sequences characteristic of CTX-M groups 1 and 9 gene sequences. The method includes performing at least one cycling step of amplification and hybridization. The amplification step includes contacting the sample nucleic acid with one or more pairs of primers to produce amplification product if the CTX-M groups 1 and 9 nucleic acid is present. The preferred primers target specific regions of the CTX-M gene of a resistant organism. The oligonucleotide probes detect the amplified target directly or indirectly. The most preferred oligonucleotide probe is a 5'-minor groove binder-fluorophore-oligonucleotide-quencher-3' conjugate that fluoresces on hybridization to its complementary amplified target. In another embodiment the preferred oligonucleotide probe is a 3'-minor groove binder-quencher-oligonuceotide-fluorophore-3' conjugate that fluoresces on hybridization to its complementary amplified target when cleaved by 5'-endonuclease activity.

[0009] Kits are also provided for the detection of CTX-M groups 1 and 9 genes in biological samples comprising at least one annealing oligonucleotide primer reagent specific for the amplification of CTX-M groups 1 and 9 sequences and comprising at least one oligonucleotide probe specific for the detection of CTX-M groups 1 and 9 sequences.

[0010] The method further includes detecting of the presence or absence of a fluorescent signal (*e.g.*, a signal resulting from FRET) of the hybridization probe of the invention. The presence of the fluorescent signal usually indicates the presence of CTX-M groups 1 and 9 gene sequences in the biological sample, while the absence of signal usually indicates the absence of CTX-M groups 1 and 9 gene sequences in the biological sample.

[0011] The method can additionally include determining the melting temperature profile between the probe and the amplification product. The melting curve further confirms the presence or absence of CTX-M groups 1 and 9 gene sequences and potential presence of CTX-M groups 1 and 9 gene sequences, with mismatch(es) in the probe sequence area.

[0012] The primers and probes of the invention allow the specific, sensitive, and rapid detection of CTX-M groups 1 and 9 gene sequences that have higher clinical prevalence rates.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1 shows real-time titration (tenfold titration (100pg – 1fg)) of CTX-M15 DNA extracted from *E. coli* ATCC BAA-2326 alone and with Internal Control.

[0014] Figure 2 shows single- and bi-plex amplification data for the CTX-M15- and CTC-M15 + IC shown in Figure 1 re-plotted as linear curves.

[0015] Figure 3 shows the real-time detection of CTX-M15 nucleic acids with probes and primers designed for 3 different locations.

DETAILED DESCRIPTION

I. General

[0016] The present disclosure provides primers and probes for use in methods for the specific amplification and/or detection of nucleic acid sequences encoding certain beta-lactamases, namely CTX-M groups 1 and 9.

II. Definitions

[0017] The term "CTX-M" as used herein refers to Extended-Spectrum β-Lactamases causing antibiotic resistance in mainly gram negative bacteria that acquired chromosomal $bla_{CTX\text{-}M}$ related genes from different species of Kluyvera. The CTX-M-1 group includes six plasmid-mediated enzymes (CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, and FEC-1) showing > 97% identity and CTX-M-9 group includes nine plasmid-mediated enzymes (CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27, and Toho-2) (Bonnet, 2004) showing >98% identity.

[0018] A "sample" as used herein refers to a sample of any source which is suspected of containing CTX-M nucleic acids. These samples can be tested by the methods described herein. A sample can be from a laboratory source or from a non-laboratory source. A sample may be suspended or dissolved in liquid materials such as buffers, extractants, solvents, and the like. Samples also include biological samples such as animal and human tissue or fluids such as whole blood, blood fractions, serum, plasma, cerebrospinal fluid, lymph fluids, milk, urine, various external secretions of the respiratory, intestinal, and genitourinary tracts, tears, and saliva; and biological fluids such as cell extracts, cell culture supernatants, fixed tissue specimens, and fixed cell specimens. Samples include nasopharyngeal or throat swabs, stools, or rectal swabs. Biological samples may also include sections of tissues such as biopsy and autopsy samples or frozen sections taken for histologic purposes. A biological sample is obtained from any mammal including, *e.g.*, a human.

[0019] The terms "flap primer" or "overhang primer" refer to a primer comprising a 5' sequence segment non-complementary to a target nucleic acid sequence (*e.g.*, a CTX-M groups 1 and 9 nucleic acid sequence) and a 3' sequence segment complementary to the target nucleic acid sequence (*e.g.*, a CTX-M groups 1 and 9 nucleic acid sequence). The flap primers of the invention are suitable for primer extension or amplification of the target nucleic acid sequence (*e.g.*, CTX-M groups 1 and 9 nucleic acid sequence).

[0020] The term "overhang sequence" refers to a non-complementary adapter, flap, or overhang sequence in a primer. "Non-complementary" sequences do not bind to a target sequence under amplification conditions. The flap portion of a flap primer can comprise nucleotides that are complementary to the target sequence provided that the three nucleotides immediately 5' to the portion of the flap are not complementary to the target sequence.

[0021] The term "fluorescent generation probe" refers either to a) an oligonucleotide having an attached minor groove binder, fluorophore, and quencher or b) a DNA binding reagent. The probes may comprise one or more non-complementary or modified nucleotides (*e.g.*, pyrazolopyrimidines as described herein below) at any position including, *e.g.*, the 5' end. In some embodiments, the fluorophore is attached to the modified nucleotide.

[0022] The term "modified bases" refers to those bases that differ from the naturally-occurring bases (adenine, cytosine, guanine, thymine, and urasil) by addition or deletion of one or more functional groups, differences in the heterocyclic ring structure (i.e., substitution of carbon for a heteroatom, or vice versa), and/or attachment of one or more linker arm structures to the base. Preferred modified nucleotides are those based on a pyrimidine structure or a purine structure, with the latter more preferably being 7 deazapurines and their derivatives and pyrazolopyrimidines (described in U.S. Patent No. 7,045,610); and also described in U.S. Patent No. 6,127,121. Preferred modified bases are 5-substituted pyrimidines and 3-substituted pyrazolopyrimidines. Examples of preferred modified bases are 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (PPG or Super G®), 4-amino-1H-pyrazolo[3,4-d]pyrimidine, 1H-pyrazolo[5,4-d]pyrimidin-4(5H)-6(7H)-dione, 6-amino-3-prop-1-ynyl-5-hydropyrazolo[3,4-d]pyrimidine-4-one, 6-amino-3-(3-hydroxyprop-1-yny)l-5-hydropyrazolo[3,4-d]pyrimidine-4-one, 6-amino-3-(3-aminoprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidine-4-one, 4-amino-3-(prop-1-ynyl)pyrazolo[3,4-d]pyrimidine, 4-amino-3-(3-hydroxyprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, 4-amino-3-

(3-aminoprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, 3-prop-1-ynyl-4,6-diaminopyrazolo[3,4-d]pyrimidine, 2-(4,6-diaminopyrazolo[3,4-d]pyrimidin-3-yl)ethyn-1-ol, 3-(2-aminoethynyl)pyrazolo[3,4-d]pyrimidine-4,6-diamine, 5-prop-1-ynyl-1,3-dihydropyrimidine-2,4-dione, 5-(3-hydroxyprop-1-ynyl)-1,3-dihydropyrimidine-2,4-dione, 6-amino-5-prop-1-ynyl-3-dihydropyrimidine-2-one, 6-amino-5-(3-hydroxyprop-1-yny)-1,3-dihydropyrimidine-2-one, 6-amino-5-(3-aminoprop-1-yny)-1,3-dihydropyrimidine-2-one, 5-[4-amino-3-(3-methoxyprop-1-ynyl)pyrazol[3,4-d]pyrimidinyl]-2-(hydroxymethyl)oxolan-3-ol, 6-amino-1-[4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-3-(3-methoxyprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidin-4-one, 4-(4,6-Diamino-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-but-3-yn-1-ol (Super A®), 6-Amino-3-(4-hydroxy-but-1-ynyl)-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one, 5-(4-hydroxy-but-1-ynyl)-1H-pyrimidine-2,4-dione (Super T®), 3-iodo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine, 3-bromo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine, 3-chloro-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine, 3-Iodo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine, 3-Bromo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine and 3-chloro-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine. Examples of universal bases can be find co-owned U.S. Application US2013/0261014 incorporated by reference herein.

[0023] The terms "fluorescent label" or "fluorophore" refer to compounds with a fluorescent emission maximum between about 400 and about 900 nm. These compounds include, with their emission maxima in nm in brackets, Cy2™ (506), GFP (Red Shifted) (507), YO-PRO™-1 (509), YOYO™-1 (509), Calcein (517), FITC (518), FluorX™ (519), Alexa™ (520), Rhodamine 110 (520), 5-FAM (522), Oregon Green™ 500 (522), Oregon Green™ 488 (524), RiboGreen™ (525), Rhodamine Green™ (527), Rhodamine 123 (529), Magnesium Green™ (531), Calcium Green™ (533), TO-PRO™-1 (533), TOTO®-1 (533), JOE (548), BODIPY® 530/550 (550), Dil (565), BODIPY® 558/568 (568), BODIPY® 564/570 (570), Cy3™ (570), Alexa™ 546 (570), TRITC (572), Magnesium Orange™ (575), Phycoerythrin R&B (575), Rhodamine Phalloidin (575), Calcium Orange™ (576), Pyronin Y (580), Rhodamine B (580), TAMRA (582), Rhodamine Red™ (590), Cy3.5™ (596), ROX (608), Calcium Crimson™ (615), Alexa™ 594 (615), Texas Red® (615), Nile Red (628), YO-PRO™-3 (631), YOYO™-3 (631), R-phycocyanin (642), C-Phycocyanin (648), TO-PRO™-3 (660), TOTO®-3 (660), DiD

DilC(5) (665), Cy5™ (670), Thiadicarbocyanine (671), and Cy5.5 (694). Additional fluorophores are disclosed in PCT Patent Publication No. WO 03/023357 and U.S. Patent No. 7,671,218. Examples of these and other suitable dye classes can be found in Haugland et al., Handbook of Fluorescent Probes and Research Chemicals, Sixth Ed., Molecular Probes, Eugene, Ore. (1996); U.S. Patent Nos. 3,194,805; 3,128,179; 5,187,288; 5,188,934; 5,227,487, 5,248,782; 5,304,645; 5,433,896; 5,442,045; 5,556,959; 5,583,236; 5,808,044; 5,852,191; 5,986,086; 6,020,481; 6,162,931; 6,180,295; and 6,221,604; EP Patent No. 1408366; Still other dyes are provided via online sites such as zeiss.com. Phosphonate dyes are disclosed in co-owned US 7,671,218, US 7,767,834 and 8,163,910.

[0024] There is extensive guidance in the art for selecting quencher and fluorophore pairs and their attachment to oligonucleotides (Haugland, 1996; U.S. Patent Nos. 3,996,345, 4,351,760 and 8,410,255 and the like).

[0025] Preferred quenchers are described in co-owned U.S. Patent Nos. 6,727,356 and 7,662,942.

[0026] In the description herein, the abbreviations M, FL, Q, CPG, and ODN refer to "minor groove binder," "fluorescent label" or "fluorophore," "quencher," "controlled pore glass" (as an example of a solid support), and "oligonucleotide" moieties or molecules, respectively, and in a manner which is apparent from context. The terms "probe" and "conjugate" are used interchangeably and preferably refer to an oligonucleotide having an attached minor groove binder, fluorophore, and quencher.

[0027] The terms "oligonucleotide," "nucleic acid," and "polynucleotide" are used interchangeably herein. These terms refer to a compound comprising nucleic acid, nucleotide, or its polymer in either single- or double-stranded form, *e.g.*, DNA, RNA, analogs of natural nucleotides, and hybrids thereof. The terms encompass polymers containing modified or non-naturally-occurring nucleotides, or to any other type of polymer capable of stable base-pairing to DNA or RNA including, but not limited to, peptide nucleic acids as described in Nielsen *et al.*, *Science*, 254:1497-1500 (1991), bicyclo DNA oligomers as described in Bolli *et al.*, *Nucleic Acids Res.*, 24:4660-4667 (1996), and related structures. Unless otherwise limited, the terms encompass known analogs of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally-occurring nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs) and any combination thereof. A "subsequence" or "segment" refers to a sequence of nucleotides that comprise a part of a longer sequence of nucleotides.

[0028] The term "internal control" refers to a control amplification reaction that monitors false negative amplification of targets due to failure of one or more reagents, failure of amplification due to thermal cycling, inhibition of amplification, failure of reporting the reaction, or similar failures. The use of Bacteriophage MS2 (Dreier 2005) and purified *Bacillus atrophaeus* subsp. *globigii* spores as internal controls (Picard 2009) have been reported. Practical considerations in design of competitive and non-competitive internal controls are also known in the field (Hoorfar 2004).

[0029] The practice of the methods described herein will employ, unless otherwise indicated, conventional techniques in organic chemistry, biochemistry, oligonucleotide synthesis and modification, bioconjugate chemistry, nucleic acid hybridization, molecular biology, microbiology, genetics, recombinant DNA, and related fields as are within the skill of the art. These techniques are fully explained in the literature. See, for example, Sambrook, Fritsch & Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory Press (1989); Ausubel, et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons (1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996); Gait (ed.), OLIGONUCLEOTIDE SYNTHESIS: A PRACTICAL APPROACH, IRL Press (1984); and Eckstein (ed.), OLIGONUCLEOTIDES AND ANALOGUES: A PRACTICAL APPROACH, IRL Press (1991).

III. Description

[0030] Preferred embodiments herein are directed to primers and probes for use in methods for specific amplifying and/or detecting CTX-M groups 1 and 9 nucleic acids. Primers and probes of the invention are suitable to be used in the methods of the invention to detect CTX-M groups 1 and 9 sequences either simultaneously in a single reaction or in separate reactions. Typically, the amplification methods are performed on CTX-M groups 1 and 9 nucleic acids. One such amplification method is the polymerase chain reaction (*see, e.g.*, U.S. Patent Nos. 4,683,195 and 4,965,188; Mullis *et al.*, *Cold Spring Harb. Symp. Quant.Biol.*, 51 Pt 1:263-273 (1986)).

[0031] Amplification procedures are those in which many copies of a target nucleic acid sequence are generated, usually in an exponential fashion, by sequential polymerization and/or ligation reactions. In addition to the more traditional amplification reactions discussed below, the present method is useful in amplifications involving three-way junctures (see, e.g., WO 99/37085), signal amplification (see, e.g., Capaldi, et al., Nuc. Acids Res., 28:E21 (2000)), T7 polymerases, reverse transcriptase, RNase H, RT-PCR, Rolling Circles, cleavase and the like. Isothermal amplification methods have been reviewed (Niemz, A. et al Trends Biotechnol., 29: 240-50 (2011)). The term "oligonucleotide primers adjacent to a probe region" refers to when 0 or one or more bases separate the primer and probe. The term "overlapping with said probe region" is defined as disclosed in U.S. Patent No. 7,319,022. The term "Ct" refers to the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold.

[0032] Accordingly, in a first aspect, the invention provides methods for detecting a CTX-M nucleic acid in a sample, comprising:

(a) contacting a sample suspected of containing the CTX-M with at least one flap primer having the formula:

$$5'\text{-}[X]_n\text{-}Y\text{-}3' \qquad (I),$$

wherein X represents the 5' portion of the flap primer that is non-complementary to the CTX-M nucleic acid, Y represents the 3' portion of the flap primer that is complementary to the CTX-M nucleic acid, wherein X is about 3-30 nucleotides in length and n=0 or 1, and;

(b) incubating the mixture of step (a) under conditions sufficient to amplify the CTX-M nucleic acid, thereby generating an amplified CTX-M nucleic acid; and

(c) detecting the amplified CTX-M nucleic acid.

[0033] In some embodiments the at least one flap primer comprises more than one primer sequence. In some embodiments a fluorescence-generating probe is used to detect the amplified CTX-M nucleic acid. The probe may contain a minor groove binder.

[0034] In carrying out the preferred methods, the reaction mixture typically comprises at least two flap primers: a forward flap primer and a reverse flap primer. The forward flap primer and the reverse flap primer can be, but need not be, of equal lengths.

[0035] In one embodiment, the 5' sequence portion of the flap primer that is non-complementary to the CTX-M nucleic acid (X) is about 1-15 nucleotides in length, usually about 4-14 or about 4-13 nucleotides in length, and more usually about 4-12 nucleotides in length. The 5' sequence portion of the flap primer that is non-complementary to the CTX-M nucleic acid (X) can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length. In preferred embodiments, the primer is about 30 nucleotides in length overall. If the complementary sequence is less than 30 nucleotides, then a flap may be used to produce a 30-mer primer.

[0036] In certain instances, the 3' sequence portion of the flap primer that is complementary to the CTX-M nucleic acid (Y) comprises a greater number of nucleotides than the 5' sequence portion of the flap primer that is non-complementary to the CTX-M nucleic acid (X). For example, the 3' sequence portion of the flap primer that is complementary to the CTX-M nucleic acid (Y) can comprise about 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% of the total length of a flap primer.

[0037] In certain other instances, the 5' sequence portion of the flap primer that is non-complementary to the CTX-M nucleic acid (X) comprises about an equal number of nucleotides as the 3' sequence portion of the flap primer that is complementary to the CTX-M nucleic acid (Y). For example, the X and Y portions each can be about 4-30, 6-25, 8-20, or 10-15 nucleotides in length, usually about 10-14 or 11-13 nucleotides in length, and more usually about 12 nucleotides in length. The X and Y portions each can be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[0038] In another embodiment, the 5' sequence portion of the flap primer that is non-complementary to the CTX-M nucleic acid (X) comprises at least about 60%, 65%, 70%, 75%, 80%, 90%, or 95% adenine or thymine nucleotide bases, or modified bases thereof.

[0039] In some embodiments, the 5' sequence portion of the flap primer that is non-complementary to the CTX-M nucleic acid (X) comprises the following sequence or a portion of it: AATAAATCATAA (SEQ ID NO:51). This non-complementary flap sequence may be considered to represent AATAAATCATAA-J (SEQ ID NO:52), in which J is an additional oligonucleotide sequence. In other embodiments, the Y portion of the first flap primer comprises sequences substantially complementary to at least a portion of the CTX-M groups 1 and 9 nucleic acids, namely SEQ ID NOs: 1, 12, 19 or 29, shown below in Table 1, namely primers having sequences with substantial identity to SEQ ID NOs: 2, 3, 6, 7, 8, 13, 14, 16, 17, 20, 21, 24, 25, 30, 31, 33 and 34. "Substantially complementary to at least a portion of" means that the sequence is complementary enough to the CTX-M sequence that it will hybridize and result in amplification of the CTX-M sequence. "Substantial identity" more specifically means about 85% complementary.

[0040] The sample is typically obtained from a mammal suspected of having a bacterial infection with potential CTX-M antibiotic resistant involvement. Preferably, the mammal is a human. Examples of samples suitable for use in the methods of the invention include, but are not limited to a rectal swab.

[0041] Generally, the methods produce a detectable signal when the probe hybridizes to the amplified target (US Patent NOs: 7,381,818 and 7,759,126). In addition this method allows the post-amplification melting curve analysis. Alternatively, the fluorescent probe is cleaved by using a nucleic acid polymerase having 5'-3' nuclease activity to yield a fluorescent signal (US Patent NO: 5,538,848). Further, the methods are particularly suited to continuous monitoring of a detectable signal ("real-time detection"). In certain embodiments, simultaneous amplification is detected using a fluorescence-generating probe, for example, a hybridization-based fluorescent probe or a nucleic acid binding fluorescent compound.

[0042] Amplified CTX-M groups 1 and 9 nucleic acid can be detected using any of the methods of detection known in the art. For example, detection can be carried out after completion of an amplification reaction (e.g., using ethidium bromide in an agarose gel) or simultaneously during an amplification reaction ("real-time detection") (McPherson et al., PCR Basics, 2000; and Rapid Cycle Real-time PCR Methods and Applications: Quantification, Wittwer et al. (eds.), Springer-Verlag (2004)). Preferably, the amplified CTX-M groups 1 and 9 nucleic acid is detected by hybridization to a probe that specifically binds to the amplified CTX-M groups 1 and 9 nucleic acids. In certain instances, the amplified CTX-M groups 1 and 9 nucleic acids is detected using one or more fluorescence-generating probes. Fluorescence-generating probes include probes that are cleaved to release fluorescence (e.g. U.S. Patent NO: 5,538,848, 7,790,385 etc.), nucleic acid binding compounds (e.g., U.S. Patent NO: 5,994,056; Bengtsson et al., Nucl. Acids Res., 31: e45 (2003)), hybridization-based probes (e.g., U.S. Patent NOs: 5,925,517, 7,205,105, 7,381,818, etc.), and the like. In certain embodiments, the CTX-M groups 1 and 9 nucleic acid is detected with one or more nucleic acid binding fluorescent compounds (e.g., SYBR® Green 1 (Molecular Probes; Eugene, OR), BOXTOX, BEBO (TATAA Biocenter; Gotenborg, Sweeden), etc.).

[0043] In one embodiment, the CTX-M groups 1 and 9 nucleic acid is detected using a fluorescence-generating probe, disclosed in Table 1, that hybridizes to the CTX-M groups 1 and 9 nucleic acids and one or more nucleotide bases of at least one flap primer sequence (typically, the complementary portion, Y). For example, the fluorescence-generating probe can hybridize to the CTX-M groups 1 and 9 nucleic acid and to one or more nucleotide bases of the forward flap primer sequence, one or more nucleotide bases of the reverse flap primer sequence, or simultaneously to one or more nucleotide bases of both the forward and the reverse flap primer sequences. The fluorescence-generating probe can optionally hybridize to the CTX-M groups 1 and 9 nucleic acid and to 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide bases of at least one flap primer sequence, particularly the complementary portion (Y) of a flap primer.

[0044] In a preferred embodiment, the fluorescence-generating probe of the invention comprises a sequence having substantial identity to a CTX-M groups 1 or 9 sequence selected from SEQ ID NOs: 4, 9, 10, 11, 18, 22, 26, 27, 28, 32, 35 and 36-46.

[0045] In another preferred embodiment, the fluorescence-generating probes of the invention comprise at least one of the following sequences:

R_a-G*TCGGCTCGGTACGG-R_b (SEQ ID NO:22) and

R_a-G*TAGGTTCAGTGCCATCC-R_b (SEQ ID NO:26)

wherein G* is the guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, and M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, with the proviso that the substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

[0046] In a preferred embodiment Fl is AquaPhluor® 593 with an excitation wavelength of 593 nm.

[0047] The primers can incorporate additional features which allow for the detection or immobilization of the primer but do not alter the basic property of the primer, e.g., to act as a point of initiation of nucleic acid synthesis. In some instances, the primers contain one or more non-natural bases or modified bases in either or both the complementary and non-complementary sequence regions of the primer.

[0048] In certain instances, amplification is carried out using a polymerase. The polymerase can, but need not, have 5' nuclease activity. In certain other instances, primer extension is carried out using a reverse transcriptase and amplification is carried out using a polymerase.

[0049] In another embodiment, the primer sequences overlap, wherein the stability of the overlapping sequence duplex is less than that of the stability of the individual primer target duplexes.

[0050] In another aspect, the invention provides methods for simultaneously detecting nucleic acids from CTX-M groups 1 and 9 in a sample, comprising:

(a) contacting a sample suspected of containing the CTX-M groups 1 and 9 nucleic acids with:

(i) at least one forward flap primer comprising at least one of the following sequences:

5'-AATAAATCATAAGCGCATCGCCCGGAATG-3' (SEQ ID NO: 21)

5'-AATAAATCATAATCGGGTCGCCGGGAATG-3' (SEQ ID NO: 25)

wherein the underlined nucleotide sequence is non-complementary to the CTX-M groups 1 and 9 sequences; and

(ii) at least one reverse flap primer comprising at least one of the following sequences:

5'-AATAAATCATAAACGAAACGTTCCGTCTCGAC-3' (SEQ ID NO:20)

5'-AATAAATCATGCGATGAGACGTTTCGTCTGGA-3' (SEQ ID NO:24),

wherein the underlined nucleotide sequence is non-complementary to the CTX-M groups 1 and 9 sequences;

(b) incubating the reaction mixture of step (a) under conditions sufficient to amplify the CTX-M groups 1 and 9 nucleic acids, thereby generating amplified CTX-M groups 1 and 9 nucleic acids from bacteria containing the CTX-M groups 1 and 9 lactamase genes that carry antibiotic resistance; and

(c) detecting the amplified CTX-M groups 1 and 9 nucleic acid.

[0051] Some embodiments comprise primer ratios that allow asymmetric amplification of the CTX-M groups 1 and 9 nucleic acids.

[0052] The sample is typically obtained from a mammal suspected of having an infection of an organism that carries extended spectrum beta-lactamase (ESBL). Preferably, the mammal is a human. Typical sample suitable for use in the methods of the invention contain ESBL containing organisms, preferably rectal swabs.

[0053] In some embodiments, continuous monitoring of a detectable signal ("real-time detection") is used to detect the signal. In certain embodiments, simultaneous amplification is detected using a fluorescence-generating probe, for example, a hybridization-based fluorescent probe, a probe with a cleaving-site or a nucleic acid binding fluorescent compound. In some embodiments, end-point fluorescent measurement using a dissociation curve analysis is used to detect the signal.

[0054] In yet another aspect, kits are provided for detecting an CTX-M groups 1 and 9 nucleic acid in a sample, comprising:

at least one forward flap primer comprising at least one of the following sequences:

5'-AATAAATCATAAGCGGATCGCCCCCAATG-3' (SEQ ID NO: 21)

5'-AATAAATCATAATCGGGTCGCCGGGAATG-3' (SEQ ID NO: 25)

wherein the underlined nucleotide sequence is non-complementary to the CTX-M groups 1 and 9 sequences; and

(ii) at least one reverse flap primer comprising at least one of the following sequences:

5'-AATAAATCATAAACGAAACGTTCCGTCTCGAC-3' (SEQ ID NO:20)

5'-AATAAATCATGCGATGAGACGTTTCGTCTGGA-3' (SEQ ID NO:24),

wherein the underlined nucleotide sequence is non-complementary to the e CTX-M groups 1 and 9 sequences.

[0055] In certain instances, the kits further comprise a fluorescence-generating probe such as a hybridization-based fluorescent probe, CTX-M groups 1 and 9 or a nucleic acid binding fluorescent compound. In a preferred embodiment, the fluorescence-generating probes comprise the following sequence:

R_a-G*TGACI*TGGATGAAAG-R_b-3' (SEQ ID NO:4)

R_a-G*TGACGTGGCTCAAAG-R_b (SEQ ID NO:9)

R_a-G*TGACI*TGGA*TGAAAGGC-R_b (SEQ ID NO:10)

R_a-G*TGACGTGGCTCAAAGGC-R_b (SEQ ID NO:11)

wherein R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, and M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, with the proviso that the substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

[0056] In another preferred embodiment, the fluorescence-generating probes comprise at least one of the following sequences:

R_a-G*TCGGCTCGGTACGG-R_b (SEQ ID NO:22) and

R_a-G*TAGGTTCAGTGCGATCC-R_b (SEQ ID NO:26)

wherein G* is 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, and M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, with the proviso that the substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

[0057] In a preferred embodiment Fl is AquaPhluor 593 with an excitation wavelength of 593 nm (ELITechgroup Molecular Diagnostics, Bothell, WA).

[0058] In certain other instances, the kits further comprise a control nucleic acid that is suitable for use as an internal control. As a non-limiting example, the control nucleic acid can comprise a nucleic acid sequence SEQ ID NO:47. Preferably, the control nucleic acid comprises the following sequence:

5'-CTGCACGGACCAGTTACTTTACGGACCACGTACCGCATTGGTACAAGAT

CTCCGGTAGAAAAAATGAG-3' (SEQ ID NO:47).

[0059] The kits of the invention can also comprise primers and probes directed against the control nucleic acid. As a non-limiting example, a control probe (e.g., a fluorescence-generating probe) and a set of control primers designed against the nucleic acid sequence SEQ ID NO:47 can be included in the kits. Preferably, the control probe and primers have the following sequences:

(i) Probe: R_a-G*ACCACGTACCGCATTG-R_b (SEQ ID NO:50)

wherein G* is the guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, and M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and

900 nm, and Q is a non-fluorescent quencher, with the proviso that the substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized; and that fluorophore has an emission wavelength different from that of the probe for detecting the nucleic acids from CTX-M groups 1 and 15;

(ii) Primers: CTGCACGGACCAGTTACTTTACG (SEQ ID NO:48);

CTCATTTTTTCTACCGGAGATCTTGT (SEQ ID NO:49).

[0060] In a preferred embodiment Fl is AquaPhluor 525 with an excitation wavelength of 525 nm (ELITechgroup Molecular Diagnostics, Bothell, WA), "M" is a minor groove binder such as, for example DPI3, "G*" is PPG, and "Q" is the Eclipse® Dark Quencher.

[0061] In an additional embodiment, the invention provides isolated nucleic acids comprising the sequences set forth in Table 1 below. Table 1 shows primers, probes, and target sequences for CTX-M groups 1 and 9 and an Internal Control.

Table 1

CTX-M groups 1, 9			
SEQ ID #	**Primer or Probe**	**Sequence** **>gi|11321257 for 1&15** **gi|16304825 for 9**	**Position**
		Location 1	
1	Group 1amplicon	CCAACGGGCGCAGCTGGTGACGTGGATGAAAGGCAATACTACCGGTGCAGCGAG	grp1 615-668
2	CTX-E1	AATAAATCATAACTCGCTGCACCGGTI*GTA	grp1,15 651-668
3	CTX-L1	AATAAATCATAACCAACGGGCGCAGCT	grp 1 615-629
4	CTX-	R_a-G*TGACI*TGGATGAAAG-R_b	grp 1

	AP593-1		631-646
5	Group 9 amplicon	GAAACCCAGCGGGCGCAGTTGGTGACGTGGCTCAAAGGCAATACGACCGGCGCAGCCAG	grp9 741-998
6	CTX-E2	AATAAATCATAATGGCTGCGCCGGTCGTA	grp9 782-798
7	CTX-L2	AATAAATCATAA*GAAACCCAGCGGGCGCAGTT*	grp9 741-760
8	CTX-L3	AATAAATCATAACCAGCGGGCGCAGTT	Grp9 745-760
9	CTX-AP593-2	R_4-G*TGACGTGGCTCAAAG-R_5	grp 9 762-777
10	CTX-AP593-7	R_4-G*TGACI*TGGA*TGAAAGGC-R_5	grp 1 631-648
11	CTX-AP-8	R_4-G*TGACGTGGCTCAAAGGC-R_5	grp9 761-779
Location 2			
12	Group 1,15 amplicon	CCGAATCTCTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAG	
13	CTX-L5	AATAAATCACTCAGCCAGTGACATCGTCCCAT	grp 1, 349-372
14	CTX-E4	AATAAATCATACCGAATCTGTTAAATCA*GCGAGT	grps 1 270-293
15	Group 9	AGCTGCTTAATCAGCCTGTCGAGATCAAGC	

	amplicon	CTGCCGATCTGGTTAACTACAATCCGATTGCCGAAAAACACGTCAACGGCACAATGACGCT	
16	CTX-L4	AATAAATCATAACGTCATTGTGCCGTTGACGT	grp9 475-494
17	CTX-E3	AATAAATCATAGCTGCTTAATCAGCCTGTCGA	grp9 407-427
18	CTX-AP593-3	R_a-G*CA*ATCGGA*T*T*I*TAGT-R_b	all grps 1&15: 451-466 9:382-397
Location 3			
19		ACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCCATTCCGGGCGATCCGC	grp 1 479-541
20	CTX-E6	AATAAATCATAAACGAAACGTTCCGTCTCGAC	grp1 479-498
21	CTX-L7	AATAAATCATAAGCGCATCGCCCGGAATG	grp 1 525-541
22	CTX-AP593-11	R_a-G*TCGGCTCGGTACGG-R_b	grp1 498-512
23	grp 1,5 amplicon	GCGATGAGACGTTTCGTCTGGATCGCACTGAACCTACGCTGAATACCGCCATTCCCGGCGACCCGAGAGAC	Group9
24	CTX-E5	AATAAATCATGCGATGAGACGTTTCGTCTGGA	grp9 607-628

25	CTX-L6	AATAAATCATAATCGGGTCGCCGGGAATG	grp9 656-672
26	CTX-AP593-4	R_a-G*TAGGTTCAGTGCGATCC-R_b	grp9 626-643
27	CTX-AP593-5	R_a-G*TCGGCTCGGTACGG-R_b	grp1,15
28	CTX-AP593-10	R_a-G*TAGGTTCAGTGCCATCC- R_b	grp9 626-643
4th location (variation of the 2nd location)			
29		ACGTCAACGGCACAATGACGCTGGCAGAACTGAGCGCGGCCGCGTTGCAGTACAGCGACAATACCGCCATGAACAAATTGATTGCCCAGC	Group 1:
30	CTX-L8	ATCATAA**CG**TG**A**GCAATCAGCTTATTCAT**C**GC	group 1 409-433
31	CTXM-E7	ATAAATCATAACGTCAACGGCACAATGACGCT	group 1 345-365
32	CTX-AP593-6	R_a-G*T*T*I*TCGCTGTA*CTGTAG- R_b	all grps
33	CTX-L9	ATCATAA**GC**TG**G**GCAATCAA**T**T**TG**TTCAT**G**GC	group 9 409-433
34	CTXM-E8	ATAAATCATAACGTCAACGGCACAATGACGCT 71	group 9 476-496
35	CTX-AP593-12	R_a-GTTI*TCGCTGTACTGTA*G-R_b	all grps
		Specific Probes	

	Group 9		
		Amplicon 1 (starting from 5' end of alignment)	
36		R$_a$-ATGGTGACAAAGAGAGT-R$_b$	
37		R$_a$-CCGCGAACATCATCCGT-R$_b$	
		Amplicon 2	
38		R$_a$-CGGCGGCGTGCATTC-R$_b$	
39		R$_a$-GCCCAGCAGCAGCG-R$_b$	
		Amplicon 3	
40		R$_a$-CCCGGGAGGCGTGA-R$_b$	
41		R$_a$-GATCGCGCGGGCAA-R$_b$	
		Amplicon 4	
42		R$_a$-TCATGCGCTGGGCGAA-R$_b$	
43		R$_a$-AGCTTACGCTGGGTCATG-R$_b$	
44		R$_a$-GAGCCACGTCACCAACT-R$_b$	
		Amplicon 5	
45		R$_a$-GGTGATCTGGCCGCA-R$_b$	
46		R$_a$-GGCGCACGACCC-R$_b$	
I* =3-(aminobutynyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one G*=6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (PPG or Super G$^{®}$) A*=4-(4,6-Diamino-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-but-3-yn-1-ol (Super A$^{®}$) T*= 5-(4-hydroxy-but-1-ynyl)-1H-pyrimidine-2,4-dione (Super T$^{®}$)			
Internal Control			
47	Amplicon	CTGCACGGACCAGTTACTTTACGGACCACGTACCGCATTGGTACAAGATCTCCGGTACAAAAAATGAG	

48	E6132-L	CTGCACGGACCAGTTACTTTACG	0.20µM
49	E6132-E	CTCATTTTTTCTACCGGAGATCTTGT	0.20µM
50	E6132-AP525-TM3	R_a-G*ACCACGTACCGCATTG-R_b	0.10µM
Fl= AquaPhluor525 with an excitation wavelength of 525 nm			

[0062] Most preferred primer and probe sequences for the detection of CTX-M groups 1 and 9 nucleic acid and most preferred IC sequences are shown in Table 2 below.

Table 2

CTX-M groups 1 and 9 **Primer and probe sequences**			
SEQ ID #	**Name**	**Sequence 5'-3'**	**1X**
26	CTXM-AP593-4	R_a-G*TAGGTTCAGTGCGATCC-R_b	0.10µM
27	CTXM-AP593-5	R_a-G*TCGGCTCGGTACGG-R_b	0.10µM
25	CTXM-L6	AATAAATCATAATCGGGTCGCCGGGAATG	0.50µM
21	CTXM-L7	AATAAATCATAAGCGGATCGCCCGGAATG	0.50µM
24	CTXM-E5	AATAAATCATGCGATGAGACGTTTCGTCTGGA	0.50µM
20	CTXM-E6	AATAAATCATAAACGAAACGTTCCGTCTCGAC	0.50µM
Ra=Fl and Fl=AquaPhluor 593 with an excitation wavelength of 593 nm Rb=Q-M where Q=Eclipse® Dark Quencher and M=DPI$_3$ and G=Super G			
Internal Control Primer and Probe Sequences			

48	E6132-L	CTGCACGGACCAGTTACTTTACG	0.20µM
49	E6132-E	CTCATTTTTTCTACCGGAGATCTTGT	0.20µM
50	E6132-AP525-TM3	R_a-G*ACCACGTACCGCATTG-R_b	0.10µM
Ra=Fl and Fl=AquaPhluor 525 with and excitation wavelength of 525 nm Rb=Q-M where Q=Eclipse® Dark Quencher and M=DPI_3 and G=Super G			
Non-complementary Flap Sequence			
51		AATAAATCATAA	NA
52		AATAAATCATAA-J	
J = an oligonucleotide sequence			

[0063] In one aspect, the present invention provides target sequences suitable for specific detection and amplification of extended beta-lactamase resistant genes that involves CTX-M groups 1 and 9.

[0064] More specifically, a method of detecting the presence or absence of extended beta-lactamase resistant nucleic acids in samples that involve CTX-M groups 1 and 15, the method comprises contacting said sample with one or more oligonucleotides designed to hybridize to any one of SEQ ID NO: 1, 12, 19 or 29 or a complement under conditions which discriminate groups 1 and 15 nucleic acid and detecting if the hybridization of said one or more oligonucleotides to SEQ ID NO: 1, 12, 19 or 29 or a complement thereof has occurred, wherein said hybridization detects the presence or absence of CTX-M groups 1 and 15 nucleic acid.

[0065] In another embodiment a method of detecting the presence or absence of extended beta-lactamase resistant nucleic acids in samples that involve CTX-M group 9 comprises contacting said sample with one or more oligonucleotides designed to hybridize to any one of SEQ ID NO: 5, 15, or 23 or a complement under conditions which discriminate group 9 nucleic acid and detecting if the hybridization of said one or more oligonucleotides to SEQ ID NO: 5, 15, or 23 or a complement thereof has occurred, wherein said hybridization detects the presence or absence of CTX-M group 9 nucleic acid.

[0066] In one aspect the detection of presence or absence of extended beta-lactamase resistant gene nucleic acids as defined in Table 1 that involves CTX-M groups 1 and 9 by hybridization comprises nucleic acid-based amplification.

[0067] In another aspect the generated amplicon is at least about 30 nucleotide bases long.

[0068] Other aspects include the one or more oligonucleotides hybridizing to the nucleic acids of extended beta-lactamase resistant genes sequences as defined in Table 1 that involve CTX-M groups 1 and 9 comprises primers and/ or probes.

[0069] The one or more oligonucleotide primers hybridizing to nucleic acids of CTX-M group 1 may be selected from sequences SEQ ID NO: 2, 3, 13, 14, 20, 21, 30 and 31.

[0070] The one or more oligonucleotide primers hybridizing to nucleic acids of CTX-M group 9 may be selected from sequences comprising SEQ ID NO: 6, 7, 8, 16, 17, 24, 25, 33 and 34.

[0071] Nucleic acid-based amplification products generated from CTX-M groups 1 nucleic acid may be detected by one or more oligonucleotide probes comprising sequences selected from SEQ ID NOs: 4, 18, 22, and 27.

[0072] In some embodiments R_a=M-Fl where M is a minor groove binder and Fl is a fluorophore and R_b– quencher.

[0073] Nucleic acid-based amplification products generated from CTX-M group 9 nucleic acid may be detected by one or more oligonucleotide probes comprising sequences selected from SEQ ID NOs: 9, 11, 26, 28, and 36-46.

[0074] The present method provides oligonucleotide primers ("overhang primers," "flap primers," or "adapter primers") which are most generally noted as 5'-$(X)_p$-

Y-3' primers where p=0 or 1. X represents the sequence portion of the primer that is non-complementary to the CTX-M groups 1 and 9 nucleic acid, and Y represents the sequence portion of the primer that is complementary to the CTX-M groups 1 and 9 nucleic acid.

[0075] Accordingly, in one group of embodiments, the primer has the formula:

$$5'\text{-}(X)_p\text{-}Y\text{-}3' \qquad (I),$$

wherein X represents the 5' sequence of the primer non-complementary to the CTX-M groups 1 and 9 nucleic acid, Y represents the complementary 3' sequence of the primer, p is 0 or 1, and X-Y represents the nucleic acid oligomer primer. In certain further embodiments, X is $[A\text{-}B]_m$ and Y is $[A\text{-}B]_n$, wherein A represents a sugar phosphate backbone, modified sugar phosphate backbone, locked nucleic acid backbone, or a variant thereof in any combination as used in nucleic acid preparation; B represents a nucleic acid base or a modified base of a base; and the subscripts m and n are integers of from about 4-30 or 5-25, 7-20, or 9-15, and more usually about 12. In certain embodiments, the values of the subscripts m and n are equal, for example, both m and n simultaneously can be an integer of from about 5-25, 7-20, or 9-15, and more usually about 12.

[0076] Primers and probes were designed to amplify and detect regions of the blaCTX-M genes blaCTX-M gene located on a transferable plasmid and more specifically of CTX-M groups 1 and 9 nucleic acids that have substantial or absolute homology between members of respective groups. In some embodiments, the primers are flap primers comprising the following formula:

$$5'\text{-}(X)_p\text{-}Y\text{-}3' \qquad (I),$$

wherein X represents the 5' portion of the flap primer that is non-complementary to the CTX-M groups 1 and 9 nucleic acid, Y represents the 3' portion of the flap primer that is complementary to the CTX-M groups 1 and 9 nucleic acid, and p is about 3-30. 5-25, 7-20, or 9-15.

[0077] The 5'-non-complementary sequences of the primers of this invention can be modified as taught in U.S. Patent Application 2007/0048758.

[0078] The primers and probes of the present invention are generally prepared using solid phase methods known to those of skill in the art. In general, the starting materials are commercially available, or can be prepared in a straightforward manner from commercially available starting materials using suitable functional group manipulations as described in, for example, March et al., ADVANCED ORGANIC CHEMISTRY – Reactions, Mechanisms and Structures, 4th ed., John Wiley & Sons, New York, NY (1992).

[0079] In one embodiment, the primers and probes of the invention can comprise any naturally occurring nucleotides, non-naturally occurring nucleotides, or modified nucleotides known in the art (see, e.g, U.S. Patent Publication No. 20050118623; and U.S. Patent No. 6,949,367, U.S. Patent Publication No. 20120244535).

[0080] The ability to design probes and primers in a predictable manner using an algorithm that can direct the use or incorporation of modified bases, minor groove binders, fluorphores, and/or quenchers based on their thermodynamic properties have been described in, e.g., U.S. Patent No. 6,683,173. Accordingly, the use of any combination of normal bases, unsubstituted pyrazolo[3,4-d]pyrimidine bases (e.g., PPG and PPA), 3-substituted pyrazolo[3,4-d]pyrimidines, modified purines, modified pyrimidines, 5-substituted pyrimidines, universal bases, sugar modifications, backbone modifications, and/or minor groove binders to balance the Tm (e.g., within about 5-8°C) of a hybridized product with a modified nucleic acid, reduce G-G self-association or to accommodate mismatches in primer or probe is contemplated by the present invention. Co-owned U.S. Patent Application 2012/0244535, incorporated by reference, provides additional explanation as to how to address primers and probes with as many as five mismatches in a primer.

[0081] Detailed descriptions of the chemistry used to synthesize oligonucleotides by the phosphoramidite method are provided in U.S. Patent Nos. 4,458,066 and 4,415,732; Caruthers et al., Genetic Engineering, 4:1-17 (1982); and Users Manual Model 392 and 394 Polynucleotide Synthesizers, pages 6-1 through 6-22, Applied Biosystems, Part No. 901237 (1991). Labeled oligonucleotides can be synthesized by chemical synthesis, e.g., by a phosphoramidite method, a phosphite-triester method, and the like, (see, e.g., Gait, Oligonucleotide Synthesis, IRL Press (1990)). Labels can be introduced during enzymatic synthesis utilizing labeled nucleoside triphosphate monomers, or introduced during chemical synthesis using labeled non-nucleotide or nucleotide phosphoramidites, or may be introduced subsequent to synthesis.

[0082] A variety of linking groups and methods are known to those of skill in the art for attaching fluorophores, quenchers, and minor groove binders to the 5' or 3' termini of oligonucleotides. See, for example, Eckstein, (ed.), Oligonucleotides and Analogues: A Practical Approach, IRL Press, Oxford (1991); Zuckerman et al., Nuc. Acids Res., 15:5305-5321 (1987); Sharma et al., Nuc. Acids Res., 19:3019 (1991); Giusti et al., PCR Methods and Applications, 2:223-227 (1993), U.S. Patent Nos. 4,757,141 and 4,739,044; Agrawal et al., Tetrahedron Letters, 31:1543-1546 (1990); Sproat et al., Nuc. Acids Res., 15:4837 (1987); Nelson et al., Nuc. Acids Res., 17:7187-7194 (1989); and the like. Still other commercially available linking groups can be used that can be attached to an oligonucleotide during synthesis and are available from, e.g., Clontech Laboratories (Palo Alto, CA.). Other methodologies for attaching a fluorophore to an oligonucleotide portion involve the use of phosphoramidite chemistry at the conclusion of solid phase synthesis by way of dyes derivatized with a phosphoramidite moiety. See, e.g., U.S. Patent Nos. 5,231,191; 4,997,928; 6,653,473; 6,790,945; and 6,972,339; and PCT Patent Publication No. WO 01/42505.

IV. Additional Amplification Reaction Components

Buffers

[0083] Buffers that may be employed are borate, phosphate, carbonate, barbital, Tris, etc., based buffers (see, e.g., U.S. Patent No. 5,508,178). The pH of the reaction should be maintained in the range of from about 4.5 to about 9.5 (see, e.g., U.S. Patent No. 5,508,178). The standard buffer used in amplification reactions is a Tris based buffer between 10 and 50 mM with a pH of around 8.3 to 8.8 (see, e.g., Innis et al., supra).

[0084] One of skill in the art will recognize that buffer conditions should be designed to allow for the function of all reactions of interest. Thus, buffer conditions can be designed to support the amplification reaction as well as any subsequent restriction enzyme reactions. A particular reaction buffer can be tested for its ability to support various reactions by testing the reactions both individually and in combination.

Salt concentration

[0085] The concentration of salt present in the reaction can affect the ability of primers to anneal to the target nucleic acid (see, e.g., Innis et al., supra). Potassium chloride can be added up to a concentration of about 50 mM to the reaction mixture to promote primer annealing. Sodium chloride can also be added to promote primer annealing (see, e.g., Innis et al., supra).

Magnesium ion concentration

[0086] The concentration of magnesium ion in the reaction can affect amplification of the target nucleic acid sequence (see, e.g., Innis et al., supra). Primer annealing, strand denaturation, amplification specificity, primer-dimer formation, and enzyme activity are all examples of parameters that are affected by magnesium concentration (see, e.g., Innis et al., supra). Amplification reactions should contain about a 0.5 to 6.0 mM magnesium concentration excess over the concentration of dNTPs. The presence of magnesium chelators in the reaction can affect the optimal magnesium concentration. A series of amplification reactions can be carried out over a range of magnesium concentrations to determine the optimal magnesium concentration. The optimal magnesium concentration can vary depending on the nature of the target CTX-M groups 1 and 9 nucleic acid and the primers being used, among other parameters.

Deoxynucleotide triphosphate concentration

[0087] Deoxynucleotide triphosphates (dNTPs) are added to the reaction to a final concentration of from about 20 μM to about 300 μM. Typically, each of the four dNTPs (G, A, C, T) are present at equivalent concentrations (see, e.g, Innis et al., supra). In some embodiments, uracil N-glycosylase is used with dUTP (instead of TTP) in PCR reactions.

Nucleic acid polymerases

[0088] A variety of DNA dependent polymerases are commercially available that will function using the present methods and compositions. For example, Taq DNA Polymerase may be used to amplify target DNA sequences. The PCR assay may be carried out using as an enzyme component a source of thermostable DNA polymerase suitably comprising Taq DNA polymerase which may be the native enzyme purified from Thermus aquaticus and/or a genetically engineered form of the enzyme. Other commercially available polymerase enzymes include, e.g., Taq polymerases marketed by Qiagen, New England Biolabs, Applied Biosystems, Promega or Pharmacia. Other examples of thermostable DNA polymerases that could be used in the invention include DNA polymerases obtained from, e.g., Thermus and Pyrococcus species. Concentration ranges of the polymerase may range from 1-5 units per reaction mixture. The reaction mixture is typically between about 5 µl and about 100 µl.

Other agents

[0089] Additional agents are sometimes added to the reaction to achieve the desired results. For example, DMSO can be added to the reaction, but is reported to inhibit the activity of Taq DNA Polymerase. Nevertheless, DMSO has been recommended for the amplification of multiple target sequences in the same reaction (see, e.g., Innis et al., supra). Stabilizing agents such as gelatin, bovine serum albumin, and non-ionic detergents (e.g., Tween-20) are commonly added to amplification reactions (see, e.g., Innis et al., supra). Additionally, betaine (Sigma-Aldrich; St. Louis, MO), an isostabilizing agent, can be added to equalize the contribution of GC- and AT-base pairing to the stability of the nucleic acid duplex.

Minor Groove Binders

[0090] Minor groove binder oligonucleotide conjugates (or "probes") are described in, e.g., U.S. Patent No. 6,312,894. These conjugates form hyper-stabilized duplexes with complementary DNA. In particular, sequence specificity of short minor groove binder probes is excellent for high temperature applications such as PCR. The probes/conjugates of the present invention will also optionally have a covalently attached minor groove binder. A variety of suitable minor groove binders have been described in the literature (see, e.g., U.S. Patent No. 5,801,155; Wemmer et al., Curr. Opin. Struct. Biol., 7:355-361 (1997); Walker et al., Biopolymers, 44:323-334 (1997); Zimmer et al., U. Prog. Biophys. Molec. Bio., 47:31-112 (1986); and Reddy et al., J. W., Pharmacol. Therap., 84:1-111 (1999)).

[0091] The minor groove binder-quencher-oligonucleotide-fluorophore conjugates can be in a linear arrangement (as suggested by the formula 5'-M-Q-ODN-Fl-3' or 5'-M-Fl-ODN-Q-3') or in a branched arrangement wherein the quencher (Q) and the minor groove binder (M) are attached to a linking group that serves to join ODN, Q, and M. Additionally, the quencher can be attached at the distal (relative to attachment to ODN) terminus of the minor groove binder (e.g., 5'-Q-M-ODN-Fl-3'). Each of the arrangements is meant to be included when the linear abbreviation (M-Q-ODN-Fl) is used. Additionally, the minor groove binder and quencher portions each can be attached at either the 3' or 5' end of the oligonucleotide, or an internal position of the oligonucleotide, so long as such attachment does not interfere with the quenching mechanisms of the conjugate. Generally, this can be accomplished through the use of a suitable linking group (see, e.g., U.S. Patent NOs: 7,205,105 and 7,381,818).

[0092] Suitable methods for attaching minor groove binders (as well as reporter groups such as fluorophores and quenchers described below) through linkers to oligonucleotides are described in, for example, U.S. Patent Nos. 5,512,677; 5,419,966; 5,696,251; 5,585,481; 5,942,610; and 5,736,626.

[0093] The minor groove binder is generally attached to the 3' or 5' position of the oligonucleotide portion via a suitable linking group. Attachment at the 5' end provides both a benefit of hybrid stability, since melting of an oligonucleotide duplex begins at the termini, but also reduces and/or prevents nuclease digestion of the probe during amplification reactions.

[0094] The location of a minor groove binder within a minor groove binder-oligonucleotide conjugate can also affect the discriminatory properties of such a conjugate. An unpaired region within a duplex will result in changes in the shape of the minor groove in the vicinity of the mispaired base(s). Since minor groove binders fit best within the minor groove of a perfectly-matched DNA duplex, mismatches resulting in shape changes in the minor groove would reduce binding strength of a minor groove binder to a region containing a mismatch. Hence, the ability of a minor groove binder to stabilize such a hybrid would be decreased, thereby increasing the ability of a minor groove binder oligonucleotide conjugate to discriminate a mismatch from a perfectly matched duplex. On the other hand, if a mismatch lies outside of the region complementary to a minor groove binder oligonucleotide conjugate, discriminatory ability for unconjugated and minor groove binder-conjugated oligonucleotides of equal length is expected to be approximately the same. Since the ability of an oligonucleotide probe to discriminate single base pair mismatches depends on its length, shorter oligonucleotides are more effective in discriminating mismatches. The primary advantage of the use of minor groove binder oligonucleotides conjugates in this context lies in the fact that much shorter oligonucleotides compared to those used in the prior art (i.e., 20 mers or shorter), having greater discriminatory powers, can be used, due to the pronounced stabilizing effect of minor groove binder conjugation.

[0095] The selection of minor groove binders and available minor groove binders have been disclosed in US Patent NOs: 5,801,155, 6,312,894 and 7,582,739.

Quenchers

[0096] Recently developed detection methods employ the process of fluorescence resonance energy transfer (FRET) for the detection of probe hybridization rather than direct detection of fluorescence intensity. In this type of assay, FRET occurs between a donor fluorophore (reporter) and an acceptor molecule (quencher) when the absorption spectrum of the quencher molecule overlaps with the emission spectrum of the donor fluorophore and the two molecules are in close proximity. The excited-state energy of the donor fluorophore is transferred to the neighboring acceptor by a resonance dipole-induced dipole interaction, which results in quenching of the donor fluorescence. If the acceptor molecule is a fluorophore, its fluorescence may sometimes be increased. The efficiency of the energy transfer between the donor and acceptor molecules is highly dependent on distance between the molecules. Equations describing this relationship are known. The Forster distance (Ro) is described as the distance between the donor and acceptor molecules where the energy transfer is 50% efficient. Other mechanisms of fluorescence quenching are also known, such as collisional and charge transfer quenching. There is extensive guidance in the art for selecting quencher and fluorophore pairs and their attachment to oligonucleotides (see, e.g., Haugland, Handbook of Fluorescent Probes and Research Chemicals, Ninth Edition, Molecular Probes, Eugene, OR (2002) and the Web Edition at www.probes.com/handbook; and U.S. Patent Nos. 3,996,345 and 4,351,760). Preferred quenchers are described in U.S. Patent Nos. 6,727,356 and

6,790,945. Additional mono- and bis-azo dyes are commercially available from Berry and Associates (Dexter, MI) and Glen Research (Sterling, VA).

Fluorophores

[0097] Fluorophores useful in the present invention are generally fluorescent organic dyes that have been derivatized for attachment to the terminal 3' or 5' carbon of the oligonucleotide probe, preferably via a linking group. One of skill in the art will appreciate that suitable fluorophores are selected in combination with a quencher that is typically also an organic dye, which may or may not be fluorescent. Examples of these and other suitable dye classes can be found in Haugland et al., Handbook of Fluorescent Probes and Research Chemicals, Sixth Ed., Molecular Probes, Eugene, Ore. (1996); U.S. Patent Nos. 3,194,805; 3,128,179; 5,187,288; 5,188,934; 5,227,487, 5,248,782; 5,304,645; 5,433,896; 5,442,045; 5,556,959; 5,583,236; 5,808,044; 5,852,191; 5,986,086; 6,020,481; 6,162,931; 6,180,295; and 6,221,604; EP Patent No. 1408366; Still other dyes are provided via online sites such as http://www.zeiss.com. Preferred phosphonate dyes are disclosed in co-owned US 7,671,218, US 7,767,834 and 8,163,910.

[0098] There is a great deal of practical guidance available in the literature for selecting appropriate fluorophore-quencher pairs for particular probes. Haugland supra and the Web Edition at www.probes.com/handbook and the like. Examples of these and other suitable dye classes can be found in Haugland et al., Handbook of Fluorescent Probes and Research Chemicals, Sixth Ed., Molecular Probes, Eugene, Ore. (1996); U.S. Patent Nos. 3,194,805; 3,128,179; 5,187,288; 5,188,934; 5,227,487, 5,248,782; 5,304,645; 5,433,896; 5,442,045; 5,556,959; 5,583,236; 5,808,044; 5,852,191; 5,986,086; 6,020,481; 6,162,931; 6,180,295; and 6,221,604; EP Patent No. 1408366; Still other dyes are provided via online sites such as http://www.zeiss.com. Methods for derivatizing fluorophores and quenchers for covalent attachment via common reactive groups are well known. See, for example, Haugland, supra; and U.S. Patent Nos. 3,996,345 and 4,351,760.

[0099] Preferred fluorophores are those based on xanthene dyes, a variety of which are available commercially with substituents useful for attachment of either a linking group or for direct attachment to an oligonucleotide. Most preferred phosphonate dyes are disclosed in co-owned US 7,671,218, US 7,767,834 and 8,163,910.

EXAMPLES

[0100] The following examples are provided to illustrate, but not to limit, the subject matter described herein.

Oligonucleotides

[0101] Primers were synthesized using standard phosphoramidite chemistry. The 5'-M-Fl-ODN-Q and Fl-ODN-Q-M probes were prepared by automated DNA synthesis on a M-FL- or M-Q-modified polystyrene solid support, respectively, using 5- β-cyaoethyl –or 3'-β-cyanoethyl phosphoramidites (Glen Research; Sterling, VA) designed for synthesis of oligonucleotide segments in the 5'→3 or 3'→5' direction, respectively. Oligonucleotide synthesis was performed on an ABI 394 synthesizer according to the protocol supplied by the manufacturer using a 0.02M iodine solution. Modified and universal bases were synthesized based on the methods disclosed in U.S. Patent No. 6,949,367, 6,127,121 and U.S. Patent Publication No. 20120244535. Fluorophore reporting dyes or quenchers were introduced at the last step of the synthesis using the corresponding phosphoramidites as required. All oligonucleotides were purified by reverse phase HPLC.

PCR

[0102] Real-time PCR was performed using the RGQ MDx real-time PCR amplification instrumentation on samples from human rectal swabs from symptomatic and asymptomatic patients material extracted with QIAsymphony SP/AS DNA extraction system (QIAGEN Inc., Valencia, CA) in a diagnostic assay to detect ESBL DNA. The assay mixture contains the following components:

[0103] **Master Solution A:** Includes the CTX-M group 1, 9-specific and Internal Control probes and primers, PCR buffer, HotStartTaq QR2 DNA Polymerase, Uracil N-Glycosylase, and deoxynucleotides (dATP, dCTP, dGTP, and dUTP). 23µL of the Master A reagent mix is used for each 40µL PCR reaction. The following formulation of Master A was selected as shown in Table 3 below.

[0104] **Master Solution B:** an 80mM $MgCl_2$ solution. 2µL of the Master B reagent mix is used for each 40µL PCR reaction for the final concentration of 4mM.

[0105] **Sample:** 15 µL of the extracted sample.

EXAMPLE 1

[0106] This example illustrates the real-time PCR detection of CTX-M15 DNA (member of CTX-M group 1) extracted from *E. coli* ATCC BAA-2326 alone and with Internal Control (biplex).

[0107] Nucleic acid was extracted with the Qiagen QIAsymphony SP/AS DNA extraction system from *E. coli* ATCC BAA-2326 according to instrument instructions (QIAsymphony® SP/AS User Manual, April 2012). The biplex MGB TaqMan assay was performed using 35 µL Master Solution A that includes the primers, probes and reagents shown in Table 3, 2 µL Master Solution B as described above and 15 µL extracted sample. Table 3 below shows the primers, probes, and reagents of Master Solution A.

Table 3

SEQ ID NO:	Component Name	Oligonucleotide Sequence	Final 1X Concen.
25	CTX-L6	AATAAATCATAATCGGGTCGCCGGGAATG	0.500 µM
21	CTX-L7	AATAAATCATAAGCGGATCGCCCGGAATG	0.500 µM
24	CTX-E5	AATAAATCATGCGATCAGACGTTTCGTCTGGA	0.500 µM
20	CTX-E6	AATAAATCATAAACGAAACGTTCCGTCTCGAC	0.500 µM
26	CTX-AP593-4	AP593-G*TAGGTTCAGTGCGATCC-EDQ-MGB	0.100 µM
27	CTX-	AP593-G*TCGGCTCGGTACGG-EDQ-MGB	0.100 µM

	AP593-5		
48	E6132-L	CTGCACGGACCAGTTACTTTACG	0.200 µM
49	E6132-E	CTCATTTTTTCTACCGGAGATCTTGT	0.200 µM
50	E6132-AP525-TM3	AP525-G*ACCACGTACCGCATTG-EDQ-MGB	0.100 µM
	100 mM dATP	N/A	0.200 mM
	100 mM dCTP	N/A	0.200 mM
	100 mM dGTP	N/A	0.200 mM
	100 mM dUTP	N/A	0.400 mM
	10X PCR Buffer	N/A	1X
	HotStarTaq QR2 DNA Polymerase	5U/µL	0.10 U/µL
	UNG	1U/µL	0.01 U/µL
	Molecular Biology Grade Water	N/A	N/A

[0108] The PCR cycling conditions are shown in Table 4 below.

Table 4

Stage	Temp (°C)	Time
UNG Treatment	50° C	2 min.
Polymerase Activation UNG Deactivation	95° C	15 Min.
Denaturation:	95° C	10 Sec.
Annealing:	56° C	30 Sec.
Extension:	72° C	15 Sec.

[0109] In Figure 1 is shown the real-time titration (the tenfold titration (100pg – 1fg)) of CTX-M15 DNA extracted from *E. coli* ATCC BAA-2326 alone and with Internal Control.

[0110] The single- and bi-plex amplification data of Figure 1 was re-plotted as linear curves in Figure 2a and 2b. As shown in Figure 2a and 2b the slopes of the CTX-M15- and CTC-M15 + IC curves are very similar, -3.188 and -3.123 respectively.

EXAMPLE 2

[0111] This example demonstrates the amplification and detection of CTX-M nucleic acid with probes and primers designed against locations 1, 2 and 3.

[0112] The primers and probes used are shown in Table 5 below. The amplification was performed similarly to that described in Example 1 and detection of CTX-M15 amplified nucleic acids is shown in Figure 3. Figure 3 shows the real-time detection of CTX-M15 nucleic acids with probes and primers designed for 3 different locations.

Table 5

Location	Primer SEQ ID NO:	Probe SEQ ID NO:

1	2, 3, 6, 7, and 8	4 and 9
2	13, 14, 16 and 17	18
3	20, 21, 24 and 25	26 and 27

EXAMPLE 3

[0113] This example illustrates the performance of primers and probes of Example 1 to detect CTX-M nucleic acids extracted and quantified from 9 different isolates as measured by Ct. Table 6 shows the Cts of different isolates in different CTXM groups as determined by the procedure described in Example 1.

Table 6

CTX-M group	Isolate Name	CTX-M Ct, no IC	CTX-M Ct, with IC	IC Ct
CTX-M 1	Misc 336	15.28	15.27	24.58
CTX-M 1	Misc 337	14.71	15.03	24.58
CTX-M 9	Ecoli 254	15.43	15.49	24.56
CTX-M 15	A15	15.32	15.32	24.65
CTX-M 15	H15	16.34	16.43	24.63
CTX-M 15	CUMC 245	16.82	16.82	24.39
CTX-M-14 (CTX-M 9 group homolog)	La14	16.36	16.23	24.36
CTX-M-14 (CTX-M 9 group homolog)	Lo14	15.11	15.17	24.28

CTX-M-14 (CTX-M 9 group homolog)	Misc 439	16.35	16.26	24.33

[0114] The results in Table 6 demonstrate the ability of primers and probes from Example 1 to detect CTX-M target nucleic acids from different isolates representing CTX-M1, 9 and 15.

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WHAT IS CLAIMED IS:

1. A method for detecting a CTX-M nucleic acid in a sample, comprising:

(a) contacting a sample suspected of containing the CTX-M nucleic acid with at least one flap primer having the formula:

$$5'\text{-}[X]_p\text{-}Y\text{-}3' \qquad (I),$$

wherein X is a 5' portion of the flap primer that is non-complementary to the CTX-M nucleic acid and Y is a 3' portion of the flap primer that is substantially complementary to at least a portion of the CTX-M nucleic acid, X has a sequence that comprises at least a portion of AATAAATCATAA (SEQ ID NO: 51), Y has a sequence that is substantially complementary to at least a portion of SEQ ID NO: 1, 12, 19 or 29, and p is 0 or 1, to produce a mixture;

(b) incubating the mixture of step (a) under conditions sufficient to amplify the CTX-M nucleic acid, thereby generating an amplified CTX-M nucleic acid; and

(c) detecting the amplified CTX-M nucleic acid.

2. The method of claim 1, wherein X and Y are each about 4 to about 30 nucleotides in length.

3. The method of claim 1, wherein the step of detecting the amplified CTX-M nucleic acid comprises using a fluorescence-generating probe having a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 4, 9, 10, 11, 18, 22, 26, 27, 28, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

4. The method of claim 3, wherein the fluorescence-generating probe further comprises a minor groove binder.

5. The method of claim 1, wherein the flap primer has a sequence having substantial identity to a sequence that is selected from SEQ ID NO: 2, 3, 6, 7, 8, 13, 14, 16, 17, 20, 21, 24, 25, 30, 31, 33, and 34.

6. The method of claim 1, wherein the flap primer comprises a sequence that is complementary to more than one portion of the CTX-M nucleic acid.

7. The method of claim 1, wherein X is $[A\text{-}B]_m$ and Y is $[A\text{-}B]_n$, wherein A represents a sugar phosphate backbone, modified sugar phosphate backbone, locked nucleic acid backbone, or a variant thereof in any combination, B represents a nucleic acid base or a modified base, m and n are integers of from about 4 to about 30.

8. The method of claim 1, further comprising the step of amplifying a control nucleic acid.

9. The method of claim 8, wherein the control nucleic acid has a sequence with substantial identity to SEQ ID NO: 47.

10. The method of claim 8, wherein the step of amplifying a control nucleic acid comprises using a fluorescence-generating probe having substantial identity to SEQ ID NO: 50, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

11. The method of claim 8, wherein the step of amplifying a control nucleic acid comprises using one or more primers having substantial identity to a sequence that is SEQ ID NO: 48 or 49.

12. A method for detecting a CTX-M nucleic acid in a sample, comprising:

    (a) contacting a sample suspected of containing the CTX-M nucleic acid with at least one primer having a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 2, 3, 6, 7, 8, 13, 14, 16, 17, 20, 21, 24, 25, 30, 31, 33, and 34, to produce a mixture;

    (b) incubating the mixture of step (a) under conditions sufficient to amplify the CTX-M nucleic acid, thereby generating an amplified CTX-M nucleic acid; and

    (c) detecting the amplified CTX-M nucleic acid.

13. The method of claim 12, wherein the step of detecting the amplified CTX-M nucleic acid comprises using a fluorescence-generating probe.

14. The method of claim 13, wherein the fluorescence-generating probe has a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 4, 9, 10, 11, 18, 22, 26, 27, 28, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

15. The method of claim 13, wherein the fluorescence-generating probe further comprises a minor groove binder.

16. The method of claim 12, further comprising the step of amplifying a control nucleic acid.

17. The method of claim 16, wherein the control nucleic acid has a sequence of SEQ ID NO: 47.

18. The method of claim 16, wherein the step of amplifying a control nucleic acid comprises using a fluorescence-generating probe having substantial identity to SEQ ID NO: 50, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

19. The method of claim 16, wherein the step of amplifying a control nucleic acid comprises using one or more primers having substantial identity to a sequence that is SEQ ID NO: 48 or 49.

20. A method for detecting a CTX-M nucleic acid in a sample, comprising:

(a) contacting a sample suspected of containing the CTX-M nucleic acid with at least one flap primer having the formula:

$$5'\text{-}[X]_p\text{-}Y\text{-}3' \qquad (I),$$

wherein X is a 5' portion of the flap primer that is non-complementary to the CTX-M nucleic acid and Y is a 3' portion of the flap primer that is substantially complementary to at least a portion of the CTX-M nucleic acid, and p is 0 or 1, to produce a mixture;

(b) incubating the mixture of step (a) under conditions sufficient to amplify the CTX-M nucleic acid, thereby generating an amplified CTX-M nucleic acid; and

(c) detecting the amplified CTX-M nucleic acid using a fluorescence-generating probe having a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 4, 9, 10, 11, 18, 22, 26, 27, 28, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

21. The method of claim 20, wherein X has a sequence that comprises at least a portion of AATAAATCATAA (SEQ ID NO: 51) and Y has a sequence that is substantially complementary to at least a portion of SEQ ID NO: 1, 12, 19 or 29.

22. The method of claim 20, wherein the flap primer has a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 2, 3, 6, 7, 8, 13, 14, 16, 17, 20, 21, 24, 25, 30, 31, 33, and 34.

23. The method of claim 20, further comprising the step of amplifying a control nucleic acid.

24. The method of claim 23, wherein the control nucleic acid has a sequence of SEQ ID NO: 47.

25. The method of claim 23, wherein the step of amplifying a control nucleic acid comprises using a fluorescence-generating probe having substantial identity to SEQ ID NO: 50, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, Ra is independently selected from (M)a-Fl and (M)a-Q, Rb is independently selected from (M)a-Fl and (M)a-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of Ra and Rb allows quenching of fluorescence when the probe is unhybridized.

26. The method of claim 23, wherein the step of amplifying a control nucleic acid comprises using one or more primers having substantial identity to a sequence that is SEQ ID NO: 48 or 49.

27. A method for simultaneously detecting CTX-M groups 1 and 9 nucleic acids in a sample, comprising:

(a) contacting a sample suspected of containing the CTX-M groups 1 and 9 nucleic acids with:

(i) at least one forward flap primer comprising at least one of the following sequences:

5’-AATAAATCATAAGCGGATCGCCCGGAATG-3’ (SEQ ID NO: 21), and

5’-AATAAATCATAATCGGGTCGCCGGGAATG-3’ (SEQ ID NO: 25), and

(ii) at least one reverse flap primer comprising at least one of the following sequences:

5’-AATAAATCATAAACGAAACGTTCCGTCTCGAC-3’ (SEQ ID NO:20), and

5’-AATAAATCATGCGATGAGACGTTTCGTCTGGA-3’ (SEQ ID NO:24), to produce a reaction mixture;

(b) incubating the reaction mixture of step (a) under conditions sufficient to amplify the CTX-M groups 1 and 9 nucleic acids, thereby generating amplified CTX-M groups 1 and 9 nucleic acids; and

(c) detecting the amplified CTX-M groups 1 and 9 nucleic acids.

28. The method of claim 27, wherein the step of detecting the amplified CTX-M groups 1 and 9 nucleic acids comprises using fluorescence-generating probes comprising at least one of the following sequences:

R_a-G*TCGGCTCGGTACGG-R_b (SEQ ID NO:22), and

R_a-G*TAGGTTCAGTGCGATCC-R_b (SEQ ID NO:26),

wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, Ra is independently selected from (M)a-Fl and (M)a-Q, Rb is independently selected from (M)a-Fl and (M)a-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of Ra and Rb allows quenching of fluorescence when the probe is unhybridized.

29. The method of claim 27, further comprising the step of amplifying a control nucleic acid having a sequence of SEQ ID NO: 47.

30. The method of claim 29, wherein the step of amplifying a control nucleic acid comprises using a fluorescence-generating probe having substantial identity to SEQ ID NO: 50, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, Ra is independently selected from (M)a-Fl and (M)a-Q, Rb is independently selected from (M)a-Fl and (M)a-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of Ra and Rb allows quenching of fluorescence when the probe is unhybridized.

31. The method of claim 29, wherein the step of amplifying a control nucleic acid comprises using one or more primers having substantial identity to a sequence that is SEQ ID NO: 48 or 49.

32. A kit for detecting a CTX-M nucleic acid in a sample, comprising one or more flap primers having a sequence with substantial identity to a sequence selected from SEQ ID NO: 2, 3, 6, 7, 8, 13, 14, 16, 17, 20, 21, 24, 25, 30, 31, 33, and 34.

33. The kit of claim 32, further comprising one or more fluorescence-generating probes having a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 4, 9, 10, 11, 18, 22, 26, 27, 28, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

34. The kit of claim 32, further comprising one or more primers or probes for amplifying a control nucleic acid having sequences with substantial identity to a sequence that is SEQ ID NO: 48, 49, or 50, or combinations thereof.

35. A kit for detecting a CTX-M nucleic acid in a sample, comprising one or more fluorescence-generating probes having a sequence with substantial identity to a sequence that is selected from SEQ ID NO: 4, 9, 10, 11, 18, 22, 26, 27, 28, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46, wherein G* is guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one, R_a is independently selected from $(M)_a$-Fl and $(M)_a$-Q, R_b is independently selected from $(M)_a$-Fl and $(M)_a$-Q, M is a minor groove binder, a is 0 or 1, Fl is a fluorophore with emission wavelength between about 400 and 900 nm, and Q is a non-fluorescent quencher, wherein substitution of R_a and R_b allows quenching of fluorescence when the probe is unhybridized.

36. The kit of claim 35, further comprising one or more flap primers having a sequence with substantial identity to a sequence selected from SEQ ID NO: 2, 3, 6, 7, 8, 13, 14, 16, 17, 20, 21, 24, 25, 30, 31, 33, and 34.

37. The kit of claim 35, further comprising one or more primers or probes for amplifying a control nucleic acid having sequences with substantial identity to a sequence that is SEQ ID NO: 48, 49, or 50, or combinations thereof.

Figure 1

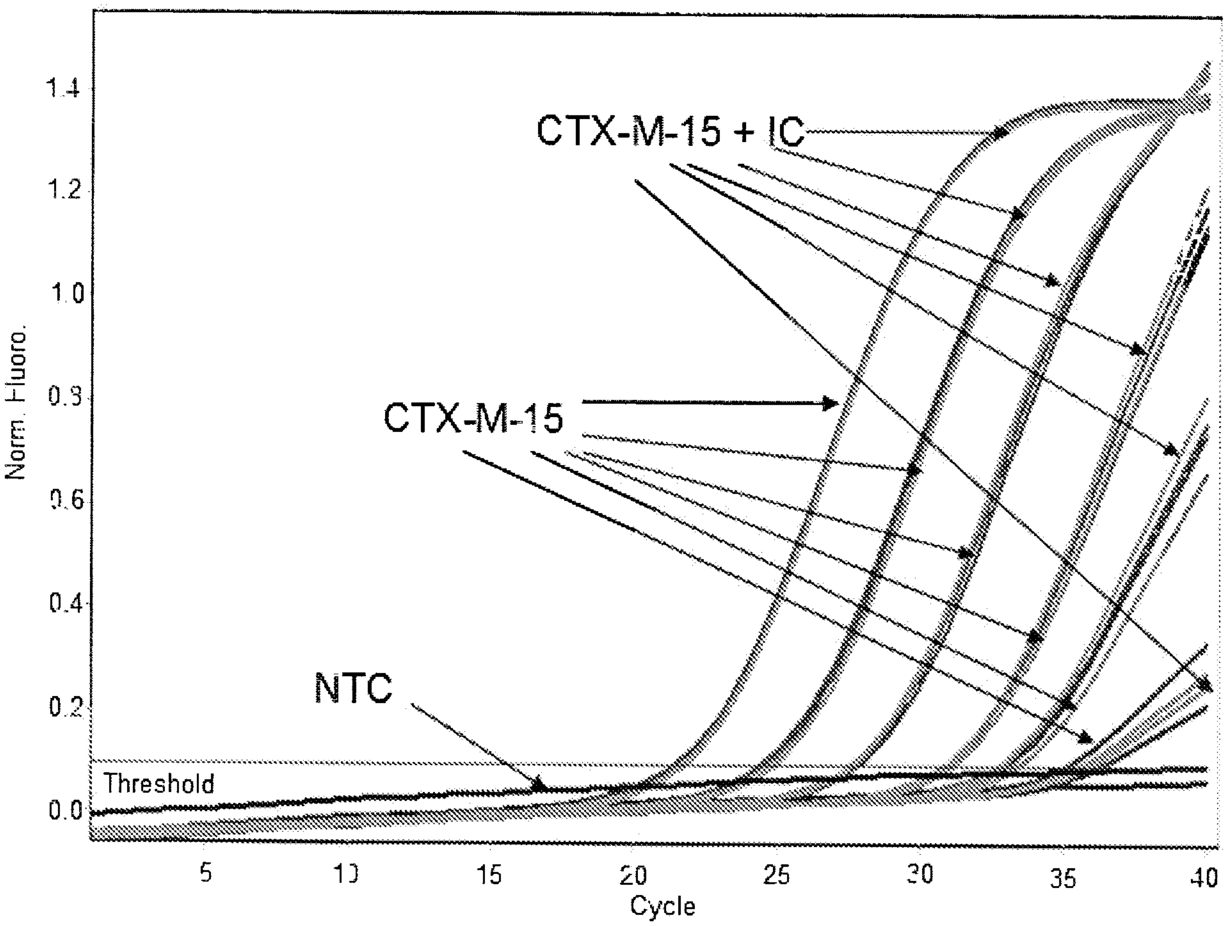

CTX-M-15 + IC
CTX-M-15
NTC
Threshold
Norm. Fluoro.
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
25
30
35
40
Cycle

Figure 2

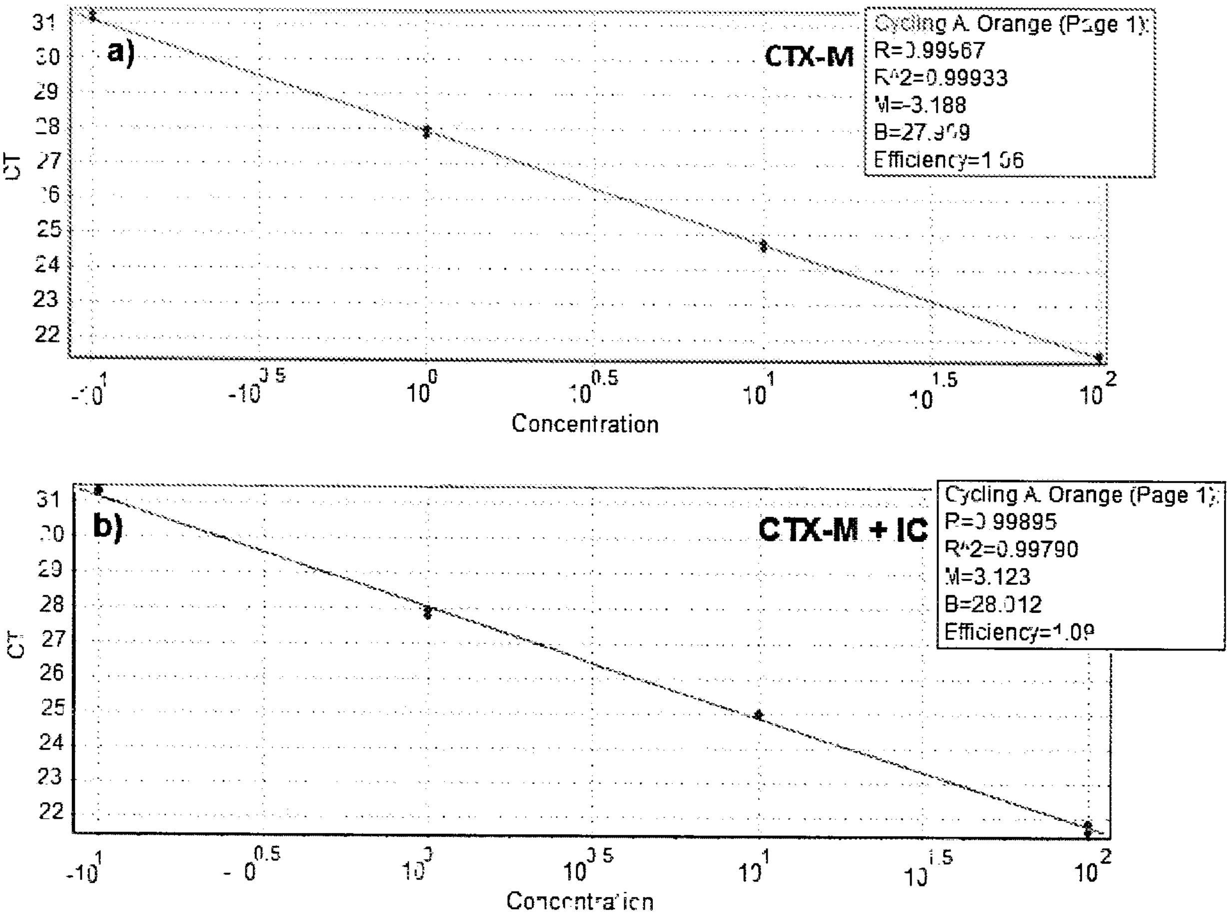
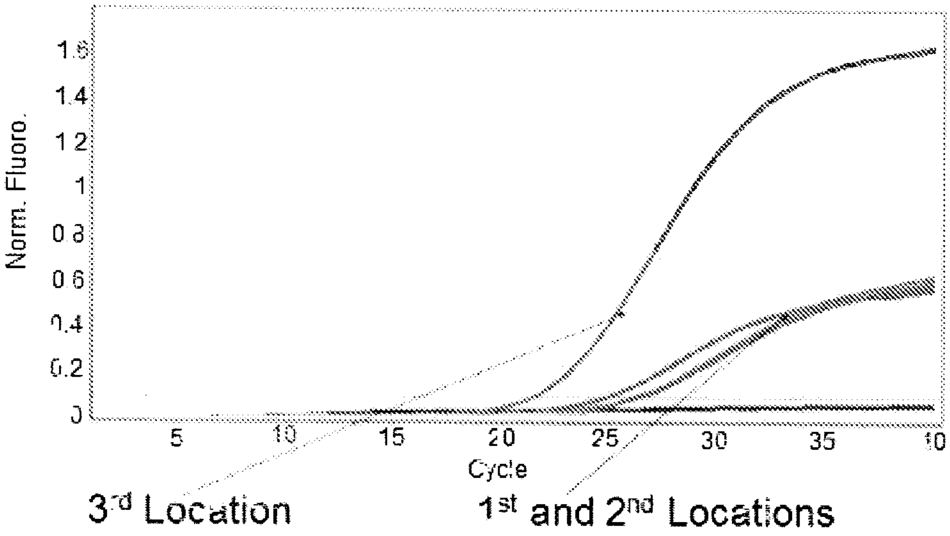

a)
CTX-M
Cycling A. Orange (Page 1):
R=0.99967
R^2=0.99933
M=-3.188
B=27.909
Efficiency=1.06
CT
31
30
29
28
27
26
25
24
23
22
-10^1
-10^0.5
10^0
10^0.5
10^1
10^1.5
10^2
Concentration
b)
CTX-M + IC
Cycling A. Orange (Page 1):
R=0.99895
R^2=0.99790
M=3.123
B=28.012
Efficiency=1.09
CT
31
30
29
28
27
26
25
24
23
22
-10^1
-10^0.5
10^0
10^0.5
10^1
10^1.5
10^2
Concentration

Figure 3


Norm. Fluoro.
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
35
40
Cycle
3rd Location
1st and 2nd Locations

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/063614

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, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/138402 A1 (CHECK POINTS HOLDING B V [NL]; VOS PIETER [NL]) 10 November 2011 (2011-11-10)	1,2,5-9, 11,12, 16,17, 19, 22-24, 26,32
Y	page 3, last paragraph; table 3; page 15, lines 4-14; examples; claims	3,4,10, 13-15, 18,20, 21,25, 33-37

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☒ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents :

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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
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"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 24 February 2016	Date of mailing of the international search report 14/03/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Hennard, Christophe

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/063614

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Form PCT/ISA/210 (continuation of second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YINGFANG WANG ET AL: "Transposition of ISEcp1 modulates blaCTX-M-55-mediated Shigella flexneri resistance to cefalothin", INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS, vol. 42, no. 6, 1 December 2013 (2013-12-01), pages 507-512, XP055252775, AMSTERDAM, NL ISSN: 0924-8579, DOI: 10.1016/j.ijantimicag.2013.08.009 table 2; item 2.3 -----	1,2,5-9, 11,12, 16,17, 19, 22-24, 26,32
Y	IRINA A. AFONINA ET AL: "<title>Hybridization-triggered fluorescence detection of DNA with minor groove binder-conjugated probes</title>", MEDICAL IMAGING 2002: PACS AND INTEGRATED MEDICAL INFORMATION SYSTEMS: DESIGN AND EVALUATION, vol. 4626, 19 June 2002 (2002-06-19), pages 322-331, XP055252776, 1000 20th St. Bellingham WA 98225-6705 USA ISSN: 0277-786X, DOI: 10.1117/12.472099 ISBN: 978-1-5106-0167-3 pages 326-329 -----	3,4,10, 13-15, 18,20, 25,33-37
Y	IRINA AFONINA ET AL: "Primers with 5' flaps improve real-time PCR", BIOTECHNIQUES, vol. 43, no. 6, 1 December 2007 (2007-12-01), pages 770-774, XP055054003, ISSN: 0736-6205, DOI: 10.2144/000112631 table 1; figure 2 -----	21

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2015/063614

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