Development of nineteen Taqman real-time PCR assays for screening and detection of highly pathogenic bacteria

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ABSTRACT

Background: Here, we describe a set of six Taqman real-time PCR assays for screening of highly pathogenic bacteria, i.e. *Bacillus*, *Brucella*, *Burkholderia*, *Coxiella*, *Francisella*, and *Yersinia* species. Twelve specific assays are subsequently performed to identify the species that are classified as highly pathogenic and a general 16S Taqman real-time PCR assay is included to see if the sample contains bacteria.

Methods: These assays were designed using all available genomes in the public database of bioterror agents. They were validated with a collection of reference strains, clinical isolates and one environmental sample.

Results: These assays were tested against all the ring trials we participate among them the ones which were coordinated by Robert Koch Institute from a repository built up in the framework of the EU funded project ‘Efficient response to highly dangerous and emerging pathogens’ (EMERGE). All bacteria were accurately identified in food, clinical and environmental matrices.

Conclusions: These assays are used routinely in our diagnostic laboratory to rapidly screen for and specifically detect select highly pathogenic bacteria of potential bioterrorism use. The platform can be used as an open array format in 96-well plates to screen for a single species or up to 6 agents in one run.

Abbreviations: ATCC; American Type Culture Collection, B; Brucella; BLAST; Basic local alignment search tool; BSL; Biosafety level; Cq; Quantification cycle; DNA; Deoxyribonucleic acid, FAM; 6-carboxyfluorescein, FOHM; the Public Health Agency of Sweden, IAC; internal amplification control, LOD; Limit of detection, MGB; Minor groove binder, NCB; National Center for Biotechnology Information, NFA; National Food Agency, PCR; polymerase chain reaction; PhHV-1; Phocine Herpesvirus 1, SVA; National Veterinary Institute, Tm; Melting Temperature.

Introduction

The U.S. Department of Health and Human Services has classified the highly pathogenic bacteria *Bacillus anthracis* (*B. anthracis*), *Francisella tularensis* (*F. tularensis*), *Yersinia pestis* (*Y. pestis*), *Brucella* species, *Burkholderia mallei* (*B. mallei*), *Burkholderia pseudomallei* (*B. pseudomallei*) and *Coxiella burnetii* (*C. burnetii*) as possible severe threats to public health [1]. These pathogens cause serious diseases such as anthrax, tularemia, plague, brucellosis, glanders, melioidosis, and Q-fever. The natural prevalence of these agents is low in most parts of the world, even though some of these agents may cause smaller outbreaks in human and animal populations, e. g. *Y. pestis* in Madagascar and *F. tularensis* in Sweden [2–5]. Some of these bacteria have the potential to be used in bioterrorist attack [6,7]. The intentional release of these agents can result in severe public health consequences as was shown 2001 in the Unites States. Letters containing anthrax spores caused illness in 22 persons, leading to 5 deaths. Contact to aerosolized *B. anthracis* spores and *F. tularensis* can lead to inhalational anthrax and tularemia, respectively. Pneumonic plague caused by *Y. pestis* may also spread from person to person. Rapid and accurate assays for microbial identification are needed to ensure proper medical intervention, prevent further dissemination of these agents and decrease anxiety among the public. In the case of assumed intentional release of such agents, the assay must be able to classify a broad panel of potential threat microorganisms in different background matrices, which may or may be not contaminated with non-pathogenic bacteria [8]. In the last decades some multiplex assays have been designed to shorten the period of identification of microorganisms responsible for these infectious diseases [9–11]. The commercially available Ibis PLEX-ID technology offered by Abbot can screen a sample for...
highly pathogenic bacteria in one step [12]. This method is a combination of PCR and electrospray ionization-mass spectrometry (PCR/ESI-MS), but requires expensive equipment and specialized laboratories [13]. In this study we describe a set of nineteen Taqman real-time PCR assays for the screening and subsequently specific identification of above mentioned bioterror agents to be used in the standard microbiology laboratories on the clinical and environmental samples. First to be run is six Taqman real-time PCR assays for the screening of them on the species level. In addition, we developed twelve Taqman real-time PCR assays for the specific identification on the species level. Furthermore, we developed one Taqman real-time PCR assay targeting the 16S RNA gene for identification of all other non-bioterror agents.

Material and methods

Bacterial strains and growth condition

The bacterial strains (see supplementary data) used in this study were grown on horse blood or chocolate agar, tularemia agar plates (Bacto™ Tryptose Phosphate broth 20 g NaCl 10 g Glucose 11 g Thiamine 5 mg of L-cysteine HCl 5 g of Na Thioglycolate 2 g Agar 10 g of defibrinated rabbit blood 50 mL, pH 7.2 for 1 L) or selective tularemia agar plates with antibiotics (Bacto™ Tryptose Phosphate broth 20 g NaCl 10 g Glucose 11 g Thiamine HCl 5 mg of L-cysteine HCl 5 g of Na thioglycolate 2 g Agar 10 g of defibrinated rabbit blood 50 mL, penicillin 600 000. Polymyxin B IU 1,000,000 IU Cycloheximide 100 mg, pH 7.2 for 1 L) or Ashdown agar in a humidified atmosphere. All other bacteria were incubated at 37°C for 48 hours, F. tularensis plates in a humidified atmosphere and Brucella in a CO2 atmosphere. All other bacteria were incubated at 37°C for 24 hours.

Internal amplification control (IAC)

As internal amplification control (IAC), Phocine Herpesvirus 1 (PhHV-1) aliquots with a known DNA concentration and a target quantification cycle (Cq) of 32 was used in all samples [16].

Clinical and environmental specimens

These Taqman real-time PCR assays runs on all clinical samples that come into our laboratories for the diagnosing of anthrax, tularemia, plague, brucellosis, glanders, melioidosis, and Q fever directly on the samples. The clinical samples could be biopsies, wound sores, blood culture bottles, glands, urine and sputum. All our environmental samples such as powder samples with anthrax issues are diagnosed with the Taqman real-time PCRs described here.

External quality assurance exercises

The assays were tested on all ring trials, external quality assurance exercises (EQAE) our laboratory participated during the last decade. The EQAE samples could be from clinical, food and environmental matrices with both viable and inactivated bacteria. Our laboratory received these matrices from the EU funded project ‘Quality Assurance Exercises and Networking on the Detection of Highly Infectious Pathogens’ (QUANDHIP, CHAFEA agreement no. 2010 21 02) and from Efficient response to highly dangerous and emerging pathogens at EU level (EMERGE, CHAFEA n 677 066). The aim of this Joint Action (JA) is to build up a stabilized consortium that links up 37 highly specialized advanced laboratories from 25 European countries, to guarantee universal exchanges of best diagnostic strategies to support a joint European response to outbreaks of highly pathogenic infectious agents. The JA will provide a supportive European infrastructure and strategy for external quality assurance exercises (EQAE), training and biosafety/biosecurity quality management.

DNA extraction

Clinical samples DNA and bacterial DNA from reference strains was extracted using the EZ1® DNA Tissue Kit from (Qiagen) according to the standard protocol from the manufacturer except that the Nucliens lysis buffer (BioMerieux) was used as inactivation step instead of the lysis buffer/proteinase K from the Qiagen kit. A volume of 195 µl of Nucliens lysis buffer with sample was used in each extraction of the total volume of 200 µl. A volume of 5 µl of seal herpes virus cell culture was used in each sample of the total volume of 200 µl in the extraction step as a process control. Each extraction was eluted in 50 µl. The DNA concentration was measured with a NanoDrop® (Thermo Scientific, Wilmington, USA) spectrophotometer and stored at 4°C until further use.

Real-time PCR

All primers and probes were designed using the AlleleID™ software (Table 1). Primers and probes were ordered from (Biomers, Germany) and MGB probes were ordered from (Applied Biosystems®). All the bacteria probes were labelled with a FAM fluorophore as reporter dye at 5’ end and BHQ-1 as quencher dye at the 3’ end, and PhHV-1 probe were labelled with VIC at 5’ end and MGB as quencher dye at the 3’ end. The Taqman real-time PCR assays were carried out in 25 µl reaction mixtures containing 5 µl template DNA, in PerfeCTa® qPCR ToughMix® ROX,
diluted in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™), 0.9 μM of each primers and 0.2 μM probes. In each well we had primers and probe for both the sample and the seal herpes. Amplification and detection were performed using PCR machine, ABI-7500/96-well plate real-time FAST PCR platform (Applied Biosystems®) or StepOne Plus real-time PCR system (Applied Biosystems®). Thermocycling parameters were as follows: inactivation 95°C for 3 min, followed by 45 cycles 95°C for 3 s, and annealing at 60°C for 30 s. The DNA was considered positive when the target amplification was detected within 40 cycles. Eluted DNA from National Counterterrorism Center (NCTC) strains was used as positive control and seal herpes virus as internal amplification control.

Table 1. Taqman real-time PCR primer and probe sequences used in the study for the detection of highly pathogenic bacteria. The first set of assays to identify to the genus level is marked in bold. The remaining assays are used to identify the subspecies. The seal herpes PCR is used as an internal control in all assays.

| Agents           | Target          | Oligonucleotide sequence                                      |
|------------------|-----------------|--------------------------------------------------------------|
| Bacillus genera  | rpoB-F          | ACCTCTTTCTTACAGTGAGG                                       |
|                  | rpoB-R          | CCGCTAAGAGTCTGCATACG                                       |
|                  | rpoB-P          | FAM-CATTTTCTGCCAANACTCATAAG                                  |
| Francisella genera | ISFtu2-F       | CCGTATTACAAGAAAGATC                                         |
|                  | ISFtu2-R        | CTTGTTATCATCTTTATCATC                                       |
|                  | ISFtu2-P        | FAM-TGATCAAATGACGAGCAGCAT                                    |
| Yersinia genera  | Inv-F           | TTAGACACAATGGTACAGGAT                                        |
|                  | Inv-R           | ACTGGTACGATGTTCCGCTAAAA                                      |
|                  | Inv-P           | FAM-CGTTACGAGATGCGAACAGAC                                    |
| All bacteria     | 16S-F           | CGGGAAGAAACCTCAAC                                            |
|                  | 16S-R           | CGTGAACGCGATGAGT                                             |
|                  | 16S-P           | FAM-CGCGATGCGATGACGGG                                        |
| Burkholderia genera | Flc-F         | GCAGACTCTCGGAAAGTG                                           |
|                  | Flc-R           | CTGGAGGATGATGCGAAC                                           |
|                  | Flc-P           | FAM-CGGTACAGATGCGAACG                                        |
| Brucella genera  | IS1117-F        | TGGCTCTATGTAATGTTAATGG                                       |
|                  | IS1117-R        | CGTGGTTTTCCTCCATAC                                           |
|                  | IS1117-P        | FAM-TGCCTCATGAGATGAG                                         |
| Coxiella genera  | IS1111a-F       | CACGACGCGGACTGCC                                             |
|                  | IS1111a-R       | CGGACGCGGACTGCC                                              |
|                  | IS1111a-P       | FAM-CGCGATGCGATGCGG                                          |
| B. mallei        | Flp-F           | GACTTCTGACTAGGACCCG                                          |
|                  | Flp-R           | AGCGACGCGGACTGCC                                             |
|                  | Flp-P           | FAM-CGCGATGCGATGCGG                                          |
| B. pseudomallei  | MrpA-F          | ACATCGAACATCGGCTGCTT                                          |
|                  | MrpA-R          | FAM-TGATCAAATGACGAGCAG                                       |
|                  | MrpA-P          | FAM-TGATCAAATGACGAGCAG                                       |
| F. tularensis spp| Tu4-F           | CTGGCCGCACTGGAAACG                                           |
|                  | Tu4-R           | CGTGGCTCTCTGGATGCAAN                                        |
|                  | Tu4-P           | FAM-TGATCAAATGACGAGCAG                                       |
| F. tul Type A & B| FTT_0524-F      | AACCAGTAACTAAAGGATG                                         |
|                  | FTT_0524-R      | CGTGGCTCTCTGGATGCAAN                                        |
|                  | FTT_0524-P      | FAM-TGATCAAATGACGAGCAG                                       |
| F. tul Type B    | junction ISFtu2-3’-region hol-F | ACTGGAAATAAAATGCAATG                                       |
|                  | junction ISFtu2-3’-region hol-R | ACTGGAAATAAAATGCAATG                                       |
|                  | junction ISFtu2-3’-region hol-P | ACTGGAAATAAAATGCAATG                                       |
| B. anthracis     | BA-5345-F       | TTCCCAATAAATGTCTGTATC                                        |
|                  | BA-5345-R       | CATGGTACTACTACAAACAAG                                       |
|                  | BA-5345-P       | FAM-TCCCTCTATGTGACGAGCATG                                    |
| pX01-pagA-F      | GAGCTGTTATACATACGGT                                      |
| pX01-pagA-R      | GAACTTATATATACATATCGT                                     |
| pX02-capD-F      | FAM-TCCCTCTATGTGACGAGCATG                                  |
| pX2-capD-R       | CTGGTTGCTAATGTTTC                                         |
| pX2-capD-P       | AAGCGACGAGATGAGT                                          |
| Y. pestis        | YPO1091-F       | CTACGCGATGCAGGAAATG                                          |
|                  | YPO1091-R       | FAM-TTGGGATGGGTC                                            |
|                  | YPO1091-P       | FAM-TTGGGATGGGTC                                            |
| pPCP1-Pla gene-F | TCTGGTACACAAATGTTAC                                      |
| pPCP1-Pla gene-R | ACCTGTCCTATATCTGAT                                          |
| pPCP1-Pla gene-P | FAM-TGCCGTATATACATCTGATG                                    |
| pMT1-caf1 gene-F | AGTGGTACGACATGTT                                              |
| pMT1-caf1 gene-R | GCTACCATATACATGTTG                                         |
| pMT1-caf1 gene-P | FAM-CGCGATGCGATGCGG                                         |
| B. abortus       | B. abortus(BruAb2-0168)-F     | GGTTGTA TATCAGACCAGC                                         |
|                  | B. abortus(BruAb2-0168)-R    | CGGCTTCCAAATGGAAGGAG                                       |
|                  | B. abortus(BruAb2-0168)-P     | FAM- ATCTATGGTGACGAGCATTACGAGCGL                              |
| Seal herpes      | gb gene-F       | GGGCGAATCAGGAGATG                                           |
|                  | gb gene-R       | CGGCGTTCAGACATGAGGAG                                      |
|                  | gb gene-P       | VIC-TTATATGCTGCGGCCAACGACATGTCGACG                          |
Determination of the real-time PCR limit of detection

The limit of detection (LOD) was defined by using the B. anthracis (NCTC 10340), B. abortus (NCTC 624) B. melitensis (NCTC 10094), B. mallei (NCTC 120), B. pseudomallei (NCTC 8708), C. burnetii Nine Mile [17], F. tularensis type A (FSC 041), F. tularensis type B (LVS) and Y. pestis (NCTC 2028) strains with 10^6, 10^5, 10^4, 10^3, 10^2, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 genome equivalents per reaction. LOD samples were analysed in six replicates for each concentration and with five runs on five days.

Results

Taqman Real-time PCR is a rapid and reliable method for the analysis of different microorganisms in clinical and environmental samples. In this study we describe a set of nineteen Taqman real-time PCR assays for the screening and subsequently specific identification of highly pathogenic bioterror agents to be used in the microbiology laboratories. The first six Taqman real-time PCR assays identify bacteria from the Bacillus, Brucella, Burkholderia, Coxiella, Francisella and Yersinia genera, respectively. In addition, a general 16S Taqman real-time PCR assay is included to see if the sample contains bacteria or not. The second set of Taqman real-time PCR assays, designed to identify the species, targeting one or more chromosomal and plasmid targets (Table 1). In case there is a positive identification of Bacillus and Yersinia genus, we run three specific assays for identification of each B. anthracis and Y. pestis respectively, which targeting both the chromosomal sequence and plasmids sequences of these two bacteria. But in case there is a positive identification of Burkholderia and Coxiella on genus level we run one specific assay for identification of B. mallei, B. pseudomallei, and C. burnetii (Table 1).

We have one specific Taqman real-time assay targeting B. abortus. But in case there is a positive identification of F. tularensis on genus level we run three other assays, one targeting F. tularensis type A, type B and F. novicida, and one targeting both F. tularensis type A and type B together and the last one is specific assay for just F. tularensis type B (Table 1). We detected F. tularensis type B from one environmental water sample with the primers and probe targeting F. tularensis type B hereafter we sequenced the isolate [18,19].

Specificity was evaluated in two steps; inclusivity to testing for false negativity using repository material, and exclusivity to test for false positivity against a panel of common clinical bacterial isolates (Table 2). The inclusivity and exclusivity of these assays was tested using a series of reference strains (Table 2). All the inclusivity runs was 100 % except for the Bacillus spp, Francisella spp and Yersinia spp assays which had 93, 94 and 83 % respectively (Table 2). B. licheniformis, F. noatunensis and one strain of Y. enterocolitica was not detected in these assays.

The assays were tested on clinical, food and environmental matrices with both viable and inactivated bacteria from QUANDHIP and EMERGE network. Our results were consistent with the results from both networking.

Applicability

No false positive or false negative result is acceptable in clinical BSL3-pathogen diagnostics. To guarantee a correct identification of BSL3-pathogens, we strongly recommend isolation of the bacteria on selective agar plates proper for the specific agent. This enables evaluation of the phenotypic properties of the strains, such as colony morphology, and

Table 2. Taqman Real-time PCR results from the validation assays. The numbers in the inclusivity and exclusivity columns are in percentages. Last column shows the result from the limit of detection experiments in genomic equivalent per reaction.

| Agents          | Target      | Strains   | Inclusivity | Exclusivity | LOD   |
|-----------------|-------------|-----------|-------------|-------------|-------|
| Bacillus spp    | rpaB        | NTCC 10340| 93          | 86          | 1.56  |
| B. anthracis    | pXO1-pagA   | NTCC 10340| 100         | 96          | 1.56  |
| B. anthracis    | pXO2-capD   | NTCC 10340| 100         | 99          | 1.56  |
| B. anthracis    | BA-5345     | NTCC 10340| 100         | 99          | 6.25  |
| Brucella spp    | IS/711      | NCTC 10094| 100         | 100         | 0.78  |
| Burkholderia spp| FIC         | NCTC 8708 | 100         | 100         | 3.12  |
| B. mallei       | FlP         | NCTC 120  | 100         | 100         | 3.12  |
| B. pseudomallei | mrpA        | NCTC 8708 | 100         | 99          | 3.12  |
| Coxiella burnetii| IS1111a    | Nine Mile | 100         | 100         | 0.04  |
| Francisella spp | ISFlu2      | LVS       | 94          | 90          | 0.78  |
| F. tularensis sp| Ftu4        | LVS       | 100         | 87          | 6.25  |
| F. tularensis type A | pdpD   | FSC 041   | 100         | 88          | 0.78  |
| F. tularensis type B | JunC3/ISFlu2 | LVS     | 100         | 88          | 0.78  |
| Yersinia spp    | inv         | NCTC 2028 | 83          | 91          | 6.25  |
| Y. pestis       | YOP1091     | NCTC 2028 | 100         | 95          | 0.78  |
| Y. pestis       | pPCP1-pla   | NCTC 2028 | 100         | 96          | 6.25  |
| Y. pestis       | pMT1-cad1   | NCTC 2028 | 100         | 96          | 3.12  |

Sterile water as negative control. All samples were analyzed in duplicate.
enriches the molecular target of the PCR, as well as reduces the concentration of potential PCR inhibitors. Even after the enrichment an internal PCR process control is recommended as described above. All strains in our assays were cultured at 37°C as bacterial strains were isolated in the clinical diagnostics workflow. The real-time PCR assays were therefore recognized as applicable in combination with the isolation of bacteria from clinical specimens.

**Specificity**

The *Brucella* assay was tested with 120 human clinical *B. melitensis* isolates. The *F. tularensis* type B assay was tested with 150 human clinical *F. tularensis* isolates. The *Burkholderia* assay was tested with 10 human clinical isolates and *C. burnetii* assay with one human clinical isolate. We tested the *B. anthracis* assay with one animal sample from a horse and with reference strains. The *Y. pestis* assays was tested with reference samples and not with clinical samples because we do not have any positive clinical samples for this agent. There was no amplification from any other bacterial species. The specificity of all assays was 100% because all agent specific samples were tested positive while all non-agent specific samples gave no amplification in the specific real-time PCR.

**Practicability**

The assays are used routinely in our diagnostic laboratory since 2010, as well as in all ring trial/EQAE we have been participated both nationally and internationally in the last ten years period. The results obtained with these assays conformed to the results of other laboratories.

**Discussion**

All these assays were validated according to the validation standards of Forum for Biopreparedness Diagnostic (FBD), involving four governmental institutes: the National Veterinary Institute (SVA), the Public Health Agency of Sweden (FOHM), the National Food Agency (NFA), and the Swedish Defence Research Agency (FOI) and according to the Broeders *et al.* 2014 [20]. The validation comprised applicability, practicability, specificity and sensitivity. A number of environmental samples, as well as the closely related species of each genus were included in the exclusivity runs in this study (see supplementary data). No amplification could be detected in testing different concentrations of purified DNA from the closely related microorganisms in all assays. Unfortunately we did not have enough DNA for all the BSL-2 microorganisms which were included in this study that’s why the majority of the exclusivity assays are not 100%. *B. licheniformis, F. noatunensis* and one strain of *Y. enterolitica* was not detected in these assays. This was accepted because these strains were not classified as BSL-3 agents.

The sensitivity of the assays was tested by serially diluting one reference strains DNA template (Table 2) from the concentration $10^6$ to 0.39 genome equivalents per reaction for all the nineteen Taqman real-time PCRs assays. Six replicates for each concentration was run with five runs on five days. When all the six replicates was amplified all the five days the concentration was counted as the limit of detection of the specific target, results are shown in the last column in (Table 2) in genome equivalents per reaction.

**Conclusion**

In summary, the study describes nineteen Taqman real-time PCR assays to rapidly screen for and specifically detect select highly pathogenic bacteria of potential bioterrorism use. The platform can be used as an open array format in 96-well plates to screen for a single species or up to 6 agents in one run. Evaluation of assays showed that the platform can be used to analyze a broad spectrum of sample materials; human, animal, food and environmental matrixes.

**Authors’ contributions**

BE carried out the sequence analysis and participated in the design of the study. TB performed the laboratory work and participated in its design. TW carried out some of the laboratory work, conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

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**Ethics and consent to participate**

Ethical Review of Research Involving Humans (http://www.epn.se/media/1205/the_ethical_review_act.pdf) is not applicable for diagnostic development and quality assessment. In this study we used our highly pathogenic bacteria historical collection from the Public Health Agency of Sweden. No human patients data were used therefore informed consent was not required.

**Availability of data and materials**

The data are accessible by the request to the corresponding author.
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Disclosure statement

No potential conflict of interest was reported by the authors.

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Dr. Tara Wahab is a microbiologist at the public health agency of Sweden. She works mainly with method development for identifying highly pathogenic bacteria.

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