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Development of a Multiplex Real-Time PCR for the Rapid Detection of the Predominant Beta-Lactamase Genes CTX-M, SHV, TEM and CIT-Type AmpCs in Enterobacteriaceae

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Abstract

Beta-lactamase resistant bacteria and especially ESBL producing Enterobacteriaceae are an increasing problem worldwide. For this reason a major interest in efficient and reliable methods for rapid screening of high sample numbers is recognizable. Therefore, a multiplex real-time PCR was developed to detect the predominant class A beta-lactamase genes bla_{CTX-M}, bla_{SHV}, bla_{TEM} and CIT-type AmpCs in a one-step reaction. A set of 114 Enterobacteriaceae containing previously identified resistance gene subtypes and in addition 20 undefined animal and environmental isolates were used for the validation of this assay. To confirm the accessibility in variable settings, the real-time runs were performed analogous in two different laboratories using different real-time cyclers. The obtained results showed complete accordance between the real-time data and the predetermined genotypes. Even if sequence analyses are further necessary for a comprehensive characterization, this method was proofed to be reliable for rapid screening of high sample numbers and therefore could be an important tool for e. g. epidemiological purposes or support infection control measures.

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Introduction

In Gram-negative bacteria, the production of beta-lactamases represents the most important contributing factor to resistance against beta-lactam antibiotics. During the last couple of years increasing numbers of antibiotic-resistant bacteria have become a problem in the field of infection control. Particularly in Enterobacteriaceae, extended-spectrum- and AmpC-type beta-lactamases play an important role [1–3]. Extended-spectrum beta-lactamases (ESBLs) have the ability to hydrolyze various types of the newer beta-lactam antibiotics, including extended-spectrum cephalosporins of the 3rd and 4th generation (e.g. cefotaxime, ceftriaxone, ceftazidime) and monobactams (e.g. aztreonam), which were assessed as "critically important antimicrobials" by the WHO [4]. Nowadays the predominant ESBL-gene families encountered are bla_{CTX-M}, bla_{TEM} and bla_{SHV} [5]. Until now 219 different TEM- and 186 SHV- sequences have been published within the Lahey database (http://www.lahey.org/Studies/; June 20th, 2014). While the DNA sequences of bla_{TEM} and bla_{SHV} are very homologues and just a few point mutations at selected loci within the gene give rise to the ESBL-phenotype [6], the bla_{CTX-M} genes show a bigger range of variability. So far 157 CTX-M variants have been described (http://www.lahey.org/Studies/; June 20th, 2014) which can be divided into five different groups, based on their amino acid sequence identities. These subgroups are specified as CTX-M-1/group; CTX-M-2/group; CTX-M-8/group; CTX-M-9/group and CTX-M-25/group among which the groups CTX-M-1, -M-2 and –M-9 are the predominant ones [7].

Also, the plasmid-encoded AmpC cephalosporinases can be arranged into four general categories among which the CIT-type AmpC beta-lactamases (e.g. CMY-2,-3,-4, LAT-1, LAT-2, BIL-1) are commonly detected [8–10]. Especially the CMY-2 type is the most frequently recovered AmpC beta-lactamase from patients in hospitals and in the community, as well as from livestock and ground meat [11] and therefore it is described as the most common plasmid-mediated AmpC beta-lactamase worldwide [12].

As the horizontal plasmid transfer is an important factor for the epidemiology of the resistance genes within the bacterial ecosystem, rapid and cost-effective methods for identification of these genes are necessary. Particularly in the field of food and livestock associated bacteria (zoonotic as well as commensal ones) the monitoring of antimicrobial resistance (ESBL, AmpC and Carbapenemases) becomes more and more important and quite recently the European (EU) legislation has been revised and specific monitoring programs are obliged for all EU member states [13].

Until now, a common method for the phenotypical confirmation of ESBL-/AmpC producing bacteria is the use of susceptibility testing and the following interpretation of the obtained results by harmonized criteria e.g. the CLSI- [14] and EUCAST guidelines [15]. But it has to be considered that the development of resistance is dependent on the mode and level of expression and furthermore it cannot be excluded that resistance genes which are not expressed *in-vitro* may show an expression in *in-vivo* surroundings [16]. Moreover, resistant strains often contain more than one ESBL and/or AmpC gene belonging to different resistance gene families. This can lead to an interference with the result of the susceptibility testing. Therefore, a reliable identification and characterization of the beta-lactamase producers often requires genotypic verification [1]. For this reason a multiplex real-time PCR was developed to detect the predominant class A beta-lactamase genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and CIT-type AmpCs in a one-step reaction. To validate the assay and make it widely accessible to diagnostic laboratories a set of 114 bacterial isolates containing previously identified resistance gene subtypes were tested. To survey the reproducibility of the obtained results, all of the real-time runs were performed analogous in two different laboratories using either the Roche Lightcycler 480II or the BioRad CFX96.

Materials and Methods

1. Bacterial strains

Bacterial strains used in this study originated from different sources (livestock and companion animals, animal housings and environment as well as a few isolates from food and humans or reference strains).

A set of 114 control isolates derived either from the strain collection of the National Reference Laboratory for Antimicrobial Resistance (Federal Institute for Risk Assessment, Berlin, Germany) or were taken from the strain collection of the Institute of Animal Hygiene and Environmental Health (Free University (FU-) Berlin, Germany) which includes the majority of the livestock associated ESBL-isolates of the German research consortium RESET (www.reset-verbund.de). The CTX-M-26 positive isolate was kindly provided by the Institute of Microbiology and Epizootics (FU-Berlin, Germany) and six strains carrying different genes (bla_{TEM}, $bla_{\text{CTX-M}}$; see Table 1) were made available by the European Reference Laboratory for Antimicrobial Resistance (EURL-AR, Lyngby, Denmark). In addition two *Klebsiella pneumoniae* isolates containing the $bla_{\text{SHV-11}}$ were provided by the Robert-Koch-Institute (Wernigerorde, Germany).

All of the control isolates were previously characterized by their particular supplier laboratories (mentioned above) and the sequence subtype of each detected resistance gene was known. Ninety-one of these isolates were positive for at least one of the four resistance gene families tested in which all of the five CTX-M subgroups were covered. A total of 56 strains contained one of the four beta-lactamase genes, 32 isolates possessed two resistance genes in different combinations and the remaining three strains showed positive results for all of the tested class A beta-lactamase genes (bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$). Twenty-three strains showed none of the tested resistance genes in conventional PCR formats and therefore served as negative controls for the establishment of the real-time PCR assay. Detailed information about the different types of beta-lactamase resistance genes which were covered within this study is shown in Table 1.

Moreover 20 animal and environmental isolates were used as undefined ("blind") test strains for the final validation of the assay. At this juncture a set of different bacteria was used: seven *Klebsiella pneumoniae* isolates, eight *Escherichia coli* strains, three *Salmonella* spp. and one isolate of *Proteus mirabilis* and *Acinetobacter* spp. each.

2. DNA extraction

Bacterial isolates were grown in liquid medium overnight. 1 mL of each culture was taken and pelletized at 14,000 rpm for 3 min. The remaining cell pellet was resuspended in 300 µL sterile TE-buffer (10 mM Tris, 0,1 mM EDTA, pH 8) and heated to 98°C for 10 min. Afterwards the suspension was cooled down on ice and the cell debris was removed by a 2 min. centrifugation step (14.000 rpm). The remaining supernatant, containing chromosomal as well as plasmid DNA fractions, was directly used for PCR reactions. For further experiments the samples were stored at −20°C.

3. Primer and TaqMan Probes

Detailed information about the already characterized beta-lactamase genes were obtained from the Lahey database (http://www.lahey.org/Studies/). To ensure that the primer/probe combinations for each beta-lactamase gene family detect the corresponding gene subtypes, a set of up to 25 different sequences was applied to generate an alignment feasible as basis for the primer/probe design.

Sequence data of the genes were downloaded from the GenBank web site (http://www.ncbi.nlm.nih.gov/genbank/) and aligned using the Lasergene DNA software package 10 (DNAStar, Madison, USA).

One exception was represented by the diverse group of CTX-M genes. In this case it was not possible to cover all of the five subgroups using just one set of primers/probe. For this reason the five groups were combined to two major clusters containing either the groups CTX-M-1 and –M-9 (Cluster A) or the groups CTX-M-2, -M-8 and -M-25 (Cluster B). For each of the two clusters a separate set of primers and probes was developed. Never the less, both of the CTX-M probes were labeled with the same fluorophor (Yakima Yellow) to ensure the detection of all five CTX-M groups in one channel of the real-time cycler.

The used primers and probes were synthesized by biomers.net GmbH (Ulm, Germany). Sequences and references of the primer pairs are summarized in Table 2.

4. Multiplex real-time PCR

Real-time amplifications were performed in 25 µL reactions containing 12.5 µL ABsolute qPCR Mix (Thermo Scientific, St. Leon Roth, Germany), 1 µL of each forward and reverse primer (10 pmol), 0.1 µL TEM TaqMan probe (5 pmol), 0.2 µL of each of the other four TaqMan probes (10 pmol), 0.6 µL of sterile water and 1 µL of DNA-mixture. If the boiled cells were stored at −20°C for several months, it could have been necessary to extend the DNA amount up to 2 µL to generate improved real-time results.

To figure out the optimal real-time PCR conditions and to confirm the specificity of the assay, all of the five primer/probe pairs were investigated by using strains containing only one single resistance gene for the particular primer/probe combination. For this purpose a set of five positive control strains (P1–5) plus one negative control strain (N1), which was known to contain none of the four tested resistance genes, were chosen from the set of the 114 previously characterized control strains (Table 3). In addition the obligatory "no template control" (NTC) was part of every single real-time run. To proof the functionality of the assay in variable settings the real-time runs were performed analogous in two different laboratories using either the Lightcycler480II (Roche