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Initial establishment of a multiplex real-time PCR assay for simultaneous detection of common colistin and carbapenemase genes

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Abstract. Carbapenem and colistin are often used as last-resort treatment for Gram-negative multi-drug resistant (MDR) bacteria. Nevertheless, co-resistance of these drugs is threatening the global healthcare system. Rapid and accurate detection of carbapenem and colistin resistant bacteria is pivotal for adequate antibiotic therapy and infection control, especially in an outbreak setting. In this study, we aim to develop a multiplex real time-PCR assay for simultaneous detection of the most prevalent genes including *bla*NDM, *bla*KPC, *bla*IMP-1 and *bla*OXA-48 responsible for carbapenem resistance, and *mcr*-1 responsible for colistin resistance. The melting curve-based multiplex real time PCR assay was established with the dissociation temperature range extended from 76 °C to 87 °C. The whole process is completed within haft and hour, allowing rapid screening of the five genes in cultured bacteria samples with a limit of detection of 10 CFU/ml. The proposed multiplex real-time PCR assay is a robust, reliable and rapid method for the detection of bacterial strains carrying *bla*OXA-48, *bla*IMP, *bla*NDM, *bla*KPC and *mcr*-1 gene in individual or in cocktail of genes. This assay will be a valuable tool for surveillance and monitoring of MDR bacteria additionally resistant to either carbapenem or colistin or both drugs.

Keywords: Multi-drug resistance, carbapenem resistance, colistin resistance, *bla*OXA-48, *bla*KPC, *bla*NDM, *bla*IMP-1, *mcr*-1, multiplex Real-time PCR

Classification numbers: 3.6.2.

1. INTRODUCTION

Antibiotics have been used successfully to treat acute infections since their discovery in 1928. They have been especially useful in lowering the morbidity and death rate among

susceptible patients recuperating from surgery, chemotherapy, and transplant treatments. Nevertheless, the spread of many multidrug resistant bacteria around the world is caused by a lack of rigorous antibiotic management policies in medicine, veterinary, aquaculture and agriculture. In 2014, for the first time, the World Health Organization (WHO) issued a warning about the approaching catastrophe of antibiotic resistance [1]. Notably, the WHO released the list of antibiotic-resistant priority pathogens in 2017, which included a catalog of 12 bacterial families that are the biggest threats to human health (https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed).

The broad-spectrum antibiotic carbapenem is well regarded for its consistency in treating bacterial infections. It belongs to the class of beta-lactam antibiotic drugs that work by attaching to penicillin-binding proteins (PBPs), a crucial protein in the construction of the bacterial cell wall, to prevent the manufacture of the cell wall by the bacteria. Regretfully, a large number of carbapenemase genes were discovered on the plasmid of Enterobacteriaceae resistant to carbapenem, which sped up the interchange of resistance genes across bacterial strains and significantly increased their rate of transmission. There are several methods to identify these microorganisms. Phenotypic methods include the modified Hodge test, disc diffusion assay, minimum inhibitory concentration (MIC) assay, and synergy test. However, genotypic approaches, such as genetic sequencing and real-time polymerase chain reaction (PCR), are reliable methods for detecting carbapenem-resistant genes with high specificity and sensitivity. On the other hand, phenotypic techniques have demonstrated constraints, such as extended detection times and restricted data outputs, and the dependability of certain methods has been questioned [2].

Colistin is used as a last-resort treatment for gram-negative bacteria resistant to antibiotics, together with carbapenem. However, the colistin-resistant bacteria have proliferated due to the widespread use of this medication, especially in low-middle income countries, as a growth enhancer and infection preventive in livestock and aquaculture [3]. The discovery of the *mcr*-1 gene, the first plasmid-bound colistin-resistance gene, in *Escherichia coli* from a pig host in China underscores this concern [4]. The *mcr*-1 gene can be found in various bacterial species such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Enterobacter aerogenes*, and *Enterobacter cloacae*, this gene encodes a LPS-modifying enzyme, capable of catalyzing the addition of phosphor ethanolamine to lipid A at position 1’ or 4’, inhibiting the binding of colistin to LPS, disallowing its antibiotic effect [5]. Particularly in Asian countries, the *mcr*-1 gene is present in more than 80 % of colistin-resistant bacteria.

Hospital infection surveillance now requires multiplex molecular detection approaches due to the multidrug-resistant bacteria crisis, which is growing more and more dangerous. In order to tackle this issue, we created a novel multiplex real-time PCR protocol that enables the identification of the five most prevalent colistin and carbapenem-resistant genes found in strains that are resistant to either colistin or carbapenems worldwide. Five common resistance genes are simultaneously detected by this technology, which is based on melting point analysis. These genes include *bla*OXA-48, *bla*KPC, *bla*NDM, *bla*IMP-1 (carbapenemase genes), and *mcr*-1 (mobilized colistin resistance gene).

2. MATERIALS AND METHODS

2.1. Materials

The standard bacterial strains including *Klebsiella pneumoniae* **(** *K. pneumoniae*) ATCC374, NCTC13438, BAA2146, *Escherichia coli* (*E. coli*) NCTC13476, NCTC13846 containing *bla*OXA-48, *bla*KPC, *bla*NDM, *bla*IMP-1 and *mcr*-1 respectively, were used as the positive control for the development of multiplex real-time PCR assay. All primer sequences were synthesized by Eurofin (Germany) (Table 1); all chemicals and reagents were purchased from Qiagen and Thermo Fisher Scientific. All the sequences of target genes collected from Genebank are aligned using the Bioedit tool to identify the highly conserved region. The OligoAnalyzer tool, IDT (https://www.idtdna.com/ pages/tools/oligoanalyzer?returnurl =%2Fcalc%2Fanalyzer) was used to assess primer specificity, including primer dimer, heterodimer, secondary structure, hairpins, mismatches and melting temperature. After evaluating the single specific PCR, the primer pairs obtained with the high amplification efficiency, specificity and the T_m values of the amplicon separated by roughly 2 - 3 degrees Celsius were selected for the establishment of an optimal multiplex assay.

Table 1. Primer sequences used in this study

No.	Primer name	Primer sequence (5‘-3‘)	Tm of amplicon	Note
1	IMP-F1	ACTGGTTGTTCCAAGTCACA	81.5	This study
	IMP-R1	CAAACTGTCCAGCCACGTA		This study
2	IMP-F2	ATCCTGCACAGCACCTTG	87.5	This study
	IMP-R2	AGCGAAGTCGAGGCATT		This study
3	IMP-F3	GGCTTAATTCTCGATCTATCCC	78	[6]
	IMP-R3	CTAGCCAATAGTTAACTCCGC		
4	MCR-F	TCCAAAATGCCCTACAGACC	85	[7]
	MCR-R	GCCACCACAGGCAGTAAAAT		
5	NDM-F1	CTTCGCATAAAACGCCTCTG	88.5	This study
	NDM-R1	GCGCCGCAATCACTCATA		This study
6	NDM-F2	ACGCTGGATAGAACACCAAC	89.5	This study
	NDM-R2	AATCAAGATTTGCGGGATCAG		This study
7	NDM-F3	TCGATCCCAACGGTGATATT	88.5	This study
	NDM-R3	CGCAACACAGCCTGACTTT		This study
8	OXA-48-F1	TGTTTTTGGTGGCATCGAT	82	[8]
	OXA-48-R1	GTTCATCCTTAACCACGCCC		[9]
9	OXA-48-F2	CTTAAACGGGCGAACCAAGC	80.5	[9]
	OXA-48-R1	GTTCATCCTTAACCACGCCC		[9]
10	OXA-48-F2	AGCAAAGGAATGGCAAGAAA	75.5	[10]
	OXA-48-R2	CGCCCTGTGATTTATGTTCA		[11]
11	OXA48-F3	AGGCACGTATGAGCAAGATG	83	[12]
	OXA48-R3	TGGCTTGTTTGACAATACGC		[12]
12	BlaKPC-F1	GATACCACGTTCCGTCTGG	91	[13]
	BlaKPC-R1	ATAGTCATTTGCCGTGCCATA		[14]
13	BlaKPC-F1	GATACCACGTTCCGTCTGG	91	[13]
	BlaKPC-R2	GCAGGTTCCGGTTTTGTCTC		[15]

2.2. Methods

Single-plex real-time PCR

All primer pairs (Table 1) were tested for specificity. The reaction mixture was first prepared following the manufacturer's instruction with 10 µl master mix FastGene IC green 2x PCR Universal mix (Nippon Genetics Europe), 0.4 µM of reverse and forward primers each, 2 µl of template and the remaining volume filled with nuclease-free water to reach the total reaction volume of 20 µl.

The primer pair candidates that can identify one amplicon from another and have a consistent Ct value will be selected for further processing. Next, the single-plex real-time PCR tests' primer concentration and annealing temperature are assessed and optimized.

Multiplex real-time PCR optimization

The multiplex real-time PCR assay was developed with two pooled PCR master mixtures A and B. The mixture A consisted of 10 µl master mix FastGene IC green 2x PCR Universal mixes (Nippon Genetics Europe), 0.2 µM of each primer targeting *bla*IMP, *mcr*-1 and *bla*KPC, 2 µl of template and nuclease-free water was added to reach the total reaction volume of 20 µl. The templates of *bla*IMP, *mcr*-1 and *bla*KPC (concentration of 10^5 CFU/ml) were used as the positive control. The mixture B consisted of 10 µl of master mix FastGene IC green 2 x PCR Universal mixes (Nippon Genetics Europe), 0.2 µM of each primer targeting *bla*NDM and 0.4 µM of each primer targeting *bla*OXA-48, 2 µl of template and nuclease-free water was added to reach a total reaction volume of 20 µl. For this reaction mixture, templates of *bla*NDM and *bla*OXA-48 (concentration of 10^5 CFU/ml) were used as the positive control.

The thermo cycle was set up using the QuantStudio 5 thermal cycler system. This includes an initial denaturation stage at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 5 seconds, annealing at 63 °C for 30 seconds and elongation at 72 °C for 30 seconds. Subsequently, the process transitions to the melting curve stage, during which the temperature increases at a rate of 0.15 °C/s within the range of 60 °C to 95 °C.

Then, we fine-tuned the concentration of each primer pair to ensure amplification efficiency of multiplex reaction. Alongside these changes, additives such as Dimethyl sulfoxide (DMSO), glycerol and betaine were introduced to the reaction mixture at varying concentrations, aiming to improve real-time PCR specificity and performance.

Finally, the data for the melting peaks for each gene were aggregated to determine the upper and lower-bound of their melting temperatures (T_m).

Limit of detection (LOD)

The limit of detection of established assay was assessed using a range of varying positive template concentrations: 10^5, 10^4, 10^3, 10^2, 10^1 CFU/ml. This evaluation employs mixtures of positive templates, including a solution containing *bla*IMP, *mcr*-1, and *bla*KPC genes, as well as a solution containing *bla*NDM and *bla*OXA-48 genes. The LOD analysis was also done with individual templates.

3. RESULTS AND DISCUSSION

3.1. Optimization of single-plex real-time PCR

The amplification efficiency and the specificity of all primer pairs were evaluated using single-plex real-time PCR (Supplementary data, Figure S1). The results showed that the single-plex real-time PCR assay using IMP-F3/IMP-R3, MCR-F/MCR-R, NDM-F3/NDM-R3, OXA48-F3/OXA48-R3, and BlaKPC-R2/BlaKPC-F1 primer pairs had high specificity with low cycle threshold (Ct) values (22-24 cycles) and melting peak as our design (Table 1). Next, the concentration of selected primer pairs was optimized. As the result, the concentrations of 0.2 µM for *bla*KPC, *bla*IMP, *mcr*-1, and *bla*OXA primers were found to yield the best results, however, for *bla*NDM, it was 0.4 µM. In general, the single-plex real-time PCR assays have been optimized successfully in order to ensure their same amplification performance which plays an important role in the development of multiplex real-time PCR assay.

3.2. Optimization of multiplex real-time PCR assay

The conditions established with single-plex real-time PCR were used as a base to design the multiplex real-time PCR protocol (Figure 1).

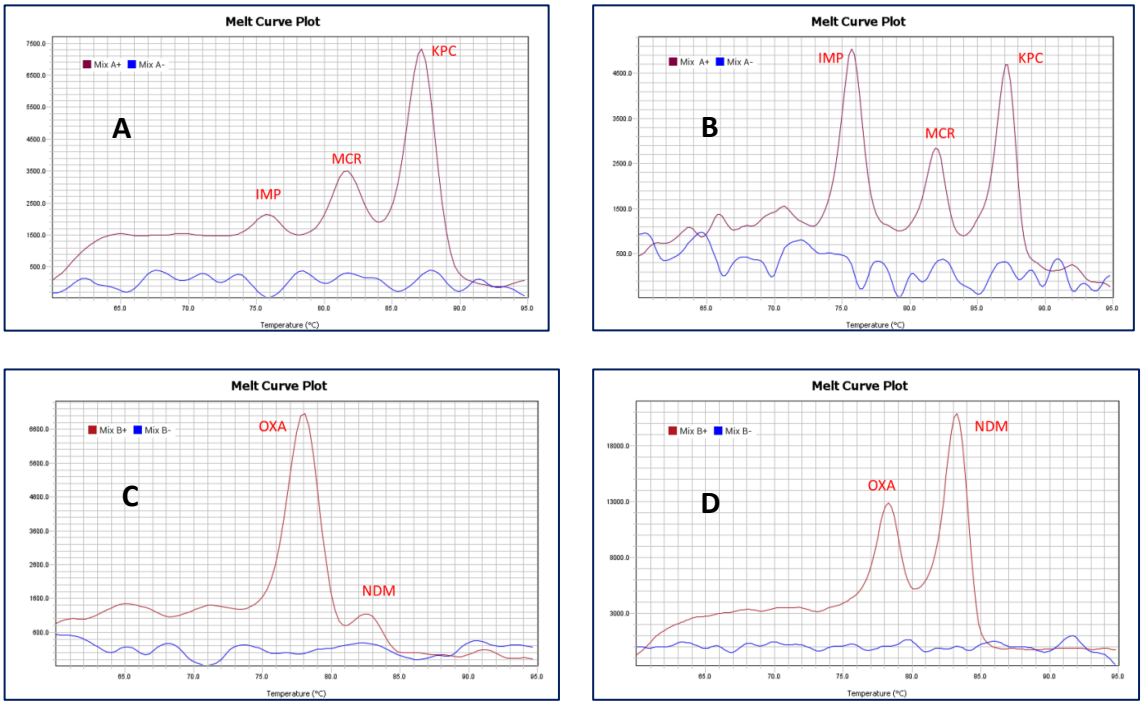

Figure 1. Optimizing primer concentration for multiplex real-time PCR. **A**) The reaction mixture A with 0.2 µM of blaIMP-F3/blaIMP-R3, MCR-F/MCR-R and blaKPC-R1/blaKPC-F2; **B**) The reaction mixture A with 0.4 µM of blaIMP-F3/blaIMP-R3 and 0.1 µM of MCR-F/MCR-R and blaKPC-R1/blaKPC-F2; **C**) The reaction mixture B with 0.2 µM of blaOXA48-F3/blaOXA48-R3 and 0.4 µM of blaNDM-F3/blaNDM-R3; **D**) The reaction mixture B with 0.1 µM of blaOXA48-F3/blaOXA48-R3 and 0.4 µM of blaNDM-F3/blaNDM-R3.

However, the combination of primer pairs might introduce unwanted interactions and influence the technique's efficiency and sensitivity in detecting these genes. Multiplex PCRs have been shown to benefit from PCR additives such as dimethyl sulfoxide and glycerol. Previous study demonstrated that these additional components avoided DNA polymerization stalling, which can occur during the extension process due to the formation of secondary

structures inside template DNA regions [16]. Therefore, specific protocol adjustments were made by incorporating additives such as DMSO, glycerol and betaine and modifying the primer concentration. For reaction mixture A, the adding of 2.5 % glycerol and 2.5% DMSO showed a reduction in the formation of undesired byproducts (Supplementary data, Figure S2). Additionally, the concentration of the blaIMP-F3/blaIMP-R3 primer was raised to 0.4 µM, while the MCR-F/MCR-R and blaKPC-R1/blaKPC-F2 primer were decreased to 0.1 µM, this is to enhance uniformity of amplification efficiency across the targets. Similarly, the reaction mixture B was changed. Optimal performance was reached with the addition of 5 % DMSO and 5 % glycerol. Within this mixture, the concentration of the blaNDM-F3/blaNDM-R3 primer was kept at 0.4 µM, while the blaOXA48-F3/blaOXA48-R3 primer concentration was reduced to 0.1 µM. Besides, the melting peak of the amplicons has changed when adding DMSO and glycerol, however, this change had no effect on the multiplex real-time PCR assay to be undistinguished between target genes that need to be detected (Table 2).

Table 1. The reference range of the T_m of multiplex real-time PCR assay

Target gene	Minimum value (°C)	Maximum value (°C)	Mean value (°C) ± Standard deviation (SD)
*bla*IMP	75.0	77.00	76.2 ± 0.52
mcr-1	81.29	83.07	82.2 ± 0.47
*bla*KPC	86.81	88.05	87.5 ± 0.41
*bla*NDM	81.46	83.63	82.8 ± 0.59
*bla*OXA	76.42	78.89	78.1 ± 0.63

Using SYBR green in combination with melting curve analysis allows for the detection of multiple target genes simultaneously in a fast and effective way. However, many parameters come in to play that can affect the melting curve analysis. Even within the same experimental setting, slight variation in the T_m can occur. Therefore, a reference range of T_m for each target gene is necessary for an accurate and reliable interpretation of the obtained data (Table 2).

3.3. The limit of detection and application potential of established multiplex real-time PCR assay

The limit detection of the established multiplex real-time PCR assay to detect *bla*OXA-48, *bla*KPC, *bla*NDM, *bla*IMP-1 and *mcr*-1 was 10 CFU/ml cultured bacteria samples in the case of single gene as well as a mixture of five genes with the same concentration (Figure 2). Therefore, our assay could detect effectively and simultaneously these antibiotic resistance genes in various concentration levels.

In 2018, Marianne Lund *et al.* [17] reported an in-house multiplex PCR performed in two tubes with *bla*NDM, *bla*VIM and *bla*IMP genes detected in one tube and *bla*OXA-48 and *bla*KPC genes in the other with the LOD ranging from 100 to 1000 CFU/ml. With this analytical sensitivity, the published assay was more sensitive than the culture in eight validated strains. Besides, the LOD of assays for rapid detection and quantification of plasmid-mediated colistin resistance genes was 100 copy numbers [18].

It is expected that our assay is a potential tool for the detection of common antibiotic resistance genes with a better LOD compared to the published assays. According to our knowledge, this is the first assay for the simultaneous detection of the common colistin (*mcr*-1)

and carbapenem (*bla*IMP-1, *bla*KPC, *bla*NDM, *bla*OXA-48) resistance genes; however, the assay needs to be further validated for its sensitivity and specificity in various sample types before being used for clinical screening.

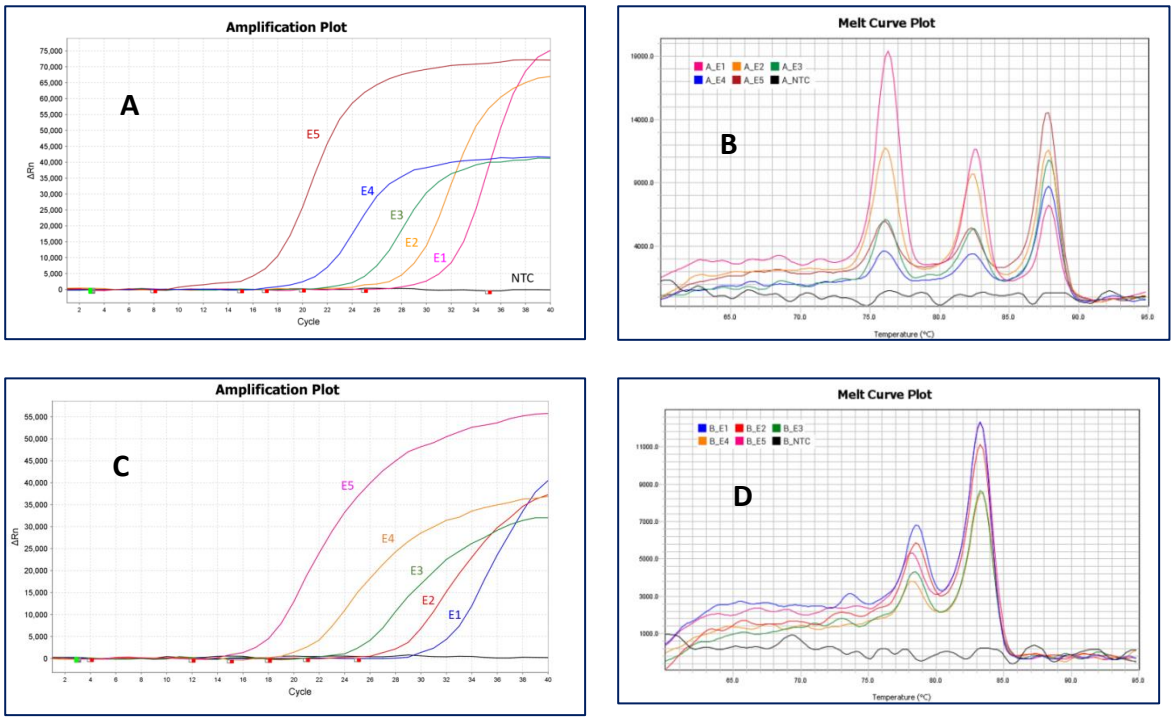

Figure 2. The limit of detection of established multiplex real-time PCR assays. **A**) The amplification plot of multiplex real-time PCR mixture A; **B**) The melting analysis of multiplex real-time PCR mixture A; **C**) The amplification plot of multiplex real-time PCR mixture B; **D**) The melting analysis of multiplex real-time PCR mixture B.

Every country has long struggled with the issue of antibiotic resistance genes, which directly affects the strategies and choices made for effective antibiotic therapy. The established assay, which is based on the multiplex principle and melting curve analysis, simultaneously detect and differentiate different genes responsible for carbapenems and colistin resistances with high levels of analytical sensitivity (10 CFU/ml). It can also produce results much more quickly, minimizing human error and lowering expenses. This assay shows potential as a useful tool for tracking the distribution and prevalence of the five common plasmid-borne genes for carbapenem and colistin resistance.

4. CONCLUSIONS

In summary, the multiplex real-time PCR assay for rapid and simultaneous detection of common colistin and carbapenem resistance genes was initially established for the first time, with a limit of detection of 10 CFU/ml cultured bacteria samples.

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CRediT authorship contribution statement. Le Thi Thuy, Do Quang Minh, Le Thi Thu Hang: Formal analysis. Anne-Laure Banuls, Dong Van Quyen: Formal analysis, Supervision. Nguyen Quang Huy: Methodology, Investigation, Supervision, Funding acquisition.

Declaration of competing interest. The authors declare no conflict of interest.

REFERENCES

1. Ventola C. L. - The antibiotic resistance crisis: part 1: causes and threats, Pharmacy and Therapeutics. **40** (4) (2015) 277-83. PMCID: PMC4378521
2. Codjoe F. S., Donkor E. S. - Carbapenem Resistance: A Review, Med. Sci. (Basel). **6** (1) (2017). DOI: 10.3390/medsci6010001
3. Kumar H., Chen B-H., Kuca K., Nepovimova E., Kaushal A., Nagraik R., Bhatia S. K., Dhanjal D. S., Kumar V., Kumar A., Upadhyay N. K., Verma R., Kumar D. - Understanding of Colistin Usage in Food Animals and Available Detection Techniques: A Review, Anima (Basel) **10** (10) 1892 (2020). https://doi.org/10.3390/ani10101892
4. Liu Y. Y., Wang Y., Walsh T. R., Yi L. X., Zhang R., Spencer J., Doi Y., Tian G., Dong B., Huang X., Yu L-F., Gu D., Ren H., Chen X., Luchao L., He D., Zhou H., Liang Z., Liu J. H., Shen J. - Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect Dis. **16** (2) 161-82016 (2016). DOI: 10.1016/S1473-3099(15)00424-7
5. Gao R., Hu Y., Li Z., Sun J., Wang Q., Lin J., Ye H., Liu F., Srinivas S., Li D., Zhu B., Liu Y-H., Tian G-B., Feng Y., - Dissemination and Mechanism for the MCR-1 Colistin Resistance, PLoS Pathog. **12** (11) e1005957 (2016). DOI: 10.1371/journal.ppat.1005957
6. Ewa K., Tomasz D., Grażyna M., - Rapid detection of NDM, VIM, KPC and IMP carbapenemases by real-time PCR, J. Bacteriol Parasitol **7** (6) (2016). DOI: 10.4172/2155-9597.1000299
7. Liu J., Zhang Z., Feng Y., Hu H., Yu Y., Qiu L., Liu H., Guo Z., Huang J., Du C., Qiu J. - Molecular Detection of the mcr Genes by Multiplex PCR, Infect Drug Resist. **13** 3463-3468 (2020). doi: 10.2147/IDR.S256320
8. Monteiro J., Widen R. H., Pignatari A. C., Kubasek C., Silbert S., - Rapid detection of carbapenemase genes by multiplex real-time PCR, J. Antimicrob Chemother **67** (4) 906-9 (2012). DOI: 10.1093/jac/dkr563
9. Jaggi N., Chatterjee N., Singh V., Giri S. K., Dwivedi P., Panwar R., Sharma A. P. - Carbapenem resistance in Escherichia coli and Klebsiella pneumoniae among Indian and international patients in North India, Acta Microbiol Immunol Hung. **66** (3) 367-376 (2019). DOI: 10.1556/030.66.2019.020
10. Park B.Y., Mourad D., Hong J. S., Yoon E. J., Kim D., Lee H., Jeong S. H. - Performance Evaluation of the Newly Developed BD Phoenix NMIC-500 Panel Using Clinical Isolates of Gram-Negative Bacilli, Ann Lab Med. **39** (5) 470-477 (2019). DOI:10.3343/ alm.2019.39.5.470
11. Mustapha A., Singh P. - Multiplex assay for detection of bacterial species in biological samples, (2015). Patent US10190177B2

12. Subirats J., Royo E., Balcázar J. L., Borrego C. M. - Real-time PCR assays for the detection and quantification of carbapenemase genes (bla (KPC), bla (NDM), and bla (OXA-48)) in environmental samples, Environ Sci. Pollut Res. Int. **24** (7) 6710-6714 (2017). DOI: 10.1007/s11356-017-8426-6
13. Zaman T. U., Aldrees M., Johani S. M. A., Alrodayyan M., Aldughashem F. A., Balkhy H. H. - Multi-drug carbapenem-resistant Klebsiella pneumoniae infection carrying the OXA-48 gene and showing variations in outer membrane protein 36 causing an outbreak in a tertiary care hospital in Riyadh, Saudi Arabia, Int J. Infect Dis. **28** 186-92 (2014).
14. Mustapha A., Singh P. - Multiplex assay for detection of bacterial species in biological samples, (2019). Patents US10190177B2
15. Tenover F. C., Kalsi R. K., Williams P. P., Carey R. B., Stocker S., Lonsway D., Rasheed J. D., Biddle J. W., McGowan J. E., Hanna B. - Carbapenem resistance in Klebsiella pneumoniae not detected by automated susceptibility testing, Emerg Infect Dis. **12** (8) 1209-13 (2006). doi: 10.3201/eid1208.060291
16. Elnifro E. M., Ashshi A. M., Cooper R. J., Klapper P. E. - Multiplex PCR: optimization and application in diagnostic virology, Clin Microbiol Rev. **13** (4) 559-70 (2000). doi: 10.1128/cmr.13.4.559-570.2000
17. Lund M., Petersen M. B., Jørgensen A. L., Paulmann D., Wang M. - Rapid real-time PCR for the detection of IMP, NDM, VIM, KPC and OXA-48 carbapenemase genes in isolates and spiked stool samples, Diagn Microbiol Infect Dis. **92** (1) 8-12 (2018). DOI: 10.1016/j.diagmicrobio.2018.04.002
18. Tolosi R., Apostolakos I., Laconi A., Carraro L., Grilli G., Cagnardi P., Piccirillo A. - Rapid detection and quantification of plasmid-mediated colistin resistance genes (mcr-1 to mcr-5) by real-time PCR in bacterial and environmental samples, J. Appl. Microbiol. **129** (6) 1523-1529 (2020). DOI: 10.1111/jam.14738