M U N I

COMMENTARY TO HABILITATION THESIS

Use of PCR-based methods in microbiology and their specific applications

Mgr. Petr Králík, Ph.D.

Molecular diagnostics has revolutionized microbiology by offering speed, sensitivity, and specificity often unattainable by traditional culture-based techniques. Among its versatile tools, PCR-based approaches utilizing in vitro amplification of DNA—particularly PCR and quantitative PCR (qPCR)—have become indispensable. Yet, as molecular diagnostics matures, its utility is increasingly shaped by how well it addresses three critical diagnostic needs: how many microbes are present (quantification), which ones are viable (viability assessment), and how many targets can be detected simultaneously (multiplexing). These three facets—quantification, viability, and multiplex detection—not only represent advanced applications of molecular methods but also offer synergistic potential for improving microbial diagnostics in both research and routine settings.

The first key application of PCR-based methods is quantification of target DNA, which is best exemplified by qPCR and partially digital PCR (dPCR). These methodologies empower researchers to make absolute determinations regarding the quantity of DNA copies present, thereby facilitating not only the qualitative assessment of microbial load but also its quantitative evaluation. However, the quantification of DNA by qPCR is a complex procedure that requires strict compliance with rigorous requirements for standards and their quantification. Failure to adhere to these principles can lead to erroneous conclusions.

Viability assessment, the second pillar, refers to the inability of PCR-based methods to assess the viability of microorganisms, as the presence of DNA/RNA alone provides no information about the physiological state of the living cell. This aspect is addressed through utilization viability PCR (vPCR), where chemical dyes such as propidium monoazide (PMA) or newer metal-based compounds like platinum or palladium salts selectively inhibit PCR amplification of DNA from dead cells. This strategy enables to selectively differentiate between live and dead cells of particular microorganism, circumventing the growth-dependency of cultures.

Finally, multiplex diagnostics with technologies such as xMAP enable the simultaneous detection of dozens of microbial targets. Conventional diagnostics procedures, such as those employed in research and routine qPCR, are capable of detecting up to four or five targets within a single analytical run. However, there exist certain applications for which a more sophisticated targeted analysis is required. MOL-PCR, a particularly noteworthy example, capitalizes on the specificity of ligation and the versatility of bead-based xMAP platforms, enabling scalable, high-throughput assays for pathogen identification, single-nucleotide polymorphism (SNP) typing, and resistance profiling.

Each of these molecular applications addresses a limitation of traditional culture diagnostics. Quantification by qPCR or dPCR provides numerical insight into microbial load, in that requires a specific interpretation criteria. For example, in the case of Mycobacterium avium subsp. paratuberculosis (MAP), quantification allows veterinarians to distinguish infected animals from passive shedders using threshold models based on DNA copy numbers.

Viability assays offer a functional improvement over culture, which fails to detect difficultly culturable microorganisms or viable but non-culturable organisms. Using MAP as the model example, the ability to detect live MAP cells in milk, even after pasteurization, highlights the importance of distinguishing DNA from dead cells and infectious agents. The optimization of metal-based dyes has enhanced the efficiency and reliability of these methods, thereby reducing the complexity of light-activated protocols, such as PMA treatment.

Multiplexing, in contrast, addresses the need for efficiency and complex targeted analysis of the sample. Conventional PCR or qPCR is constrained in its capacity to target a limited number of genes, whereas MOL-PCR facilitates the concurrent detection of up to 50 targets, including specific DNA regions, resistance genes, and virulence factors. The assay's modular design facilitates rapid updates, scalability, and integration into diverse workflows, ranging from food safety to biothreat surveillance.

Together, these three diagnostic capabilities offer a complementary toolkit: quantification delivers clarity on microbial burden, viability testing ensures biological relevance, and multiplexing enhances diagnostic breadth without increasing resource use.

To date, I have authored or co-authored 75 publications in peer-reviewed journals. A selection of 17 articles related to the three aspects of utilization of PCR based methods in microbiology I consider to be my the most significant contribution to the field. The following tables offer a synopsis of my contributions to these articles, with particular emphasis on my roles in experimental work, student supervision, manuscript preparation, and research guidance.

1. Slana, I., Kralik, P., Kralova, A., Pavlik, I., 2008. On-farm spread of Mycobacterium avium subsp paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY 128, 250-257.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**
50	-	40	20

2. Kralik, P., Slana, I., Kralova, A., Babak, V., Whitlock, R.H., Pavlik, I., 2011. Development of a predictive model for detection of Mycobacterium avium subsp paratuberculosis in faeces by quantitative real time PCR. VETERINARY MICROBIOLOGY 149, 133-138.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**
80	100	80	50

3. Kralik, P., Pribylova-Dziedzinska, R., Kralova, A., Kovarcik, K., Slana, I., 2014. Evidence of passive faecal shedding of Mycobacterium avium subsp paratuberculosis in a Limousin cattle herd. VETERINARY JOURNAL 201, 91-94.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**
50	50	50	50

4. Kralik, P., Ricchi, M., 2017. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. FRONTIERS IN MICROBIOLOGY 8.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**

80	100	70	50

5. Beinhauerova, M., Babak, V., Bertasi, B., Boniotti, M.B., Kralik, P., 2020. Utilization of Digital PCR in Quantity Verification of Plasmid Standards Used in Quantitative PCR. FRONTIERS IN MOLECULAR BIOSCIENCES 7.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	20	100

6. Beinhauerova, M., Beinhauerova, M., McCallum, S., Seller, E., Ricchi, M., O'Brien, R., Blanchard, B., Slane, I., Babak, V., Kralik, P., 2021. Development of a reference standard for the detection and quantification of Mycobacterium avium subsp. paratuberculosis by quantitative PCR. SCIENTIFIC REPORTS 11.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	30	100

7. Kralik, P., Nocker, A., Pavlik, I., 2010. Mycobacterium avium subsp paratuberculosis viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY 141, S80-S86.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
100	60	80	70

8. Pribylova, R., Kubickova, L., Babak, V., Pavlik, I., Kralik, P., 2012. Effect of short- and long-term antibiotic exposure on the viability of Mycobacterium avium subsp paratuberculosis as measured by propidium monoazide F57 real time quantitative PCR and culture. VETERINARY JOURNAL 194, 354-360.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
60	80	50	90

9. Kralik, P., Babak, V., Dziedzinska, R., 2014. Repeated cycles of chemical and physical disinfection and their influence on Mycobacterium avium subsp paratuberculosis viability

measured by propidium monoazide F57 quantitative real time PCR. VETERINARY JOURNAL 201, 359-364.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
80	100	50	100

10. Ricchi, M., De Cicco, C., Kralik, P., Babak, V., Boniotti, M.B., Savi, R., Cerutti, G., Cammi, G., Garbarino, C., Arrigoni, N., 2014. Evaluation of viable Mycobacterium avium subsp paratuberculosis in milk using peptide-mediated separation and Propidium Monoazide qPCR. FEMS MICROBIOLOGY LETTERS 356, 127-133.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
10	50	40	40

11. Kralik, P., Babak, V., Dziedzinska, R., 2018. The Impact of the Antimicrobial Compounds Produced by Lactic Acid Bacteria on the Growth Performance of Mycobacterium avium subsp paratuberculosis. FRONTIERS IN MICROBIOLOGY 9.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
80	100	50	80

12. Cechova, M., Beinhauerova, M., Babak, V., Slana, I., Kralik, P., 2021. A Novel Approach to the Viability Determination of Mycobacterium avium subsp. paratuberculosis Using Platinum Compounds in Combination With Quantitative PCR. Frontiers in Microbiology 12.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	50	100

13. Cechova, M., Beinhauerova, M., Babak, V., Kralik, P., 2022. A viability assay combining palladium compound treatment with quantitative PCR to detect viable Mycobacterium avium subsp. paratuberculosis cells. Scientific Reports 12.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)

-	100	50	100

14. Reslova, N., Michna, V., Kasny, M., Mikel, P., Kralik, P., 2017. xMAP Technology: Applications in Detection of Pathogens. FRONTIERS IN MICROBIOLOGY 8.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	50	30	100

15. Reslova, N., Huvarova, V., Hrdy, J., Kasny, M., Kralik, P., 2019. A novel perspective on MOL-PCR optimization and MAGPIX analysis of in-house multiplex foodborne pathogens detection assay. SCIENTIFIC REPORTS 9.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	50	20	100

16. Jelinkova, P., Hrdy, J., Markova, J., Dresler, J., Pajer, P., Pavlis, O., Branich, P., Borilova, G., Reichelova, M., Babak, V., Reslova, N., Kralik, P., 2021. Development and Inter-Laboratory Validation of Diagnostics Panel for Detection of Biothreat Bacteria Based on MOL-PCR Assay. MICROORGANISMS 9.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	30	20	100

17. Hrdy, J., Vasickova, P., Nesvadbova, M., Novotny, J., Mati, T., Kralik, P., 2021. MOL-PCR and xMAP Technology A Multiplex System for Fast Detection of Food- and Waterborne Viruses. JOURNAL OF MOLECULAR DIAGNOSTICS 23, 765-776.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	20	20	40