Warsaw, August 22, 2018

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Referee Report on the Dissertation Thesis of MUDr. Anna Šrámková
at the Charles University, Faculty of Medicine in Plzeň

Mrs. Anna Šrámková studied in the Faculty of Medicine in Plzeň of the Charles University in 2008-2014, and graduated with specialization in general medicine. In 2014 she commenced her Ph. D. studies in the Department of Clinical Microbiology, the Biomedical Center of the same Faculty, and one year later she took up also the position of the Doctor of Medicine in the Department of Clinical Microbiology of the University Hospital in Plzeň. In 2016 Mrs. Šrámková spent one month on postgraduate training in the Department of Clinical and Experimental Medicine of the University Hospital "Careggi" in Florence, Italy. These briefly presented educational and professional records, especially when analysed in time course, indicate high motivation of Mrs. Šrámková for work and individual progress. The combination of medical background, routine practice in clinical microbiology and hospital epidemiology, and research activity in epidemiology of bacterial infections demonstrates extraordinary ambition and self-organisational skills. I would like to highlight the value of Mrs. Šrámková's specific professional and personal profile that seems to be relatively rare in Central European countries.

The dissertation thesis of Mrs. Šrámková contains five original papers published or accepted for publication in peer-reviewed international journals, consistent on basis of merit as a whole, and having a common methodological denominator. The papers are preceded by an introductory material, including opening remarks, a hypothesis and aim of the thesis, a review of the current knowledge in the scope of the main topic, and a list of methods used in the experimental part. On the other side, the research articles are followed by a separate discussion, an overview conclusion, and attachments with the CV and publication parameters. In general the thesis has an appropriate structure; some specific comments on the composition and/or content of the introduction and discussion sections will be presented below.

As mentioned just above, the core of the thesis has been the set of five scientific reports published in 2017-2018. The research described refers to epidemiology of antimicrobial resistance (AMR) in pathogenic bacteria that is a critical issue in clinical microbiology,

infectious diseases and public health today, owing to the non-controlled AMR spread during the recent decades. Given that AMR compromises all antibiotics available now and that the antibacterial pipeline has been in deep crisis for the last 30 years, the situation is alarming with the clear vision of the era of untreatable infections (Cohen, 1992). This has been reflected by innumerable reports, position papers or recommendations by the scientific milieu and public health authorities, as well as diverse legal acts by national and international political bodies. Each significant document points out the necessity of multi-level and multi-sectorial efforts to be undertaken, including antimicrobial stewardship, infection control, development of new antiinfectives and rapid diagnostics solutions, and epidemiological monitoring of AMR combined with early warning systems. All these activities must be based on high-quality data provided by up-to-date scientific investigations.

Since the 1980/1990s the epidemiology of infections and AMR has been revolutionized by the adoption of molecular biology techniques, allowing for the unprecedented resolution, precision and reliability of comparative analyses of bacterial isolates recovered from clinical samples and other sources. The introduction of DNA sequencing into typing approaches in the 1990/2000s, soon followed by the development of online data processing tools and databases, has been another milestone in this process, resulting in full inter-laboratory procedure standardization and data exchange in real time. Finally, the rapid progress in the next-generation sequencing technologies has brought about another radical gain of quality in epidemiology in last years that was the implementation of whole-genome sequencing (WGS) for isolate characterization. This currently top-level methodology has been superior in the amount of data provided and non-limited flexibility in discriminatory power of the analysis, complying so with a large variety of scientific objectives. Molecular techniques have opened new horizons in epidemiology of infections, addressing both the vertical and horizontal components of bacterial evolution. These allowed revealing factual clonal structures of bacterial populations, with unambiguous identification and the most precise description of epidemic "high-risk" clonal lineages of pathogenic organisms. The molecular approach has been the only way to uncover the major role of mobile genetic elements, including transposable units, plasmids or phages in the dissemination of AMR, virulence or epidemicity factors. Owing to all the above, today we understand much better the basis (*i. e.* origins, ways and mechanisms) of the emergence and spread of pathogens of the highest clinical and epidemiological relevance. Remarkably contributing to modern clinical microbiology or bacterial genetics, molecular epidemiology provides also the key data for the monitoring, surveillance and intervention actions aimed at controlling the AMR spread.

The five study reports contained in the Mrs. Šrámková's Ph. D. thesis are located in state-of-the-art molecular epidemiology of AMR as it has been described briefly just above. These all refer to Gram-negative rods of the family *Enterobacteriaceae* (4 papers) or the species *Pseudomonas aeruginosa* (1 paper) with resistance to carbapenems, *i. e.* last-resort drugs in the treatment of severe nosocomial infections caused by Gram-negative microorganisms. The studies were focused on clinical isolates with the essential type of carbapenem resistance mechanisms, namely carbapenem-hydrolysing β-lactamases or carbapenemases. The carbapenemase-producing *Enterobacteriaceae* (CPE) and *P. aeruginosa* (CPP) belong to the key factors of the aforementioned AMR crisis which is due to their resistance to almost all or all β-lactam antibiotics, notorious co-resistance to multiple other antimicrobial classes, and dramatically rapid proliferation in bacterial populations, mainly in hospitals but more

and more so in the community world-wide. This dissemination has been due to extensive clonal spread of CPE and CPP strains, often representing the high-risk genotypes with enhanced epidemicity, horizontal diffusion of multiple mobile genetic platforms carrying carbapenemase and other AMR genes, and continuously on-going *de novo* selection events generating newer and newer carbapenemase types or variants. Huge environmental reservoirs in several geographic areas of the world combined with massive travelling and migration enormously contribute to the situation, especially in case of CPE. The highest significance of CPE and CPP in current epidemiology of bacterial infections fully justifies the choice of these organisms as the study material in the projects included in the dissertation.

The focus on CPE and CPP is explained also by the scientific interests of the team in which Mrs. Šrámková has been working since the end of her university studies. Doc. Ing. Jaroslav Hrabák, the group leader and supervisor of Mrs. Šrámková's Ph. D. studies, has been conducting molecular epidemiology investigations on multi-drug-resistant Gram-negative pathogens in the Czech Republic since 2006. One of the most important elements of the activity of Doc. Hrabák and his team is the mutual collaboration with the National Reference Laboratory for Antibiotics of the National Institute of Health in Prague, led consecutively by Dr. Pavla Urbášková and Doc. Dr. Helena Žemličková. Providing his expertise in molecular biology and biochemical methodologies, Doc. Hrabák has full access to one of the best European collections of bacterial clinical isolates, collected for years by the continuous AMR surveillance in hospitals all over the Czech Republic. This kind of setting, consisting of a well-organised national AMR monitoring system and high-quality molecular epidemiology research, has been rather rare, and in my opinion, our Czech Colleagues demonstrate one of the best examples of such capacity. Owing to that, the study material of Mrs. Šrámková has been unique and representative for the whole country, and allowed so the Czech scientific data on the critical AMR microorganisms to circulate internationally and contribute to the view of the CPE/CPP epidemiology in Europe. Moreover, such data are the inestimable circumstance and support for the national medical-care authorities in their efforts counteracting one of the most alarming public health threats.

According to what was mentioned above, the value of the Mrs. Šrámková's Ph. D. research relies on the combination of the fine material with the high-level study design and analysis, and the state-of-the-art methodology used. Each of the studies included in the dissertation has addressed adequate scientific questions and each was logically conducted. What should be underlined is that Mrs. Šrámková and her colleagues were able to formulate consecutive hypotheses in real time according to the observations made in the course of their experiments, and modify their work plans correspondingly. As mentioned just above, they applied a comprehensive and appropriate set of microbiological, biochemical, genetic and molecular biology techniques. These included culture-based reference assays in antimicrobial susceptibility testing, MALDI-TOF MS assays in bacterial species identification and carbapenemase detection, isoelectric focusing in β-lactamase profiling, conjugation and transformation tests in plasmid horizontal transfer analysis, and PCR, DNA sequencing, pulsed-field gel electrophoresis and hybridization in strain typing and characterization of AMR genes and mobile genetic elements. Each of the five projects reported comprised the most modern WGS approach that provided the deep characteristics of selected organisms, including complete structures of their carbapenemase genetic determinants (transposons, integrons, integrative conjugative elements, genomic islands, plasmids), as well as full

information on their clonal identity, phylogenetic position, AMR repertoire (resistome) or taxonomy in case of the *Enterobacter cloacae* complex isolates. The WGS analysis allowed Mrs. Šrámková and her colleagues to understand better the CPE and CPP epidemiology in the Czech Republic than ever before, estimate probable numbers of independent import or selection events of these pathogens in the country, reveal cases and mechanisms of their spread, and precisely track evolution of the critical AMR genetic determinants in the context of the international data. The implementation of WGS into the projects was done in a competent and adequate way, and has remarkably increased their scientific impact.

The most important part of the thesis has been the study on *Enterobacteriaceae* producing OXA-48-like carbapenemases in the Czech Republic, which soon may become the major CPE type and a significant public health challenge in this country. Mrs. Šrámková is the first author of the report published in 2017 in a renowned journal *Antimicrobial Agents and Chemotherapy*, indicating her main role in the research team. The analysis comprised 24 isolates from 2013-2015, recovered from 18 patients in seven hospitals. The comprehensive and elegant analysis demonstrated two key epidemiological phenomena, being a clonal outbreak of *Klebsiella pneumoniae* ST101 OXA-48 in one of the hospitals, and dissemination of IncL-group pOXA-48-like plasmids in enterobacterial populations. Like in numerous other countries, the plasmid spread has been identified to be the essential factor of the increase of OXA-48 CPE cases in the Czech Republic, and this hypothesis was based on the observation of molecules with identical sequences in multiple bacterial species and clones, including *K. pneumoniae* ST15, ST101, ST395, ST461 and ST1520, *Escherichia coli* ST216 and ST4956, and *E. cloacae* complex ST109. Diverse organisms with the same plasmids were recovered from three patients, likely documenting the *in vivo* transmission cases, finally the conjugation potential of the pOXA-48-like molecules was evidenced by mating assays. In my opinion the study has been of high quality and provided a remarkable set of data, relevant for the health-care specialists and authorities in the Czech Republic, and for the international scientific community. However, I would suggest not excluding totally the possibility of repeated independent imports of microorganisms with identical plasmids from abroad; pOXA-48-like plasmids have been extremely broadly dispersed in *Enterobacteriaceae* populations and are characterized by a relatively stable and conserved structure.

Of the four remaining studies, carried out with the participation of Mrs. Šrámková, two are similar to the OXA-48 report by the scale of analysis, being works on larger groups of clinical isolates collected from the entire Czech territory. Like the previous one, these two have been conducted in the state-of-the-art way and provided very interesting epidemiological observations, especially regarding the species and/or clonal distribution of carbapenemase producers. The group of 18 *Enterobacteriaceae* producing metallo-β-lactamases (MBLs) of the NDM type were dominated by NDM-4-producing *Enterobacter xiangfangensis* ST182, first identified in one hospital in a sporadic case in 2012, and then as an outbreak organism in the same centre in 2016. The Authors considered the possibility of a hidden circulation of this unusual organism in the hospital environment for four years, especially that the isolates carried the same NDM-4-encoding plasmid of the IncX3 group. However, the WGS analysis revealed significant differences in chromosomes of the isolates from 2012 and 2016, indicating two independent imports of the specific genotype into a single medical institution. The study has shown also the prominent role of the IncX3-like plasmids in the NDM CPE epidemiology in the Czech Republic, having been observed in three subtypes, coding for

NDM-4, NDM-5 or NDM-1 carbapenemase variants. Apart from the outbreak *E. xiangfangensis* ST182 isolates, the NDM-4-encoding plasmid was identified in *Enterobacter asburiae*, *Enterobacter intermedius* and *E. coli* ST69 from the same hospital, demonstrating again the significance of the plasmid horizontal transfer in the CPE epidemiology. The study on carbapenem-resistant *P. aeruginosa* from 2015 revealed that out of all carbapenemase-producing Gram-negative pathogens, CPP has been the most important epidemiological threat in the Czech Republic so far. The group of 136 CPP clinical isolates from 22 hospitals, being mainly MBL-positive organisms, contained as many as 115 *P. aeruginosa* ST357 isolates with the IMP-7 enzyme, encoded by integron In-p110 or related elements, located in a LESGI-3-type genomic island. The extensive exploitation of the WGS approach has resulted in one of the best molecular epidemiology studies on MBL-producing *P. aeruginosa* ever performed, including fine phylogenetic analysis that demonstrated clearly the dissemination of the ST357 IMP-7 genotype over the whole territory, commenced in 2008. Another remarkable added value of the *P. aeruginosa* study was the inclusion of carbapenemase-negative carbapenem-resistant and carbapenem-susceptible isolates, identified in the same surveillance system. This has been rare and allowed evidencing the tight association of certain *P. aeruginosa* lineages with various AMR determinants, defining these as particularly dangerous high-risk clones. Regarding the two papers commented just above, the only element which in my opinion has not been sufficiently exposed in their discussion sections, has been the specificity of the NDM CPE and CPP epidemiological situations in the Czech Republic when compared to other European countries. Whereas the NDM CPE history has been in an early phase and may be changed soon easily, the CPP epidemiology seems to be well-established and its major characteristics, namely the vast and so far stable predominance of the ST357 IMP-7 genetic combination is striking in Europe where ST235 or ST111 clones and VIM-type enzymes definitely prevail in MBL-producing *P. aeruginosa* subpopulations. The two last publications included in the dissertation are brief reports on single *Enterobacteriaceae* isolates producing relatively rare types of carbapenemases, GES-5 and IMI-2, recovered in sporadic infection cases. Resuming my opinion on the original part of the Mrs. Šrámková's Ph. D. thesis, I would like to underline again the high scientific level of the studies described, which in total provide the almost complete and actual view of the epidemiology of carbapenemase-producing Gram-negative bacteria on a country-wide scale. The Czech Republic belongs to the European countries with the deepest and most comprehensive knowledge on these alert pathogens.

The Introduction is a good review of literature concerning carbapenem resistance mechanisms and epidemiology of CPE and CPP. Mrs. Šrámková has shown a higher-than-average knowledge on these issues, and an ability of composing longer texts of this type. Still prior to possible publication of this material as a separate article it would require some more editorial attention. For example, I would suggest limiting the fragments that contain details concerning numerous individual variants of carbapenemases, *e. g.* IMPs or VIMs, and dates, countries and species in which these were identified. In my opinion these parts have been slightly overloaded with information of not the highest importance. On the other hand, the subsection on IMP-type MBLs lacks the presentation of major phylogenetic lineages of these enzymes and their epidemiological context. The text describes some incidentally reported carbapenemases, like SHV-38 and SFC-1, but does not mention several recent types which soon may turn out to spread on larger scale, *e. g.* BKC- or FRI-like class A β-lactamases or SMB-, FIM-, LMB- or HMB-like MBLs. One may also debate on accents put on some

information, like *e. g.* the role of the horizontal spread of plasmids in epidemiology of KPC-type carbapenemases; indeed, KPC-encoding plasmids do circulate in bacterial populations, nevertheless, the far more important phenomenon is the global expansion of the *K. pneumoniae* clonal group (CG) 258. The Introduction might also be improved in terms of style and expression. For example, Mrs. Šrámková should be careful when refers to enzymes or to genes; enzymes cannot be "harboured" by plasmids or "localized" on plasmids. I would also suggest checking for references; for example, those that are cited in legends to figures do not seem to be the proper ones. In my opinion the Discussion is slightly too repetitive when compared to the discussion sections in the corresponding publications, and would also require some fine-tuning. For example, in the part referring to NDM CPE there is a species "*Kluyvera intermedia*", whereas "*Enterobacter intermedius*" is in the paper. In the part concerning *P. aeruginosa*, Mrs. Šrámková mentions a "study performed by Woodford et al.", with a citation to a review article published once by these authors. I would like to stress that my critical remarks regarding the Introduction and the Discussion of the thesis are of the minor type, and that in general I am impressed by the knowledge of Mrs. Šrámková, especially in the context of her age and relatively short time she has been involved in research on β-lactamase-producing Gram-negative bacteria.

Considering all the above, in my opinion Mrs. Anna Šrámková is a well-educated, highly motivated, capable and intelligent young researcher, starting a promising scientific career. I recommend her Ph. D. dissertation thesis for the public defense.

Once the dissertation becomes a matter of the defense, I would suggest Mrs. Šrámková to respond to my comments regarding the original part of her thesis, namely to: i) discuss the possibility of repeated introductions of pOXA-48-carrying *Enterobacteriaceae* into the Czech Republic in the context of the international data on the pOXA-48-like plasmids, and ii) debate on the specificity of the NDM CPE and CPP circulating in the Czech Republic, with broader presentation of the current international knowledge on major genotypes of these organisms prevailing in other European countries and other parts of the world. Apart from those, I would suggest the Candidate to summarize what is known on consequences of specific amino-acid polymorphisms (mutations) in selected β-lactamases of the families OXA-48 (OXA-48 vs. OXA-163), NDM (NDM-1 vs. NDM-4 vs. NDM-5) and GES (GES-1 vs. GES-5 or GES-3 vs. GES-4) for the catalytic efficiency of hydrolysis, especially of carbapenems and oxyimino-cephalosporins, and for the minimal inhibitory combinations (MICs) of these compounds against bacterial strains expressing these enzymes.