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Gdańsk, 25.01.2025

PhD Thesis Acceptance Report
for the Research Discipline Council of Biological Sciences
of Jagiellonian University in Kraków

Candidate's name and surname: Laurensius Kevin Lie

PhD Thesis Title: Coronavirus – cross-tissue and cross-species infectivity

Thesis Supervisor: Prof. dr hab. Krzysztof Pyrć

Reviewer: dr hab. Andrea Lipińska

The background and the scope of the thesis

"Facing new threats, do not forget about the old ones" – this aptly describes the research interest of the Ph.D. Candidate presented in his dissertation, focusing on a low-pathogenicity human coronavirus HCoV-229E (hereafter referred to as 229E), one of the seven human coronaviruses isolated so far. The study also addresses to a small extent three other low-pathogenicity hCoVs: HCoV-OC43, HCoV-NL63, and HCoV-HKU1. However, the data obtained from their infection in enteroids seems inconsistent, so the thesis should concentrate on 229E alone. 229E was first described in the USA in the mid-1960s, and it has remained prevalent in human population, contributing significantly to seasonal common colds. While it typically causes mild symptoms, as with all viral infections, the severity can increase depending on the host's immune competence. Despite a long, albeit not very intense, history of basic research, no specific antivirals or preventive vaccines are available. The author was motivated by the scarcity of optimal virus propagation models, especially for clinical strains of 229E. One cited reference, Shirato et al., also noted that "no reliable cell model was available for HCoV-229E, unlike MDCK cells for influenza." This justifies the primary aim of the thesis: to identify an optimal virus propagation model, which would also provide new insights into its biology, including its entry and infection kinetics, dependence on receptors/co-factors such as TMPRSS2 or the cathepsin pathway, extrapulmonary infections, and potentially virus genetic diversity and evolution. The model could also be used to test Candidate antivirals, among other research goals.

This thesis has clearly two interconnected parts: 1) generation of a cell line model that would yield high titres of genetically stable 229E virus for further research, and 2) to optimize the human enteroid model for low-pathogenicity hCoVs, with a focus on enteroid permissiveness for 229E.

The importance of such an optimal cell model is further highlighted by the difficulty in propagating other hCoVs, other than 229E, to high titers, which underscores the need for the development of suitable cell lines for these viruses as well.

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General description and the structure of the thesis

This 117 pages long dissertation is rather compact, but it contains all the information necessary to undestand the study and its achievements, as well as to demonstrate the Candidate's broad knowledge on the presented topic. The single-page print, in my opinion, was unnecessary.

The dissertation is written in clear English and it is composed of the following sections: Introduction, Abstract (in both Polish and English), Introduction, Thesis Objectives, Materials, Methods, Results, Discussion, and References. Each of the two parts of the Discussion concludes with a short summary highlighting the most important achievement of the study. The reviewer has noticed some editorial mistakes, especially in the Discussion section. The list of Abbreviations contains common biology terms like RNA and DNA, which do not need to be listed. The Polish translation of the abstract contains errors in the use of scientific vocabulary, but this can be expected from a Candidate who does not (presumably) use Polish on a daily basis. The Introduction provides unified and clear information on the coronavirus life cycle, pinpointing features common to all coronaviruses. This demonstrates the author's ability to view the topic in a general and broad way. The section also covers clinical manifestations, the innate immune response against hCoVs, ex vivo models, and gastrointestinal tract models. However, the reasoning behind the inclusion of innate immunity, without reference to restriction factors and with no clear link to the main experimental part, seems unclear.

Figues are properly referenced in the text. The Materials section is very basic and lacks some important details, such as information on the solvents used for the inhibitors and plasmid details. These details are provided in the Results section, but the information on solvents is still missing, which creates a small issue in understanding which cells should be regarded as the proper negative control: DMSO-treated or mock-treated (e.g., in Figure 9, "Mapping the entry route of HCoV-229E clinical isolate"). Regarding the References, their number is impressive, confirming the high level of knowledge in biological sciences demonstrated by the author. However, the reference system could be more uniform.

The study was conducted as part of several large grant projects (OrganoVir by EU Horizon 2020, EU4Health, ERA-ICRAD, among others), and I am sure the results contributed to achieving the projects' goals. The first part of the thesis was published in Virology (IF 2024: 2.8), and the second part is presented as a ready manuscript, although it has still not reached accepted status. Mr. Lie is also a co-author of two other research papers, including a review partially related to the topic of the dissertation, on SARS-CoV-2.

The Candidate's knowledge and independence

The Ph.D. Candidate has demonstrated his scientific skills by designing and conducting advanced research and analyzing the results. The description of the materials, methods, and the execution of the experiments showcases his broad knowledge of cell culture, virus culture techniques, and cell genetic modifications.

The Introduction clearly explains that, compared to the extensive knowledge we now have on highly pathogenic hCoVs, much less has been learned about the common cold hCoVs over the decades, and this knowledge has been largely neglected. Therefore, pushing the limits of knowledge on 229E should be appreciated. I also commend the introduction and optimization of the enteroid models in the laboratory, as organoids are valuable tools for studying viral infections, offering more accuracy than monolayer cell cultures. The description of the experimental workflow demonstrates the Candidate's experimental proficiency, systematicity and accuracy, particularly in viral kinetics. I also understand that the descriptions may not fully reflect the amount of work behind the experiments—e.g., cell clonal selection may sound straightforward, but it involves weeks of laborious work.

I am convinced that the candidate has reached the appropriate level of independence and experimental proficiency expected at this stage of a scientific career.

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Originality of the dissertation

The majority of the results affirm what has already been described in the literature, such as the significance of CD13 and TMPRSS2 for 229E entry. The use of lentiviral vectors for receptor overexpression is not a novel approach; though it is currently the method of choice, it is valuable to have another study confirming the applicability of this method. It has been previously suggested that 229E prefers TMPRSS2+ cells, and the results obtained by Mr. Lie do not contradict this, showing that 229E can use alternative pathways if TMPRSS2 is not detected. The 229E entry and its dependence on TMPRSS2 were previously studied in different models (e.g., Shirato et al. used pseudotyped VSV virus). I fully agree with the author that the A549++ cell line has potential for further studies on the 229E virus.

Extrapulmonary tissue infections of 229E have been poorly studied, despite prior reports on gastrointestinal symptoms, especially in children. Therefore, the second part of the study introduces more novelty. The comparison of low-pathogenicity hCoVs in human enteroids derived from donors of different "age" (fetal, pediatric, and adult) was performed for the first time, according to the literature. The author identified gastrointestinal cell types in the organoids susceptible to 229E. I find this part of the dissertation particularly valuable, as it may help explain why certain age groups (like children) are more prone to extrapulmonary coronavirus infections than others. The developed enteroid model is general and can be applied to similar studies for all hCoVs. Regarding other low-pathogenicity hCoVs in enteroids, the conclusion on the need to repeat the experiments with higher virus loads to obtain more conclusive data, is justified, especially since some of those viruses were reported in patient stool samples. We should agree that extrapulmonary infections of these hCoVs remain an open question.

Questions and/or criticisms to which the Reviewer expects the Candidate to respond during the defence

The following remarks and comments related to the thesis should be explained in detail:

1. The issue of solvents: This is a small but important factor for the proper interpretation of the results. As mentioned earlier, I expect some of the entry inhibitors to be soluble in DMSO, and others in water. However, in the statistical analyses, in Figure 9, only medium-treated, mock cells were set as the 100% control, and in Figure 15 ("Entry of HCoV-229E clinical isolate into intestinal organoids requires TMPRSS2"), only untreated organoids were used as controls. Was there a reason why DMSO was omitted? Would the statistics in Figure 9 differ if the data was compared to the DMSO control? Additionally, in the same Figure: did using a combination of camostat and E64d require applying a higher volume of DMSO solvent to the cells? Was a proper control with the same DMSO volume used to exclude the toxic effects of DMSO in the treatment with mixed inhibitors?

2. Regarding the first part of the study: Full appreciation of the new A549++ cell model is hampered by the lack of comparison with existing models, namely the MRC-5 and Huh-7 cell lines, which were used for propagating both the laboratory and clinical strains in this study. There is no comparison, even with the literature data, regarding the titers that can be obtained, kinetics, or, for example, the size of plaques. If the author propagated 229E in various cell lines, could he comment on whether the new A549++ cells allow better visualization of plaques, which can be problematic in coronavirus *in vitro* infections? Another related question is whether the candidate tested the stability of the transgenes in the A549++ cells by culturing them without the selection antibiotics? Since the SIN lentivirus was used for TMPRSS2 and CD13 gene incorporation, it should provide stable expression even in the absence of antibiotics. The use of antibiotics would increase costs and might interfere with the experiments. Next, Huh-7 cell line was described by the author as TMPRSS2-negative. Was this assumption made only based on the literature, or the author has actually tried to detect the protease or protease mRNA there? There is a report from 2021 by Saccon et al. (DOI: 10.1016/j.isci.2021.102420), demonstrating TMPRSS2 expression in Huh7 cells and explaining that this is the reason why these cells can maintain SARS-CoV-2.

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3. During the interpretation of the results on cell permissiveness, it should be remembered that it is governed not only by the presence of proper receptors but also by the level of restriction factors regulating the infection negatively. Bertram et al. [ref. 213] have suggested that TMPRSS2 rescues 229E-S-dependent cell entry from inhibition by IFITM proteins. Is the dependence of 229E on any of the IFITM proteins described in the literature? Also, are the levels of IFITM1, 2, and 3 known for A549 cells?

4. A general question, out of curiosity: Are the mechanisms behind the adaptation of coronavirus biology to 32°C, the temperature of the airway epithelium, known? For example, why can SARS-CoV-2 grow equally well at 32°C or 37°C, whereas 229E, as the author showed, prefers lower temperatures?

5. One of the reasons for the generation of A549++ provided by the author is the risk of adaptive mutations in coronaviruses propagated in non-optimal models (like SARS-CoV-2 in VeroE6). However, the author provides no data on mutation frequency during 229E propagation in A549++, even though genetic analysis of the virus is known to the author. The question is whether such a study was conducted to further appreciate the generation of A549++?

6. I would like to ask the author, as a person much more experienced with organoids, about the limitations of such a system.

7. A comment: In my opinion, the conclusion presented in the Discussion that CD13 overexpression may contribute to the activation of TMPRSS2 by S1 peptidase domain cleavage is not convincingly supported by the results and should be explored further.

8. A comment: We should remember that cancer cell lines, with all their uncharacterized features, are still not the most optimal models for virus propagation.

Summarizing my evaluation: I, hereby, declare that the reviewed PhD thesis by **Laurensius Kevin Lie** meets the criteria pursuant to art. 187 of Act of 20 July 2018 The Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended) and request that the Research Discipline Council of Biological Sciences of the Jagiellonian University in Kraków accepts **Laurensius Kevin Lie** for further stages of doctoral proceedings in the field of exact and biological sciences, in the discipline of biological sciences.

date **25.01.2025**

Andrea Lipińska

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Reviewer's signature

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