Metagenomic Analysis for Identifying Co-Infections in Covid-19 Patients: Review

Rina Isnawati[1*], Anna Lystia Poetranto[1], Nona Rahmaida Puetri[1], Setyo Adiningsih[1]
[1]*Center for Biomedical Research, Research Organization for Health, National Research and Innovation Agency, Cibinong, 16911, Indonesia*
rina029@brin.go.id

Keywords: COVID-19, co-infection, metagenomics, SARS-CoV-2

Abstract: The COVID-19 pandemic continues and fluctuates posing unprecedented challenges to global healthcare systems, requiring a comprehensive understanding of viral and microbial interactions within infected individuals. Metagenomics, a powerful tool for analyzing genetic material from diverse microorganisms, has emerged as a promising approach for uncovering co-infections in COVID-19 patients. This review aims to assess the current state of metagenomics-based studies focused on identifying co-infections in COVID-19 patients. Methods: This study is a review of the literature collected from the search engine Google Scholar and PubMed Electronic Database with the keywords: "COVID-19", "Co-infection", and "Metagenomics". We looked for publications published in any language between 2020 and 2023. We obtained the original articles of each relevant and reported research design, and 21 eligible articles were found for inclusion in the analysis. Co-infection has been shown in several studies to exacerbate patient clinical problems to varying degrees. Metagenomic next-generation sequencing (mNGS) technology can simultaneously show all infections present in organisms. Metagenomics analysis has increased the diagnostic level of the pathogen and provided valuable insights into the microbial landscape of COVID-19 patients, uncovering the prevalence and impact of co-infections.

1 INTRODUCTION

Corona Virus Disease 2019 (COVID-19) is an infection with the SARS-CoV-2 virus, which has become a global pandemic that continues to this day and fluctuates. The spectrum of disease that appears in COVID-19 infection can be divided into asymptomatic or presymptomatic infections with mild, moderate, severe, and critical symptoms (Esakandari et al., 2020). About 20% of SARS-CoV-2 infections would worsen the prognosis noticeably by progressing to a severe or critical stage. Previous research showed that admission to an intensive care unit, a greater likelihood of secondary infection, and a higher risk of invasive operations were all linked to severe SARS-CoV-2 pneumonia. The dangers and characteristics of secondary infections in severe COVID-19 have not yet been discussed (Zhang et al., 2020).

Several research studies have investigated the role of the microbiome in the emergence of COVID-19, posing the possibility of a connection between COVID-19 and the gut, lungs, nasopharynx, or oral microbiota (Bao et al., 2020). It has been discovered that several taxa of bacteria in the mouth or gut microbiome are connected to disease severity (Zuo et al., 2020) and may be utilized to forecast the clinical course of COVID-19. The human upper respiratory tract, which acts as a main entry route for SARS-CoV-2, has an airway microbiome that is typical of its milieu and plays a crucial role in the airway epithelial barrier (Man, de Steenhuijsen Piters, & Bogaert, 2017). When a virus is present, the epithelial barrier is crucial. Influenza virus infection can be impacted by the bacterial respiratory microbiome (Tsang et al., 2020). Bacterial colonization may be associated with the way that viral infection alters the balance of the airway microbiome to strengthen the host's innate immune response (Pittet, Hall-Stoodley, Rutkowski, & Harmsen, 2010). We can get a comprehensive understanding of the balance of the microenvironment and functional gene alterations by studying the microbiomes of COVID-19 patients.

One of the greatest advantages of mNGS is its ability to obtain a snapshot of a patient's microbiome at a specific sampling site to detect co-infections and determine other organisms that may impact patient outcomes. Understanding co-infections is important because they can worsen COVID-19 (Mostafa et al., 2020). There are limited reports of co-infections in

COVID-19 and so far, results have varied, possibly due to limitations of different methods used to detect co-infections, poor recovery, or detection based on standard-of-care methods due to broad-spectrum empirical coverage, lack of testing to understand co-infections, and the diverse geographic regions where the research was conducted.
The aim of the review was to explore the value of applying sequencing techniques in the diagnosis and treatment of secondary infections in COVID-19 patients.

2 METHODS

This study is a review of the literature collected from the search engine Google Scholar and PubMed Electronic Database with the keywords: "COVID-19", "Co-infection", and "Metagenomics". We looked for publications published in any language between 2020 and 2023.

3 RESULT

Based on the results of the analysis of 21 articles that met the criteria, the results showed that the countries that use NGS the most are from Asia, especially China at 66%, with the Illumina platform at 72%. The most widely used sample is the nasopharyngeal sample at 48% (Table 1). Sequencing results with several different platforms show pathogenic bacteria, viruses, fungi, a mixture of bacteria and fungi, a mixture of bacteria and viruses and a mixture of all three.
This study investigates the utility of nanopore sequencing technology in detecting pathogens in clinical samples of patients with severe COVID-19 infection to aid clinical diagnosis and treatment. These results demonstrate that compared with traditional clinical microbiology testing, nanopore sequencing reports test results more quickly (the day after sample collection). Compared with quantitative multiplex PCR assays, nanopore sequencing is not limited by the number of target pathogens and can detect unknown pathogens. The metagenomic sequencing technology developed in this research has high application value and can quickly and effectively detect potentially pathogenic bacteria in clinical samples of patients with severe COVID-19 infection, thereby helping the diagnosis and treatment of patients (Xiaofang et al., 2021).

4 DISCUSSION

Identifying newly or recently re-emerging viral infections at the point of care is critical to notifying the appropriate health authorities and enabling a prompt response before serious outbreaks take hold. This can be difficult since several viral agents can cause comparable symptoms, and new viruses cannot be predicted. If highly specialized knowledge of symptoms and infections is not accessible at the point of care, mNGS enables non-biased in-investigation and detection of viral agents. It has been demonstrated that the provided approach provides accurate identification of the pertinent pathogen (Fomsgaard et al., 2022).

Our research represents, as far as we are aware, a analysis to assess the prevalence of co-infections in patients with SARS-CoV-2 infection. These results, which are based on the early SARS-CoV-2 pandemic cases, indicate that bacterial co-infections are less common in COVID-19 patients than in influenza patients. In the 2009 influenza pandemic, bacterial infections seemed to be associated with morbidity and death in 1 in 4 severe or fatal cases of influenza A. The low fraction of viral NA in a particular clinical sample compared to the large levels of non- viral NA is one of the main obstacles to unknown pathogen identification with mNGS. As a result, the pathogen may not be found in a person who has been exposed to it. Depletion of host sequences with nucleases is a well-known method of resolving this issue, and filtering treatments, along with centrifugation, are other often utilized methods (Hall et al., 2014).

Importantly, mNGS also identified viruses in four samples that were not detected by routine clinical testing, HCoV NL63, RSV, hMPV was identified by mNGS but not by clinical multiplex PCR testing. Current broad-spectrum molecular testing algorithms identify most respiratory viral infections among SARS-CoV-2 PUIs, when available and implemented consistently. In addition, these results illustrate the potential of mNGS to streamline and expand clinical testing for respiratory viruses, which may augment strategies to surveil for unexpected viral coinfections or the emergence of divergent strains during periods of high transmission (Babiker et al., 2021).

Viral coinfections in COVID-19 patients have been reported globally, and are critical during early misdiagnosis. Possibly due to their immunity status, the middle-aged and the elderly are more prone to viral coinfection. However, it may not be true, healthy

Table 1: Characteristics of 21 included studies

No	Author	Country	Age group	Study type	N Partici-cipant	Method	Sample	References
1	Mostafa *et al.* 2020	USA	Adult, Elderly	Retrospective	50	GridION Nanopore	Nasopharyngeal	(Mostafa et al., 2020)
2	Ma *et al*., 2021	China	Adult,Elderly	Cohort	88	MGISEQ-2000 Shotgun	Oropharyngeal	(Ma et al., 2021)
3	Gao *et al*., 2023	China	Elderly	Case-series	42	Hiseq Illumina	Nasopharyngeal	(Gao et al., 2023)
4	Guo *et al*., 2021	China	Elderly	Retrospective	43	Novaseq 6000 Illumina	Sputum, bronchoalveolar	(Guo et al., 2021)
5	Xiaofang *et al*., 2021	China	NA	Case-series	3 (77 speci-mens)	MinION Nanopore	plasma, nasopharyngeal, sputum, fluids alveolar lavage, and bile.	(Xiaofang et al., 2021)
6	Al-Emran *et al*, 2022	Bangladesh	NA	Case-Control	11	Ion S5 systems (Thermo Fisher Scientific)	nasopharyngeal	(Al-Emran et al., 2023)
7	Iša *et al*., 2022	Mexico	NA	Retrospective	120	NextSeq 500 Illumina	Oro- and/or nasopharyngeal	(Iša et al., 2022)
8	Devi *et al*., 2022	India	Adult,Elderly	Retrospective	198	Miseq Illumina	nasopharyngeal	(Devi et al., 2022)
9	Babiker et al, 2020	USA	Adult,Elderly	NA	75	Illumina	nasopharyngeal	(Babiker et al., 2021)
10	Chong *et al*., 2021	Malaysia	Adult	Retrospective	198	NextSeq 500 Illumina	oro-nasopharyngeal	(Chong et al., 2020)
11	Yasir *et al*., 2023	Saudi Arabia	NA	NA	140	Miseq Illumina	Nasopharyngeal	(Yasir et al., 2023)
12	Faridl *et al*., 2022	Indonesia	Juvenile, Adult	Case Series	4	NextSeq 550 Illumina	Nasopharyngeal	(Faridl, Mellyani, Khoirunnisa, & Septiani, 2020)
13	Rouchka *et al*., 2021	USA	Adult,Elderly	Cohort	32	NextSeq 500 Illumina	Nasopharyngeal	(Rouchka et al., 2021)
14	Charalampous *et al*., 2021	UK	Elderly	NA	34	GridION Nanopore	Tracheal aspirates, BALs and NDLs	(Charalampous et al., 2021)
15	Molina-Mora et al., 2022	Costa Rica	NA	Retrospective	12	Miseq Illumina	Nasopharyngeal	(Molina-Mora, Cordero-Laurent, Calderón-Osorno, Chacón-Ramírez, & Duarte-Martínez, 2022)

16	Chen et al., 2021	China	Adult,Elderly	Retrospective	408	BGISEQ-50	blood, urine, and respiratory tract	(Chen et al., 2021)
17	Shah *et al*., 2020	San Francisco	Adult	retrospective cohort	316	Novaseq 6000 Illumina	oropharyngeal and/or nasopharyngeal	(Shah et al., 2020)
18	Mehta *et al*., 2021	India	Adult	Case series	100	Illumina	nasopharyngeal and/or throat	(Mehta, Sahni, Siddiqui, Mishra, & Sharma, 2021)
19	Miao *et al*., 2021	China	Adult,Elderly	Retrospective	100	Illumina	Nasal	(Miao et al., 2021)
20	Castaneda-Mogollon et al, 2021	Canada	NA	Retrospective	125	Novaseq 6000 Illumina	nasopharyngeal and Throat	(Casta et al., 2021)
21	Hoque *et al*., 2022	Bangladesh	NA	Retrospective	22	NextSeq 550 Illumina	nasopharyngeal	(Hoque et al., 2022)

people may also be coinfected. Bacterial pathogen coinfection is a worrying problem in COVID-19 management and also is the major cause of morbidity and mortality in other respiratory infections. *Klebsiella pneumoniae* was generally found in the majority of samples in several studies that have been reported.

Fungal co-infection based on our analysis revealed that COVID-19 patients showed different fungal microbial community composition compared to recovered humans and healthy controls. For example, only Saccharomyces cerevisiae was detected as the most dominant species in COVID-19 patients, whereas in controls several associated fungi were found. Due to the very low content of actual pathogen sequences in clinical specimens, the positive rate of metagenomic sequencing of clinical specimens in this article is low. To overcome this problem, in the future improvements can be made to the following aspects. First, targeted amplification of the 16S ribosomal RNA gene can be used to improve the accuracy and sensitivity of identification across clinically important bacterial species. Targeted amplification of the 16S ribosomal RNA gene can greatly reduce human genome reading interference and improve the sensitivity of bacterial detection, especially for specimens with high host cell content. The development of nanopore sequencing protocols for targeted sequencing of bacterial and fungal ribosomal RNA sequences would also be an effective complement. Second, higher pathogen detection sensitivity and genome coverage can be achieved by combining probe-targeted sequence capture and enrichment methods. Third, we continuously improve data sequencing analysis methods, striving to achieve effective and unbiased analysis while ensuring high reproducibility of test reports. In summary, this study used nanopore sequencing technology to detect Clinical specimens from severe COVID-19 patients were collected to achieve rapid identification of the pathogen. After further improvement, this detection method can be extended to detect other sudden infectious diseases in clinical practice and aid diagnosis.

5 CONCLUSIONS

Metagenomics analysis has increased the diagnostic level of the pathogen and provided valuable insights into the microbial landscape of COVID-19 patients, uncovering the prevalence and impact of co-infections. This review attempts to summarize previous studies that used metagenomic analysis to describe the viruses, bacteria, and fungi involved in COVID-19 co-infection. Therefore, after recognizing the possible pathogens causing co- infections among COVID-19 patients, and the molecular mechanisms underlying co-infections, appropriate curative and preventive interventions can be recommended.

REFERENCES

Al-Emran, H. M., Rahman, S., Hasan, M. S., Ul Alam, R., Islam, O. K., Anwar, A., … Hossain, A. (2023). Microbiome analysis revealing microbial interactions and secondary bacterial infections in COVID-19 patients comorbidly affected by Type 2 diabetes. *Journal of Medical Virology*, *95*(1), 1–12. https://doi.org/10.1002/jmv.28234

Babiker, A., Bradley, H. L., Stittleburg, V. D., Ingersoll, J. M., Key, A., Kraft, C. S., … Piantadosi, A. (2021). Metagenomic sequencing to detect respiratory viruses in persons under investigation for COVID-19. *Journal of Clinical Microbiology*, *59*(1), 1–6. https://doi.org/10.1128/JCM.02142-20

Bao, L., Zhang, C., Dong, J., Zhao, L., Li, Y., & Sun, J. (2020). Oral Microbiome and SARS-CoV-2: Beware of

Lung Co-infection. *Frontiers in Microbiology*, *11*, 1840. https://doi.org/10.3389/fmicb.2020.01840

Casta, D., Kamaliddin, C., Oberding, L., Liu, Y., Mohon, A. N., Faridi, R. M., … Pillai, D. R. (2021). A metagenomics workflow for SARS- CoV-2 identification, co-pathogen detection, and overall diversity. *Journal of Clinical Virology*, *145*. https://doi.org/10.1016/j.jcv.2021.105025

Charalampous, T., Alcolea-Medina, A., Snell, L. B., Williams, T. G. S., Batra, R., Alder, C., … Edgeworth, J. D. (2021). Evaluating the potential for respiratory metagenomics to improve treatment of secondary infection and detection of nosocomial transmission on expanded COVID-19 intensive care units. *Genome Medicine*, *13*(1), 1–16. https://doi.org/10.1186/s13073-021-00991-y

Chen, S., Zhu, Q., Xiao, Y., Wu, C., Jiang, Z., Liu, L., & Qu, J. (2021). Clinical and etiological analysis of co-infections and secondary infections in COVID-19 patients: An observational study. *Clinical Respiratory Journal*, *15*(7), 815–825. https://doi.org/10.1111/crj.13369

Chong, Y. M., Chan, Y. F., Jamaluddin, M. F. H., Hasan, M. S., Pang, Y. K., Ponnampalavanar, S., … I-Ching Sam. (2020). Detection of respiratory viruses in adults with suspected COVID-19 in Kuala Lumpur, Malaysia. *Journal of Clinical Virology*, (January).

Devi, P., Maurya, R., Mehta, P., Shamim, U., Yadav, A., Chattopadhyay, P., … Pandey, R. (2022). Increased Abundance of Achromobacter xylosoxidans and Bacillus cereus in Upper Airway Transcriptionally Active Microbiome of COVID-19 Mortality Patients Indicates Role of Co-Infections in Disease Severity and Outcome. *Microbiology Spectrum*, *10*(3), 1–19. https://doi.org/10.1128/spectrum.02311-21

Esakandari, H., Nabi-Afjadi, M., Fakkari-Afjadi, J., Farahmandian, N., Miresmaeili, S. M., & Bahreini, E. (2020). A comprehensive review of COVID-19 characteristics. *Biological Procedures Online*, *22*(1), 1–10. https://doi.org/10.1186/s12575-020-00128-2

Faridl, M., Mellyani, K., Khoirunnisa, K., & Septiani, P. (2020). RNA sequence analysis of nasopharyngeal swabs from asymptomatic and mildly symptomatic patients with COVID-19. *International Journal of Infectious Diseases*, (January).

Fomsgaard, A. S., Rasmussen, M., Spiess, K., Fomsgaard, A., Belsham, G. J., & Fonager, J. (2022). Improvements in metagenomic virus detection by simple pretreatment methods. *Journal of Clinical Virology Plus*, *2*(4), 100120. https://doi.org/10.1016/j.jcvp.2022.100120

Gao, Z., Yu, L., Cao, L., Yang, M., Li, Y., Lan, Y., … Deng, S. (2023). Analysis of coexisting pathogens in nasopharyngeal swabs from COVID-19. *Frontiers in Cellular and Infection Microbiology*, *13*(June), 1–11. https://doi.org/10.3389/fcimb.2023.1140548

Guo, M., Gao, M., Gao, J., Zhang, T., Jin, X., Fan, J., … Zhu, Z. (2021). Identifying Risk Factors for Secondary Infection Post-SARS-CoV-2 Infection in Patients with Severe and Critical COVID-19. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.715023

Hall, R. J., Wang, J., Todd, A. K., Bissielo, A. B., Yen, S., Strydom, H., … Peacey, M. (2014). Evaluation ofrapid and simple techniques for the enrichment of viruses prior to metagenomic virus discovery. *Journal of Virological Methods*, *195*, 194–204. https://doi.org/10.1016/j.jviromet.2013.08.035

Hoque, M. N., Rahman, M. S., Hossain, A., Khan, M. S., Islam, T., Mujibur, B. S., … Officer, P. S. (2022). Metagenomic analysis reveals the abundance and diversity of opportunistic fungal pathogens in the nasopharyngeal tract of COVID-19 patients. *BioRxiv Preprint*.

Iša, P., Taboada, B., García-López, R., Boukadida, C., Ramírez-González, J. E., Vázquez-Pérez, J. A., … Arias, C. F. (2022). Metagenomic analysis reveals differences in the co- occurrence and abundance of viral species in SARS-CoV-2 patients with different severity of disease. *BMC Infectious Diseases*, *22*(1), 1–12. https://doi.org/10.1186/s12879-022-07783-8

Ma, S., Zhang, F., Zhou, F., Li, H., Ge, W., Gan, R., … Huang, Z. (2021). Metagenomic analysis reveals oropharyngeal microbiota alterations in patients with COVID-19. *Signal Transduction and Targeted Therapy*, *6*(1). https://doi.org/10.1038/s41392-021-00614-3

Man, W. H., de Steenhuijsen Piters, W. A. A., & Bogaert, D. (2017). The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nature Reviews. Microbiology*, *15*(5), 259–270. https://doi.org/10.1038/nrmicro.2017.14

Mehta, P., Sahni, S., Siddiqui, S., Mishra, N., & Sharma, P. (2021). Respiratory Co-Infections: Modulators of SARS-CoV-2 Patients' Clinical Sub-Phenotype. *Frontiers in Microbiology*, *12*(May),1–16. https://doi.org/10.3389/fmicb.2021.653399

Miao, Q., Ma, Y., Ling, Y., Jin, W., Su, Y., Wang, Q., … Hu, B. (2021). Evaluation of superinfection, antimicrobial usage, and airway microbiome with metagenomic sequencing in COVID-19 patients: A cohort study in Shanghai. *Journal of Microbiology, Immunology and Infection*, *54*(180). https://doi.org/10.1016/j.jmii.2021.03.015

Molina-Mora, J. A., Cordero-Laurent, E., Calderón-Osorno, M., Chacón-Ramírez, E., & Duarte- Martínez, F. (2022). Metagenomic pipeline for identifying co-infections among distinct SARS-CoV-2 variants of concern: study cases from Alpha to Omicron. *Scientific Reports*, *12*(1), 1–10. https://doi.org/10.1038/s41598-022-13113-4

Mostafa, H. H., Fissel, J. A., Fanelli, B., Bergman, Y., Gniazdowski, V., Dadlani, M., … Simner, P. J. (2020). Metagenomic next-generation sequencing of nasopharyngeal specimens collected from confirmed and suspect covid-19 patients. *MBio*, *11*(6), 1–13. https://doi.org/10.1128/mBio.01969-20

Pittet, L. A., Hall-Stoodley, L., Rutkowski, M. R., & Harmsen, A. G. (2010). Influenza virus infection decreases tracheal mucociliary velocity and clearance of Streptococcus pneumoniae. *American Journal of Respiratory Cell and Molecular Biology*, *42*(4), 450–460. https://doi.org/10.1165/rcmb.2007-0417OC

Rouchka, E. C., Chariker, J. H., Alejandro, B., Adcock, R. S., Singhal, R., Ramirez, J., … Chung, D. (2021). Induction of interferon response by high viral loads at early stage infection may protect against severe outcomes in COVID-19 patients. *Scientific Reports*cc*11*(1). https://doi.org/10.1038/s41598-021- 95197- y

Shah, S. J., Barish, P. N., Prasad, P. A., Kistler, A., Neff, N., Kamm, J., … Phelps, M. (2020). illness: a comparison of patients with and without COVID-19. *EClinicalMedicine.*

Tsang, T. K., Lee, K. H., Foxman, B., Balmaseda, A., Gresh, L., Sanchez, N., … Gordon, A. (2020). Association Between the Respiratory Microbiome and Susceptibility to Influenza Virus Infection. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *71*(5), 1195–1203. https://doi.org/10.1093/cid/ciz968

Xiaofang, J., Xiaonan, Z., Yun, L., Xinyu, Z., Di, T., Yixin, L., … Hongzhou, L. (2021). Application of nanopore sequencing in diagnosis of secondary infections in patients with severe COVID-19. Journal of Zhejiang University (Medical Sciences) , 2–8.

Yasir, M., Al-Sharif, H. A., Al-Subhi, T., Sindi, A, A., Bokhary, D. H., El-Daly, M. M., … Azhar, E. I. 2023). Analysis of the nasopharyngeal microbiome and respiratory pathogens in COVID-19 patients from Saudi Arabia. *Journal of Infection and Public Health*, *16*(5), 680–688. https://doi.org/10.1016/j.jiph.2023.03.001

Zhang, H., Zhang, Y., Wu, J., Li, Y., Zhou, X., Li, X.,… Zhang, W. (2020). Risks and features of secondary infections in severe and critical ill COVID-19 patients. *Emerging Microbes and Infections*, *9*(1), 1958–1964. https://doi.org/10.1080/22221751.2020.18124 37

Zuo, T., Zhang, F., Lui, G. C. Y., Yeoh, Y. K., Li, A,Y. L., Zhan, H., … Ng, S. C. (2020). Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization. *Gastroenterology*, *159*(3), 944-955.e8. https://doi.org/10.1053/j.gastro.2020.05.048